Development and novel application of an *in vitro* fish gill cell assay to elucidate the ichthyotoxic mechanism of the microalga *Chattonella marina* (Raphidophyceae)

by

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Submitted in fulfillment of the
requirements for the degree of

Doctor
of
Philosophy

University of Tasmania

January, 2012

Declaration of Originality

I declare that the material presented in this thesis is original, except where due acknowledgement is given, and has not been accepted for the award of any other degree or diploma

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Dedication

To my parents,
Juan José Dorantes Mondragón and Aurora Aranda Elizalde,
for their love, support, patience and guidance in life

To my sisters, nephews and nieces,
Jackeline J. Dorantes Aranda
Delsy Dorantes Aranda
Ariadna Dorantes Aranda
J. Alejandro Dorantes Dorantes
K. Jahaziel Arroyo Dorantes
Axel J. Cisneros Dorantes
Angélica S. Arroyo Dorantes
Alexa Dorantes Dorantes
Ian A. Cisneros Dorantes
Joshua Arroyo Dorantes
Valeria Dorantes Dorantes
for their love, support and all good moments of joy and laughter

Acknowledgements

To my main supervisor, Professor Gustaaf M. Hallegraeff, for his direction, teaching, support and shared knowledge to perform and make the best of this project.

To my supervisors, Dr. Peter D. Nichols and Professor T. David Waite, for their teaching, collaboration, support, advices and guidance given to my research.

To Dr. Ben D. Mooney and Professor Allen R. Place for the gift of fatty acid fractions and karlotoxin, respectively, for the development of chapter 5.

To Mrs. Helen Bond for all her support and help in the laboratory.

To the Australian Government for the IEPRS scholarship that covered the full tuition fees and health cover (OSHC) for my PhD.

To the Mexican National Council for Science and Technology, CONACyT (190179), and the Mexican Secretary for Public Education, SEP-DGRI, for the living expenses scholarships.

To the University of Tasmania and Institute for Marine and Antarctic Studies for the top-up scholarship and funding to attend and present at the 14th International Conference on Harmful Algae.

To The University of New South Wales, UNSW, and Dr. Aurélie Godrant for letting me do an internship and for sharing her knowledge to learn new techniques, respectively.

To The Commonwealth Scientific and Industrial Research Organisation, CSIRO-Hobart, for making possible to perform the lipid work in their facilities.

To the Australian Antarctic Division, AAD, Rick van den Enden and Jennifer Penschow, for their availability and help for the electron microscopy work.

To the Menzies Research Institute, Professor Gregory M. Woods and César D. Tovar for the training regarding the maintenance and culturing of the gill cells. And to

Professor David Small and Dr. Robert Gasperini for letting me use their plate reader during the early stage of this research.

To the International Society for the Study of Harmful Algae, ISSHA, for the travel award to attend and present at the 14th International Conference on Harmful Algae.

To the School of Plant Science (UTAS), Dr. Adam Smolenski, Dr. Valérie Hecht and Dr. Evan Evans for letting me use their facilities to perform key assays for my research.

To Professor Andrew L. Rose for his ideas, advices and collaboration in part of this thesis.

To my Mexican colleagues, Dr. Rosalba Alonso Rodríguez, Dr. Lourdes Morquecho Escamilla, Dr. Ana Carolina Ruíz Fernández. Dr. Fedérico Páez Osuna and Dr. Luz M. García de la Parra, for their teaching and great input in my academic formation, encouragement and recommendations to continue my postgraduate studies.

To my friends in Mexico, Elvira Castro Andriano, Karina C. Lugo Ibarra, Eric Bautista Guerrero, Luis F. Vásquez Bedoya, Elizabeth Frausto Sotelo, Gerardo Gálvez Salinas, Alexis Rodríguez Suárez, Korynthia López Aguiar, Francisco Flores de Santiago, Carlos Sierra Becerra and Leonardo Camacho Cruz for all the great moments of happiness, and for their help by mailing me the forms and letters I needed in order to apply for this PhD. I have missed you heaps.

To my friends in Australia, César Tovar, Byron Smith, Steve Billingham, Gregor Sanders, Susanne Kennedy, Carl Snyman, Steven Smith, Richard Dearden, Hannah Smith, Nick O'Leary, Karen S. Luna Ramírez, Alexandre Kreiss, Marcus Ryan, Franco Solis, Fabiola Aburto, Joana Cubillos and Pamela Walker for their friendship, care and warm company.

Contents

Abstract

Chattonella marina algal blooms have been associated with major farmed and wild fish mortalities in tropical (Mexico), subtropical (South Australia) and temperate regions (Japan). However, the precise toxic mechanisms involved remain incompletely known and disputed. A novel sensitive *in vitro* assay using the rainbow trout cell line RTgill-W1 was developed to assess toxicity of lipid extracts, ruptured cells and intact cultures of 6 strains of *C. marina. Chattonella* was found to be less toxic than the haptophyte *Prymnesium parvum*, but more toxic than the raphidophytes *Heterosigma akashiwo, Fibrocapsa japonica* and dinoflagellate *Karenia mikimotoi*. Ruptured cells from Australian CMPL01 and Japanese N-118 *C. marina* strains were the most toxic, decreasing gill cell viability by 71 and 65%, respectively. The Mexican CMCV-1 strain was the least toxic (≤35%), possibly because it is larger and less fragile than other strains. *Chattonella marina* is unique among ichthyotoxic microalgae in its high production of superoxide anion (≤19 pmol $cell^{-1}$ hr^{-1}). Sonicated cultures showed higher levels of superoxide than intact cultures (19.0 vs 9.5 pmol $cell^{-1}$ hr^{-1}, respectively). However, O_2^- on its own did not appear to be the main cause of toxicity (only 14% loss of cell viability). Superoxide production was highest when grown in medium enriched with 1 µM Fe(III) compared to the 5 µM standard medium (25 vs 9.5 pmol $cell^{-1}$ hr^{-1}), especially after a sudden change from dark adaptation to light conditions (200 µmol photons m^{-2} s^{-1}) (up to 37.6), but lowest (4.1) at 100 nM Fe(III). The three major fatty acids in *C. marina* were palmitic (PA), eicosapentaenoic (EPA), and octadecatetraenoic (OTA) acids. Higher levels of EPA were found in ruptured cells (21.4 – 29.4% of total fatty acids) compared to intact cells (8.5 – 25.3%). Gill damaging effects from free fatty acid (FFA) fractions were conclusively demonstrated (LC_{50} at 1 hr of 0.44 µg mL^{-1}), and remain candidates of ichthyotoxicity when well-defined toxins such as brevetoxin or karlotoxin can be ruled out as causative factors. The aldehydes 2*E*,4*E*-decadienal and 2*E*,4*E*-heptadienal, which may be generated by FFA oxidation, also showed high impacts on gill cell viability, with LC_{50} (at 1 hr) of 0.34 and 0.36 µg mL^{-1}, respectively. In conclusion, *C. marina* was more toxic after cell disruption and when switching from dark to light conditions, associated with higher EPA levels and a

higher production of superoxide anion. Implications of this work for finfish aquaculture management and mitigation are discussed.

Chapter 1

Introduction | Hypotheses | Rationale | Objectives

1. Introduction

1.1. Overview of ichthyotoxic microalgae and their impacts on finfish mariculture

Harmful algal blooms (HABs), also known as red tides, are natural phenomena occurring in coastal waters and freshwater bodies. HABs have been classified into three categories according to their effects on living organisms (Hallegraeff, 2003):

1) Species that are harmless but can cause mortalities of aquatic animals through oxygen depletion, which happens when algae grow so dense and have high respiration at night or low light; this is more likely to happen due to bacterial respiration during decay of the bloom.

2) Species that do not affect humans but are harmful to finfish (ichthyotoxic) and some invertebrates with gills the main target organ.

3) Microalgal species that produce potent toxins and can affect humans through the food web (i.e. by eating contaminated shellfish and fish) causing different poisonings, and in some cases, leading to death.

The third type of HABs is usually produced by dinoflagellates and some diatoms. Filter-feeding organisms, like shellfish, tend to accumulate these toxins (paralytic, neurotoxic, diarrhetic, amnesic) and affect humans who eat contaminated

bivalves. This type of toxins and their mechanisms are now well characterized (Daranas et al., 2001; Landsberg, 2002). In contrast, the precise toxic compounds of ichthyotoxic phytoplankton (group two), especially raphidophytes, haptophytes and dinoflagellates, are not yet well understood. Mitigation practices have been often undertaken during these events, but huge economic losses to marine finfish and shellfish industries can result.

At least sixty phytoplankton species are ichthyotoxic, including raphidophytes, dinoflagellates, dictyochophytes and a few haptophytes (Landsberg, 2002; Aure et al. 2001; Backe-Hansen et al., 2001). The top ten harmful phytoplankton species with the highest impacts on marine finfish farming, and their suggested toxic compounds, are shown in table 1.1. Major fish mariculture developments occur on the coasts of Japan, Korea, China, the United States, Canada and the North Sea (Norway and Sweden area). Farmed fishes that are commonly affected by HABs on the East Pacific Ocean and North Sea are salmon, and yellowtail on the west side of the Pacific Ocean. The main species causing blooms and biggest impacts in these areas are *Chattonella antiqua*, *C. marina*, *Heterosigma akashiwo* (Raphidophyceae)*, Karenia mikimotoi* and *Cochlodinium polykrikoides* (Dinophyceae) (Table 1.1).

The two dictyochophytes *Pseudochattonella verruculosa* and *P. farcimen* have formed blooms since 1998 and 2001, respectively, in the North Sea region and New Zealand killing tons of farmed salmon. The economic losses caused by these blooms have not been reported but these species have killed about 1600 tons of farmed fish in four events (Aure et al., 2001; Backe-Hansen et al., 2001; Riisberg and Edvardsen, 2008; MacKenzie et al., 2011; Skjelbred et al., 2011).

Table 1.1. Top ten noxious marine phytoplankton causing substantial impacts on finfish mariculture.

Species	Places being affected	Fish species affected	Economic impacts US$ (Year)	Reference
Chattonella antiqua[a]	Japan	Yellow tail	1.9 million (’64)	Okaichi et al. (1989)
	Japan	Yellow tail	7 million (’70)	Hallegraeff (2003)
	Japan	Yellow tail	500 million (’72)	
Chattonella marina[a]	Japan	Yellowtail	190 million (’70-‘88)	Okaichi (1989)
	Australia	Bluefin tuna	40 million (’96)	Hallegraeff et al. (1998)
+ *C. antiqua*	Japan	Flatfish, yellowtail	10.7 million (’03)	Imai et al. (2006)
	China	Farmed fish	41 000 (’03)	Song et al. (2009)
Cochlodinium polykrikoides	Korea	Farmed fish	95.5 million (’95)	Kim (1997), Kim et al. (2002)
	Japan	Farmed fish	7 million (’99)	
	Canada	Salmon	2 million (‘99)	Whyte et al. (2001)
	Japan	Farmed fish	1.4 million (’00)	www.cearac-project.org
	Korea	Farmed fish	19 million (’03)	
	Japan	Farmed fish	1.5 million (’04)	Imai et al. (2006)
	Korea	Rockfish, parrotfish, red sea bream, bass	10 million (’07)	
	United Arab Emirates	Goldlined seabream	3 million (’08-’09)	Al Gheilani (pers. comm.), Richlen et al. (2010)
	Oman	Goldlined seabream	350,000 (’08-’09)	
Karenia mikimotoi (Dinophyceae)	Japan	Farmed fish	47.6 million (’80-’02)	Imai et al. (2006)
	Korea	Farmed fish	2.6 million (’81)	www.cearac-project.org
	Australia (Tas	Farmed fish	?	Hallegraeff (1992)
	China	Farmed fish	10.3-32 million (’88)	Dickman & Tang (1999)
	China (Zhuhai)	Amberjack, red sea bream, grouper Farmed fish	14.5 million (’98)	Qi et al. (2004) www.cearac-project.org
+ *K. digitata* →	China (HK)	Pufferfish and red sea bream	40 million (’98)	Lu and Hodgkiss (2004)
	Japan	Yellowtail, tiger puffer, amberjack, red	146,000 (’06)	www.cearac-project.org
	Japan	sea bream Salmon Farmed fish	260,000 (’07)	
(as *G. aureolum*)	Norway	Sea trout	665,000 (’82)	Dahl & Tangen (1993)
	China	Salmon	32 million (97-98)	Yin et al. (1999)

	Norway		? (’66)	Dahl & Tangen (1993)
	Scotland		? (’80)	Ayres et al. (1982)
Karenia digitata	China (Mirs Bay)	Cod, trout, groupers, snappers, sea-perch seabream,	32 million (’98)	Yang and Hodgkiss (2004)
Heterosigma akashiwo	Japan	Yellowtail and red sea bream	20 million (’72-’87)	Smayda (1998)
	Canada	Salmon	14 million (’86-’91)	Whyte (1999)
	Chile	Salmon	11 million (’88)	Avaria et al. (1999)
	New Zealand	Salmon	9 million (‘89)	Chang et al. (1990)
	USA	Farmed fish	4 million (’89)	www.nwfsc.noaa.gov/hab
	USA	Farmed fish	5 million (’90)	
	Canada	Salmon	40 million (‘93-‘97)	Rensel (2007)
	USA	Salmon	2 million (’97)	O’Halloran et al. (2006)
	USA	Salmon	2 million (’06)	
	Australia (Tas)	Salmon	?	Honjo (1993)
Karlodinium veneficum	USA		43 million (’97)	Lopez et al. (2008)
Prymnesium parvum[b]	Norway	Atlantic salmon & rainbow trout Farmed fish	5 million (’89)	Johnsen & Lein (1989) Kaartvedt et al. (1991)
	Texas, USA	Carp, Tilapia	17.5 million (’81-2010)	Sengco and Anderson (http://www.tpwd.state.tx.us/publications/pwdpubs/media/pwd_rp_t3200_1177.pdf)
	Israel		? (‘47)	
	Sweden	Farmed fish	5 million (’91)	
	Denmark			
Cerataulina pelagica (+ *Prymnesium calathiferum*)	New Zealand	Salmon	17 million (’82-’83)	Bruslé (1995)
Gonyaulax polygramma	China	Farmed fish	7 million (’88)	Landsberg (2002)
	Japan	Farmed fish	9 million (’94)	Imai et al. (2006)

[a] There has been uncertainty about the proper classification of the three species *C. antiqua*, *C. marina* and *C. ovata* since they are genetically identical but possess slightly different morphology. However, it is claimed that the classification based on morphology is insufficient to justify their distinction at the species level (Mikihide et al., 2009).

[b] This haptophyte forms blooms in brackish aquatic systems due to its wide salinity tolerance, including lakes, rivers, estuaries, reservoirs and ponds. Therefore, it is important to include this species since it is invading more inland aquatic systems causing enormous economic impacts.

Economic impacts arise to millions of dollars per event, with some HABs species showing a seasonal pattern and recurring annually. For instance, the raphidophytes *C. antiqua* and *C. marina* are considered to have occurred in low concentrations ("hidden flora") before the occurrence of the first red tide (1969) in the Seto Inland Sea, Japan. But since then, blooms of this species have been recorded almost every year from 1970 to 1989, causing a loss of US$ 190 million to fish farming in this period of time (Okaichi, 1989). These events followed the growth of the Japanese economy (1960-1965), and eutrophication has been suggested as one of the causes of the increase of HABs. The highest number of red tides (299) occurred in 1976, and although in 1973 the "Law Concerning Special Measures for Conservation of the Environment of the Seto Inland Sea" aimed to reduce the total quantity of organic pollutants of COD (Chemical Oxygen Demand) to half the level of 1972 (1700 tons per day), a decrease of HABs incidents was not evident until 1976. *Chattonella* blooms were not observed in the 1990's, but it has bloomed again since 2003 affecting finfish farms (Imai et al., 2006).

Finfish mariculture appears to be contributing, in part, to the occurrence, persistence and increase in number of HABs. The load of unconsumed food (i.e. pellets) and faeces of fish to the environment are another problem since they provide nutrients, and thus create eutrophication of the surrounding water and seabed (Folke, 1994). Fish farms are usually established in shallow and semi-enclosed coastal waters, like embayments, this is because fish pens and cages need to be sheltered from strong waves and currents (Lee et al., 2003); however fish-killing flagellates accumulate in these areas as well. Additionally, it has been suggested that water residence times or flushing rates can be affected by the presence of fish pens and cages since they may cause an increase in frictional drag and slow down of tidal currents (San Diego-McGlone et al., 2008).

Overutilisation of coastal waters for fish farming can create changes in water quality through time. It has been proven for instance, that the increase of organic load has created eutrophic conditions in a period of 10 years in Bolanio, Philippines, where an increase of ammonia (56%), nitrite (35%), nitrate (90%) and phosphate (67%) was observed together with a change of structure, composition, dominance and biomass of phytoplankton in this area, followed by the first devastating algal

bloom caused by the dinoflagellate *Prorocentrum minimum*, killing thousands of kilos of cultured milkfish valued at US$ 9 million. Mortalities were suggested to have been caused by hypoxic conditions of the area created by high oxygen consumption of waste feeds and the decay of the algal bloom (San Diego-McGlone et al., 2008). This coincides with the findings of Hodgkiss and Ho (1997), who showed that long and short term changes in N:P ratio were accompanied by increased blooms of non-siliceous phytoplankton groups, as observed in data from Japan, Hong Kong and North Europe. Additionally, a change in phytoplankton community was observed in Tolo Harbour, Hong Kong in a period of just 9 years, with red tide causative species, mainly dinoflagellates, taking over from diatoms.

The suggested harmful compounds and mechanisms for ichthyotoxic microalgae may vary among microalgal groups, and sometimes among species; however, microalgae with highest impacts on mariculture seem to share similar harmful compounds, including reactive oxygen species (ROS), such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and fatty acids with haemolytic properties (Lee, 1996) (Table 1.2). This topic remains controversial, however since some studies have claimed that production of ROS and fatty acids are involved in fish mortalities, but others discard this hypothesis and deny the role of ROS in ichthyotoxicity. Marshall et al. (2005) found that the raphidophyte *Chattonella marina* is the highest ROS producer; investigations from Oda et al. (1997) also suggested high ROS production rates by this and other raphidophytes. Similar to *Chattonella marina*, Kim et al. (1999) found ROS generation by the dinoflagellate *Cochlodinium polykrikoides* (Korean strain) and suggested that its toxic mechanism is through oxidative damage. However, Kim et al. (2002; 2010) detected trace levels of O_2^- and H_2O_2 in *C. polykrikoides* (isolated from Japan), suggesting that polysaccharides rather than ROS were the responsible for ichthyotoxicity of this dinoflagellate, but supporting the hypothesis of ROS toxicity by *C. marina*.

Table 1.2. Toxic or noxious compounds produced by ichthyotoxic microalgal species involved in finfish mortalities.

Group Species	**Suggested toxic or noxious compounds**	**Suggested effects and mechanisms and/or cause of finfish mortalities**	**References**
Raphidophytes	Reactive oxygen species (ROS): • superoxide anion (O_2^-) • hydrogen peroxide (H_2O_2) • hydroxyl radical (OH^-) Brevetoxin-like compounds? Fatty acids (haemolysins*): EPA (C20:5n3)	Lipid peroxidation Inactivation or induction of enzymes Edema in gill lamellae Mucus production Cardiac disorder Osmoregulatory disturbance Induction of Na^+,K^+-ATPase	
Chattonella antiqua *Chattonella marina* *Heterosigma akashiwo* *Fibrocapsa japonica**			Oda et al. (1997) Marshall et al. (2003) Haque and Onoue (2002) Tang et al. (2007)
Dinoflagellates			
Cochlodinium polykrikoides	ROS, FA, neurotoxic, haemolytic, hemagglutinative substances	Mucus production, respiratory arrest	Lee (1996), Kim et al. (1999; 2002)
Karenia mikimotoi	Haemolytic glycolipids and fatty acid: OPA (C18:5n3), gymnocins	Oxygen depletion, blood hypoxia	Hallegraeff (1992), Parrish et al. (1993), Satake et al. (2002)
Karenia digitata	unknown	Skin and gills severely damaged	Yang et al. (2000)
Karlodinium veneficum	Karlotoxins, ROS, fatty acids	Fish membrane disruption, gill structure breakdown. Mucus production, asphyxia	Mooney et al. (2007; 2009), Deeds et al. (2006), Van Wagoner et al. (2008)
Pyrodinium bahamense var. *compressum*	Saxitoxins (paralytic toxins)	Blockage of Na^+ channels	Maclean (1989)
Karenia brevis	Brevetoxins (neurotoxins)	Activation of Na^+ channels, loss of balance and appetite	Steidinger (2009)
Noctiluca scintillans	Ammonia (NH_3)?	Possible irritation, fish usually avoid blooms but mortalities have occurred	Okaichi and Nishio (1976)
Pfiesteria piscicida	Copper-containing toxins that generate free radicals	Narcosis	Moeller et al. (2007)
Gambierdiscus toxicus	Maitotoxin/Gambierol Ciguatoxins	Activation of Ca^{2+} channels/blockage of K^+, pathological changes in gill, liver, intestines Open Na^+ channels. Increase refractory	Diogenè et al. (1994), Cuypers et al. (2008)

		period of the lateral line nerve.	Lewis (1992), Flowers et al. (1992
Diatoms			
Cerataulina pelagica	Mucilage	Clogging of gills, anoxia	Taylor et al. (1985)
Chaetoceros wighami	Mechanical damage in gills caused by diatom spines	Haemorrhage, mucus secretion, suffocation	Bruno et al. (1989)
Chaetoceros concavicornis	(setae) =	=	
Chaetoceros convolutus			Bell (1961)
Haptophytes			
Chrysochromulina polylepis	Haemolytic compounds, glycolipids	Affect gill permeability, ion balance disturbance, osmoregulatory failure	Underdal et al. (1989), Lindahl and Dahl (1990), Meldahl et al. (1994),
Prymnesium parvum	Prymnesin (polyether compounds), glycolipids, fatty acid: OTA (C18:4n3)	=	Johansson and Granéli (1999), Henrikson et al. (2010)
Phaeocystis globosa	Acrylic acid, mucilaginous substances,	Reduction in food intake	Sieburth (1960)
Phaeocystis poucheti	dimethylsulfide, polyunsaturated aldehydes (PUA): decadienal		Hansen et al. (2004)

Interesting results, regarding the role of ROS, have been reported when performing bioassays using whole fish. Kim et al. (2009) found mortality on fish exposed to *C. polykrikoides* and fish still died upon exposure to the dinoflagellate plus ROS scavengers (i.e. catalase and superoxide dismutase). Woo et al. (2006) did not find any changes in antioxidant defences nor was lipid peroxidation detected in fish upon exposure to *C. marina*. Furthermore Tang et al. (2005) did not find comparable effects on fish after exposure to H_2O_2 itself and *C. marina*. Therefore, all these authors claimed that other mechanisms rather than ROS toxicity are responsible for *C. marina* and *C. polykrikoides* toxicity. Similarly, Marshall et al. (2003) did not find any mortality to fish exposed to O_2^-, but mortalities occurred upon exposure to eicosapentaenoic acid (EPA) on its own, and the combination of both O_2^- and EPA accelerated fish mortality, suggesting a synergy between ROS and EPA.

According to table 1.2, only a few phytoplankton species produce known toxins, whereas some others not producing toxins may cause fish mortalities due to oxygen depletion or mechanical damage (i.e. diatoms). The dinoflagellate *Karenia brevis* produces brevetoxins with neurotoxic effects. This species forms blooms almost every year and causes substantial impacts in the Gulf of Mexico and Florida Atlantic affecting mainly shellfish aquaculture and tourism (aerosol toxins) with estimated losses of US$ 15 – 25 million per year in Florida (Steidinger et al., 1999). Although affecting mostly shellfish, this species has also been associated with finfish and marine mammal mortalities (Steidinger and Melton-Penta, 1999).

Together with investigation of toxic mechanisms, control and mitigation techniques for management of HABs are also being studied. Imai et al. (2006) have listed the following as possible techniques:

- Clay spraying. Addition of clay minerals to HABs traps algal cells by flocculation despite the organisms' motility and buoyancy, causing them to sink and settle in the bottom. However, further studies are required to determine the fate and effects of flocculates containing the algal cells and toxins on benthic organisms (Sengco and Anderson, 2004).

- Towing net-pens to refuge areas. Moving net pens from areas affected by red tides to "clean" or refuge areas seems to be an effective technique and

preferred by some fish farmers. However, it can be an expensive measure and can create disadvantages if fish suffer stress and die, or if structural damages to facilities occur (Rensel and Whyte, 2003).

- Virus infection. Infective viruses have been isolated and have been able to attack strains of the species *Heterosigma akashiwo* and *Heterocapsa circularisquama*, causing mortality of these microalgae. However, further studies are needed since these viruses are strain specific rather than species specific. Thus a single virus clone can't terminate a bloom of a specific microalgal species composed of different ecotypes of clones with different sensitivity to viruses.

- Algicidal bacteria. Increases in bacteria in coastal areas associated with termination of algal blooms have been recorded, suggesting that bacteria with algicidal effects contribute to the rapid decline of red tides. Apart from viruses, many algicidal bacteria have also been isolated and are currently studied to find out their potential for control of algal blooms.

- Seaweed beds. Algicidal bacteria haven been isolated from macroalgae since they grow attached to their surface, especially from seaweeds of the genus *Gelidium*, *Ulva* and *Sargassum*. Based on these findings, Imai et al. (2002) proposed co-culturing fish with macroalgae as a prevention strategy against harmful algal blooms.

Of the techniques mentioned above, clay spraying and moving net-pens to safe areas, have already been implemented during HAB events with success. Whereas some others still remain under experimentation and are expected to be promising techniques (Table 1.3).

Apart from the control measures summarised in Table 1.3, there are some others that have been considered or are performed in few areas. Oxygenation and aeration seem to be a good technique when the bloom creates hypoxic conditions. Additionally, vigorous aeration can break *Chaetoceros concavicornis* cells into smaller sections that appear to be less harmful, as has been proven in laboratory conditions (Rensel, 1992). Airlift pumping of deep water, is effective for species that remain near the surface and it has been successfully implemented in blooms of

Heterosigma akashiwo when the pens are surrounded by perimeter tarps or skirts. This technique has proven to be a good method for preventing development of threatening HABs in Korea when low concentrations of harmful species are present (Kim, 2010). Submerging net-pens is becoming more viable as mariculture evolves; at the moment it is implemented in Hawaii for aesthetic reasons and to avoid waves (Rensel and Whyte, 2003). Finally, addition of ROS scavengers, like the antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase, has proven to give protection to fish upon exposure to ROS producers under laboratory conditions (Yang et al., 1995). This may be a good method for raphidophyte blooms like *Chattonella antiqua*, *C. marina*, *Heterosigma akashiwo* and *Fibrocapsa japonica*, but as far as known, it has not been applied to field situations.

From all the mitigation methods, it appears that the use of algicidal bacteria associated with seaweeds are a good way to prevent HABs. Therefore, development of seaweed beds in fish pens may control or avoid HABs since they provide bacteria with algicidal effects. This technique has two important advantages since it requires less economic investment and it is environmentally friendly by inflicting minimal or no harm to the marine ecosystem. However, nobody has dared to seed algicidal bacteria in nature so far; and the best mitigation success that has been applied is with clay flocculation in Korea and Japan (Kim et al., 2002, Wada et al., 2002), although this method is forbidden in Europe.

Table 1.3. Techniques for control and mitigation of HABs produced by ichthyotoxic phytoplankton.

Technique	Tested on / Applied to		Important findings or statements	Reference
	Experimental	HAB events (place)		
Clay spraying	*Karenia brevis* *Prymnesium parvum** *Cochlodinium polykrikoides* *Heterosigma akashiwo*	*Cochlodinium polykrikoides* (Japan and South Korea)	This seems to be an efficient and promising mitigation method, however further studies to elucidate the toxicity of flocculants (containing the algae) to benthic organisms are needed *Efficient removing cells but not for toxins	Kim et al. (2002) Wada et al. (2002) Pierce et al. (2004) Sengco et al. (2005) Song et al. (2010)
Towing net-pens to refuge areas		*Heterosigma akashiwo* (Canada and USA) *Karenia mikimotoi* (Norway) *Chattonella antiqua* (Japan)	This might be one of the most effective mitigation methods but it involves risks like high cost, damage to net pens, escape or death (i.e. by stress) of fish	Horner et al. (1998) Whyte (1997) Anderson et al. (2001) Rensel and Whyte (2003)
Virus infection	*Heterosigma akashiwo* *Heterocapsa circularisquama*		Virus-induced mortality important for termination of blooms Infection can be strain rather than species specific. Viruses have high replication ability and host specificity	Nagasaki et al. (1994; 2003) Tomaru et al. (2004)
Algicidal bacteria	*Heterosigma akashiwo* *Chattonella marina* *Chattonella antiqua* *Cochlodinium polykrikoides* *Fibrocapsa japonica* *Karenia mikimotoi*		Algicidal bacteria have been associated with termination of HABs Most algicidal bacteria have been classified into two groups: 1) γ-proteobacteria (*Alteromonas* sp. and *Pseudoalteromonas* sp.), and 2) *Cytophaga* / *Flexibacter* / *Bacteroides* (CFB)	Yoshinaga (2002) Mayali and Azam (2004) Imai et al. (2006) Kim et al. (2008b) Kim et al. (2009)
Seaweed beds	*Karenia mikimotoi* *Fibrocapsa japonica* *Heterosigma akashiwo*		Huge number of algicidal bacteria grow attached to surfaces of seaweeds (*Ulva* sp., *Gelidium* sp. and *Sargassum* sp.)	Imai et al. (2002; 2006)

1.1.2. *Chattonella marina*

A big problem for finfish mariculture industry

Chattonella marina (Fig. 1.1) is a marine raphidophyte causing mass mortalities in finfish farms (Table 1.1). This species has been distinguished from *Chattonella ovata* and *C. antiqua* based only on their morphology; however, they are genetically undistinguishable (Hosoi-Tanabe et al., 2006). Mikihide et al. (2009) reassessed the three species and found three groups based on morphology and phylogeny, but they were genetically identical. Therefore, these authors proposed taxonomic priority to *C. marina*, and renaming the others as *C. marina* var. *antiqua* and *C. marina* var. *ovata*. *Chattonella subsalsa* is also closely related to *C. marina* based on their morphological characteristics, but cultures from Texas identified as *C. subsalsa* do exhibit genetic differences compared to *C. marina* (Hallegraeff and Hara, 2003). A re examination of *C. subsalsa* from the French Mediterranean type locality (Mignot, 1976) is needed to resolve this.

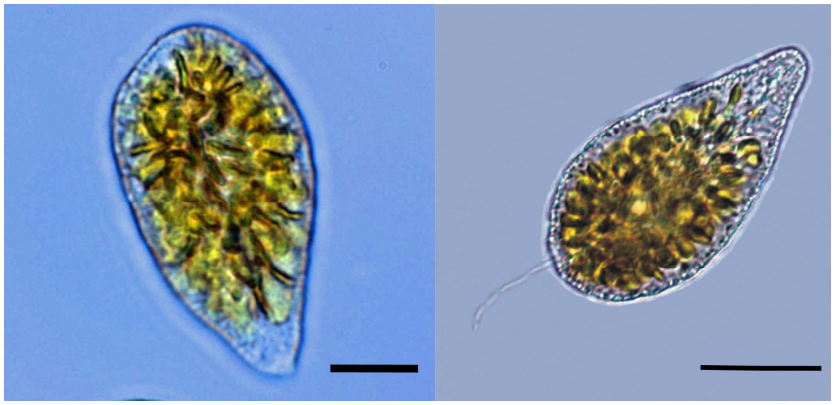

Figure 1.1. The raphidophyte *Chattonella marina* is a potent fish killer that is affecting finfish mariculture with substantial economic losses almost annually in Japan, and it has also affected tuna mariculture in Australia. Mexican strain CMCV-1 (left) and Australian CMPL01 (right). Scale bars: 40 µm.

Blooms of *Chattonella marina* have been widely reported and often are associated with wild and farmed fish mortalities in India, Australia, Japan, China, New Zealand, The Netherlands, Norway, Egypt, Canada, The United States, Brazil

and recently in Mexico (Okaichi, 1989; Imai et al., 1991; Taylor and Horner, 1994; Odebrecht and Abreu, 1995; Vrieling et al., 1995; Hallegraeff et al., 1998; Chang, 2000; Lu and Göbel, 2000; Mikhail, 2001; Songhui and Hodgkiss, 2001; Tiffany et al., 2001; Naustvoll et al., 2002; Band-Schmidt et al., 2004).

Mass mortality of cultured bluefin tuna (*Thunnus maccoyii*) was associated with a bloom formed by *C. marina* in South Australia in April/May 1996, and this was the first record of this raphidophyte in Australian waters (Hallegraeff et al., 1998; Munday and Hallegraeff, 1998). On the other hand, this alga along with other raphidophytes (e.g. the closely related *C. antiqua*) had previously impacted finfish aquaculture in Japan, where it continues to cause problems for fish farming (Okaichi, 1989; Imai, 1989).

Chattonella marina has been studied due to its high toxicity and impacts on fisheries during the last two decades. Australia and Japan are the places where this species has produced more toxic blooms and caused major aquaculture damage, therefore most of the studies on this microalga have been carried out by these countries. However, several investigations have also been carried out by Chinese researchers in the last five years. Some works have studied the growth and ecology of this species, whereas others focused on its toxicity. Thus bioassays have been performed, but most of these studies have used whole fish (from larvae to juvenile).

It has been proven that *Chattonella* species exhibit the highest rate of superoxide production among 37 microalgae analysed (Marshall et al., 2005). However, there exists controversy about the process of ROS production and their role on *Chattonella marina* toxicity. It was reported that H_2O_2 production in Japanese (NIES-3) *C. marina* could be increased by iron limitation and high pH, whereas growth rate was affected by nutrients, salinity, pH and photoperiod (Liu et al., 2007). Therefore, it was suggested that growth rate and ROS production are independent and regulated by different factors. In contrast, both Australian (CMPL01) and Japanese (N-118) *C. marina* strains produced high levels of O_2^- as influenced by photosynthesis and irradiance, with a significant superoxide decrease in dark conditions (Marshall et al., 2002a).

Susceptibility of marine fish to *C. marina* seems to be related to their osmoregulatory ability since euryhaline fishes (such as marine medaka *Oryzias melastigma* and green grouper *Epinephelus coioides*) suffer less or no mortality accompanied with minimal cytological damage and biochemical/physiological changes compared to stenohaline fishes (such as goldlined seabream *Rhabdosargus sarba*) (Xu et al., 2008). The suggested osmoregulatory disturbances in fish gills included induction of the enzyme Na^+,K^+-ATPase and the proteins in charge of the regulatory process of cystic fibrosis transmembrane (Tang et al., 2007).

Another toxic factor of *C. marina* contributing to fish kills could be the polyunsaturated fatty acid eicosapentaenoic acid (EPA), since it affects the respiratory activity, gill damage and loss of balance in fishes subjected to EPA, suggesting a synergistic effect of ROS and EPA (Marshall et al., 2003). EPA has also been claimed as a haemolytic component in *Cochlodinium polykrikoides* (Lee, 1996), whereas light-dependent haemolytic cytotoxin and brevetoxin-like compounds have been claimed from different Japanese *C. marina* strains (Onoue and Nozawa, 1989; Onoue et al., 1990; Haque and Onoue, 2002; Kuroda et al., 2005).

Onoue and Nozawa (1989) reported the production of neurotoxins in Japanese *C. marina*; these brevetoxin-like compounds have also been isolated from Japanese strains by Haque and Onoue (2002). Furthermore, Endo et al. (1992) described the effect of neurotoxins with a major impact on fish due to a cardiac disorder leading to death. Kuroda et al. (2005) suggested that Japanese *C. marina* contained a haemolytic cytotoxin, after haemolysis occurred in erythrocytes exposed to ruptured *C. marina* cells, but this did not occur with intact cell suspensions or cell-free supernatant.

Production of brevetoxin-like compounds by *C. marina* remains controversial since no significant levels have been detected in Australian strains (Hallegraeff et al., 1998; Marshall et al., 2003) nor could they be confirmed by LC-MS analyses of two different *Chattonella antiqua* strains by McNabb et al. (2006). This coincides with the most recent findings of Shen et al. (2010) after exposure of fish to *C. marina* and the well-known brevetoxin producer, the dinoflagellate, *Karenia brevis*. Fish exhibited different behavioural symptoms, hyperventilation or hypoventilation were observed upon exposure to *C. marina* and *K. brevis*, respectively. Moreover, no fish

mortality was observed after exposure to organic extracts of *C. marina*, but all fish died in *K. brevis* extracts (containing brevetoxins). In conclusion the major compounds responsible for fish mortalities by *C. marina* were claimed to be not brevetoxins, and since this raphidophyte produces a wide variety of fatty acids, they might have been mistaken as brevetoxin-like compounds.

1.2. Use of *in vivo* and *in vitro* assays for ichthyotoxicity tests of red tide phytoplankton

The main tool used to determine toxicity of harmful species is bioassays, where living organisms are studied to measure the impact and toxic effects of the experimental materials. Different techniques are usually used depending on whether responses of a whole or parts of an organism want to be determined, and this also depends on the toxicokinetics of the experimental material. For *in vivo* experiments, whole organisms (usually animals and plants) are used, whereas for *in vitro* assays only parts of the organism are used, such as organs, tissues or cells.

In vitro assays are commonly used when target organs or cells that are attacked by the experimental material are already known, or at least, they are expected to be the targets. A wide variety of endpoints can be measured depending on the characteristics of the experimental material. In toxicology, mortality (i.e. median lethal concentration LC_{50}, median lethal time LT_{50}, median lethal dose LD_{50}), biochemical and physiological markers are usually assessed.

In aquatic toxicology, the effects of HABs are mainly measured on shellfish, brine shrimp, fish and mice. In order to determine lethal concentrations or doses and propose regulations, the standard mouse bioassay (MBA) of the AOAC first developed for PSP[1] has been widely used as the reference method in many countries, and it is still used in the USA (Inami et al., 2004). Modification of this assay is currently used to detect and monitor algal toxins that produce the syndromes PSP,

[1]Paralytic Shellfish Poisoning

DSP, NSP, ASP and AZP[2], representing the phytoplankton groups that produce potent toxins and can affect humans through the food web. However, disadvantages of this method have been discussed and a variety of methods, including analytic (i.e. HPLC, LC/MS) (Louppis et al., 2010), enzyme immunoassays, protein phosphatase inhibition assays, ligand-receptor binding assays and *in vitro* assays (Jellet et al., 1992; Okumura et al., 2005) to replace MBA have been proposed, and some of these are currently used as the primary detection tool for PSP in countries like the United Kingdom, Germany, Sweden and Canada. Despite these methods showing good results, some countries (i.e. Norway, Ireland, The Netherlands) usually compare and complement their results against MBA. However, the European Union has recently validated and approved chromatographic methods (i.e. HPLC, LC-FLD) as alternative non-animal methods for detection of PSP toxins in shellfish (Hess, 2010; Turner et al., 2011). Mice are used to represent mammalian organisms since the poisonings mentioned previously are able to affect, and in some cases kill, humans. On the other hand, it is usually convenient to use whole fish for bioassays when testing ichthyotoxicity of microalgae.

As seen in Table 1.4, the most studied microalgae in order to elucidate their ichthyotoxic effects are the raphidophytes *Chattonella marina* and *Heterosigma akashiwo*, and the dinoflagellate *Cochlodinium polykrikoides*. Chosen animals for *in vivo* assays are usually target organisms (i.e. yellowtail *Seriola quinqueradiata* and red sea bream *Pagrus major*), especially for *C. marina* experiments, since it is mainly affecting these species in Asia. Most of the investigations report gill damage, and ROS involvement for the raphidophytes. By contrast, no conclusive results have been found for *C. polykrikoides* toxicity, and only few *in vitro* experiments have been performed with this dinoflagellate.

In vitro assays commonly involve mammalian cells or animal erythrocytes due to their simple handling and availability. Additionally, a wide variety of fish cell lines[3] are available nowadays, including cells initiated from ovary, muscle, caudal

[2]DSP=Diarrhetic Shellfish Poisoning, NSP=Neurotoxic Shellfish Poisoning, ASP=Amnesic Shellfish Poisoning, AZP=Azaspiracid Poisoning

[3]A cell line arises from a primary culture at the time of the first successful subculture. This term implies that cultures from it consist of lineages of cells originally present in the primary culture (Schaeffer, 1990).

trunk, heart, liver, kidney, embryo, eye, peripheral blood, skin and gills, that can be purchased from biological collections. And in some cases researchers have started their own primary cultures[4] and successfully performed *in vitro* assays. Since *in vitro* assays have shown reproducible and good results, there has been special attention in the development of primary cultures and cell lines in the last two decades, and bibliography to start primary cultures and isolation of tissues and/or cells is available (Bradford et al., 1994; Monod et al., 1998; Segner, 1998a; Ganassin et al., 2000; Wood et al., 2002; Mazon et al., 2004; Bickley et al., 2009). For instance, fish brain cells have been successfully isolated and cultured in laboratory conditions (Hinsch and Zupanc, 2006), and thus effects and mechanisms of neurotoxins produced by phytoplankton can also be studied.

[4]A culture started from cells, tissues or organs taken directly from organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a cell line (Schaeffer, 1990).

Table 1.4. Comparison of *in vivo* and *in vitro* assays to assess ichthyotoxicity of marine microalgae.

Microalgal species:	**Organisms, organs, tissues or cells used for assay** a. *In vivo* Living algae or **algal extracts**	b. *In vitro*	**References**
Chattonella marina	1a. Marine medaka 2a. Goldlined sea bream 3a. Damselfish 4a. Goldlined sea bream 5a. Goldlined sea bream 6a. Yellowtail 7a. Red sea bream, carp 8a. Yellowtail 9a. Yellowtail 10a. Yellowtail	**1b**. Rabbit, cattle and sheep erythrocytes **2b**. Cell lines: • human epithelial carcinoma (HeLa) • monkey kidney (Vero) • canine kidney (MDCK) • rat sarcoma (XC) • hamster ovary (CHO) **3b**. HeLa cells	Endo et al. (1985; 1992) Kim et al. (2001; 2002) Lee et al. (2003) Marshall et al. (2003) Kuroda et al. (2005) Tang et al. (2005; 2007) Woo et al. (2006) Kim and Oda (2010) Shen et al. (2010)
	Findings: 1a. Fish suffered hyperventilation. Organic extracts were not toxic to fish but algal cultures were. Brevetoxins are unlikely to be responsible for fish kills by *C. marina*. 2a. LT_{50} (8000 cells/mL) = 3 hrs. H_2O_2 itself showed much lower toxicity than *C. marina*. 3a. Superoxide anion on its own did not cause fish mortality. EPA killed fish similar to *C. marina*. EPA+O_2^- killed fish three times faster than EPA alone. 4a. *Chattonella marina* killed fish but oxidative stress did not occur in fish. ROS are not the principal cause of fish kills. 5a. Induction of two ion transporters in chloride cells of gills occurred (osmotic distress). 6a. Pavement cells from gills were swollen after exposure. Mucous cells with mucous decreased. Decrease of carbonic anhydrase activity in gills. 7a. Decrease of heart rate, resulting in anoxia from reduced blood circulation in gills. Neurotoxin fractions of *C. marina* depolarized the vagal nerve of fish. 8a. Drop of arterial oxygen partial pressure in fish. Heart rate was maintained until shortly before death. 9a. Surface of gill lamellae accumulated glycocalyx that *C. marina* discharged upon contact with fish gills. This glycocalyx accumulation may be responsible for ROS-mediated severe gill damage leading to death. 10a. *Chattonella marina* increased O_2^- production in the presence of fish mucus. 1b. Marked hemolysis occurred just in light conditions, especially in rabbit erythrocytes. 2b. Hemolytic extract showed significant loss of viability at >0.1μg/mL just in light conditions. 3b. Cell-free aqueous extract of *C. marina* had no effect on HeLa cells.		
Heterosigma akashiwo	1a. Copepod 2a. Oyster 3a. Scallop: eggs, larvae and juveniles 4a. Rotifer 5a. Artemia larvae, abalone larvae, spat	**1b**. Rabbit erythrocytes **2b**. Sf9 insect cells **3b**. Rat osteoblastic sarcoma (UMR-106) and human embryonic kidney (HEK-293) **4b**. Rabbit erythrocytes	Botes et al. (2003) Twiner et al. (2004; 2005) Keppler et al. (2005) Kuroda et al. (2005) Wang et al. (2006) Zhou et al. (2008)

			Ling and Trick (2010) Yu et al. (2010)
	1a. Inhibition of ingestion rates, proportions of gravid females, egg production, growth efficiencies and recruitment. 2a. Increase of oyster hepatopancreas lysosomal destabilization rates (irreversible). 3a. Decreased embryo development and arrested when reached early larval stage; swimming inhibition in post larvae and interruption of hatching process. Juvenile scallops were survived the exposure. 4a. LC_{50} at 24 hr was 1.21×10^4 cells/mL, showing inhibitory effects upon exposure to filtrate and cell contents. 5a. Mortality was always below 10%. 1b. Marked hemolysis occurred just in light conditions, comparable to *C. marina*. 2b. Extracellular organic compounds of *H. akashiwo* increased [Ca^{2+}] in Sf9 cells by inhibiting the plasma membrane Ca^{2+}-ATPase transporter and ultimately inducing apoptotic cell death. 3b. Extracellular organic compounds of four (out of nine) strains of *H. akashiwo* increased metabolic activities of mammalian cells. 4b. Hemolysis occurred only in light conditions in extracts with ultrasonic ruptured algal cells but not in intact culture or supernatant without algal cells. Haemolytic agents of many raphidophytes are uniformly light dependent.		
Cochlodinium polykrikoides	1a. Sheepshead minnow , bay scallops 2a. Black scraper, red sea bream, beakperch, seaperch, flounder, rockfish 3a. Sheepshead minnow, striped killifish, Atlantic silverside, common mummichog, bay scallops, American oysters 4a. Larvae of Eastern oyster, bay scallop and Northern quahog 5a. Damselfish 6a. Damselfish 7a. Flatfish 8a. Spotted snapper	**1b**. HeLa cells **2b**. Fish and human erythrocytes **3b**. HeLa cells	Tang and Gobler (2009a; 2009b) Kim et al. (1999) Kim et al. (2000) Kim et al. (2002; 2009) Gobler et al. (2008) Dorantes-Aranda et al. (2009; 2010) Yamasaki and Oda (2009)
	1a.Cultures more potent than raw bloom water. Early exponential growth cultures were more toxic. Cell-free culture medium caused slower ichthyotoxicity than intact cell cultures. Ichthyotoxicity can be caused by non-hydrogen ROS-like chemicals[a] since H_2O_2 did not kill fish but peroxidase and catalase reduced toxicity of live cultures. 2a. Pelagic fishes were more vulnerable than benthic fishes. Inactivation of gill transport-related enzyme activities, fall in blood oxygen partial pressure and secretion of gill mucus were observed. 3a. High mortality of finfish and shellfish. Epithelial proliferation and fusion of gill lamellae occurred. Moribund bivalves showed hyperplasia, haemorrhaging, squamation and apoptosis in gill and digestive tissues. 4a. Cultures and bloom water were highly toxic to larvae with 80-100% mortality during 24 to 72-hr exposures at $1\text{-}2\times10^3$ cells/mL. Toxicity depended on cell density, growth phase, exposure time and age of larvae. 5a & 6a. All fish died within 90 min in an intact cell culture but ruptured cells did not affect fish. Catalase and superoxide dismutase did not reduce toxicity of *C. polykrikoides*. No significant levels of ROS were detected. 7a. *C. polykrikoides* produced ROS and caused lipid peroxidation in fish gills, which increased with cell density. 8a. Hepatic catalase activity was inhibited, lipid peroxidation in liver and polysaccharide production in gills increased with cell density. 1b. Cell-free aqueous extract of *C. polykrikoides* had toxic effect on HeLa cells. Levels of ROS in this dinoflagellate were trace levels. Accumulation of polysaccharides in the		

	growth medium was observed. The medium became viscous. 2b. 50% hemolysis (HE_{50}) occurred at 4.88 and 5.27×10^3 cells/mL for fish and human erythrocytes, respectively. 3b. Aqueous extract of *C. polykrikoides* showed toxicity towards HeLa cells.

[a] The term "non-hydrogen ROS-like chemicals" refers to species that do not contain hydrogen but may have similar or stronger effects causing oxidative stress. This may include highly reactive oxygen species (i.e. singlet oxygen, superoxide anion, hydroxyl radical), lipid radicals or peroxides (Tang and Gobler, 2009).

In general, there are important societal and ethical concerns against the use of laboratory animals. Therefore replacement of whole-animal experiments by *in vitro* assays (when possible) is strongly recommended, fulfilling Russell and Burch's (1959) concept of the 3 R's (replacement, reduction, refinement). Additionally, working *in vitro* rather than *in vivo* offers several advantages, including:

- Much smaller sample volume required
- No ethics approval required (when working with cell lines), but if primary cultures will be done, the number of animals sacrificed is significantly reduced
- More replicates can be done
- More reproducibility (cell lines even more than primary cultures) (Lee at al., 2008)
- No special facilities required (i.e. aquariums, seawater filtration/sterilization equipment), lowering the cost
- Better control of experimental conditions by reducing unavoidable stress responses (Lee et al., 2009)
- Precise control of the physical and chemical environment (Bickley et al., 2009)
- Allows study toxic mechanisms in isolation from the multiple physiological systems that regulate normal *in vivo* activity (Bickley et al., 2009)
- The homogeneity of cell lines makes responses to toxicants easier to detect and with less variability than whole organisms (Castaño et al., 2003; Schirmer, 2006)

The limitation of working with cell lines can be that damage to cells does not necessarily mean fish mortality or human oral potency; especially when target cell lines are not chosen properly. Therefore selection of suitable cell lines and endpoints for *in vitro* assays are critical to achieve results with good correlation with *in vivo* tests (Schirmer, 2006).

1.3. RTgill-W1 cell line

An *in vitro* model for ichthyotoxic assessment of marine microalgae

All *in vivo* experiments for ichthyotoxicity of marine phytoplankton testing either specific algal extracts or living algae have been carried out using mostly invertebrate or vertebrate animals (Table 1.4). However, when *in vitro* approaches have been done, most of the studies only tested algal extracts because they are easier to handle due to their solubility in seawater or organic solvents (when they are lipophilic) (Kosovel et al., 1988; López et al., 2011), or in buffers as in haemolytic assays (Deeds et al., 2002).

Katsuo et al. (2007) successfully used Vero cells for direct exposure of marine toxic microalgae due to their short-time resistance to seawater compared to other mammalian cell lines that did not resist seawater, and proposed this cytotoxic assay for screening and detection of algal-derived toxic substances.

Vero cells were initiated from the kidney of an African green monkey, and hence using a fish cell line is a much better approach for ichthyotoxicity, offering potential advantages over mammalian cells since (i) fish-killing microalgae are thought to attack mainly the gills, thus a fish gill cell line is more suitable for testing toxicity of ichthyotoxic algae, and (ii) fish cell lines reduce the cost since they are commonly grown in a normal sterile atmosphere at room temperature (18-22°C). By contrast, mammalian cell lines need to be grown at 37°C in a 5%-CO_2 atmosphere, for which a special incubator is required (valued at ~AU$6000).

The cell line RTgill-W1 (Fig. 1.2) was initiated from a 15-month-old primary culture of gill fragments of a juvenile rainbow trout *Oncorhynchus mykiis* by Bols et al. (1994), and it is now available from the American Type Culture Collection (ATCC). It forms a confluent monolayer within 1 week. The cells have a polygonal shape and usually form colonies. This cell line represents a good model since it was originated from a salmonid fish, and salmon farms (including rainbow trout) are being widely developed in temperate coastal areas, and thus they are susceptible to algal blooms. Additionally, fish gills appear to be the main organ that is affected by toxic phytoplankton, representing a suitable cell line for ichthyotoxic assessment.

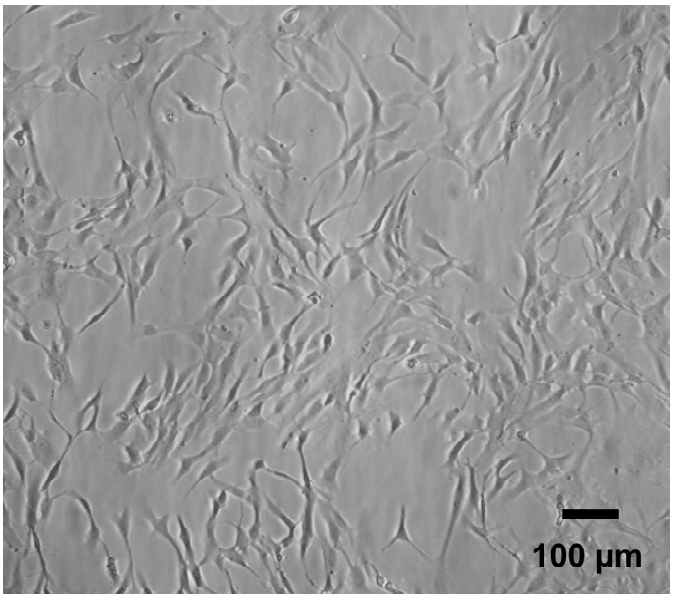

Figure 1.2. RTgill-W1 cells as seen under a light microscope. These epithelial cells grow attached to the surface of the tissue culture flask and grow in a normal atmosphere at 18-20°C.

2. Hypotheses

a) Since it has been widely claimed that the main organ affecting fish during harmful algal bloom events and leading to death is the gills, the ichthyotoxic microalga *Chattonella marina* is able to cause damage on RTgill-W1 cells.

b) *Chattonella marina* produces high amounts of eicosapentaenoic acid, and this depends on the nutrient conditions where the algae grow and condition of the cells (intact versus ruptured).

c) Iron and light influence production of superoxide anion in *C. marina*, suggesting an interaction between eicosapentaenoic acid and superoxide that play an important role in gill function and damage.

3. Rationale

There are three major problems regarding the study of ichthyotoxic phytoplankton. First, their toxic mechanisms and compounds are not well understood yet; especially the raphidophyte *Chattonella marina*, which is one of the most potent fish killers. Second, experimentation usually involves whole fish bioassays and there are societal and ethics concerns about the use of animals for laboratory assays. Finally, a simple standard *in vitro* assay using a fish cell line to study toxicity of microalgae is not available. Therefore, this study proposes an *in vitro* assay using the cell line RTgill-W1 as a model. This assay offers important advantages over *in vivo* experiments. It satisfies societal demands since it reduces and replaces whole animals, it possesses high reproducibility and can be used for any marine phytoplankton species. Therefore, the use of this assay will help us to elucidate precise toxic mechanisms of red tide microalgae.

4. Objectives

4.1. General

Develop and use of an *in vitro* assay to assess and elucidate the toxicity of the fish-killing marine microalga *Chattonella marina*

4.2. Specifics

- Develop an *in vitro* assay using the fish cell line RTgill-W1 as a model
- Assess toxicity of six different geographic strains of *Chattonella marina* and other ichthyotoxic phytoplankton species
- Measure production rates of superoxide anion in six different strains of *C. marina* under different culture conditions
- Determine lipid profiles and strain variability of *C. marina* grown and harvested under different conditions
- Extract and test the toxicity of major fatty acids from *C. marina*

Chapter 2*

Development of an *in vitro* assay using the fish cell line RTgill-W1 as a model

Abstract

Since use of cell lines offers significant advantages compared to working with whole organisms, a simple *in vitro* assay for microalgal toxicity is described using the rainbow trout cell line RTgill-W1. This application of a microplate based assay is useful for testing the toxicity of algal extracts to gill cells in a time-dependent manner. Additionally, a modification of this assay using an insert support with permeable membranes is presented to test the toxicity of living marine microalgae. The endpoint is measurement of cell viability using alamarBlue as an indicator dye. Gill cells remained 100% viable for 60 hrs in the modified version of the basal growth medium, L-15/ex, without fetal bovine serum and with 1% methanol (in which fatty acids were dissolved). In contrast, gill cells survived for 3 hrs in microplates with insert supports upon exposure to algal seawater-based growth media. Toxicity of palmitic acid was less than 10%, as observed by loss of gill cell viability, during the first 12 hrs at all concentrations. However, the highest toxicity was observed at 48 – 60 hrs with a reduction of cell viability of 50% after exposure to 140 mg L^{-1}. The ichthyotoxic raphidophyte *Chattonella marina* and dinoflagellate *Karlodinium veneficum* had a negative effect on gill cells, causing up to 70 and 38% loss of viability, respectively, when exposed to high algal concentrations for 2 hrs. These two simple and reproducible screening tests represent a sensitive and suitable alternative to whole fish ichthyotoxic bioassays for fish-killing marine microalgae.

* Published as: Dorantes-Aranda, J.J., Waite, T.D., Godrant, A., Rose, A.L., Tovar, C.D., Woods, G.M. and Hallegraeff, G.M. 2011. Novel application of a fish gill cell line assay to assess ichthyotoxicity of harmful marine microalgae. Harmful Algae 10, 366-373.

1. Introduction

The development of primary cultures and animal cell lines circumvents problems faced when using whole organisms. Cell lines are easy to handle, they grow quickly and experiments are more reproducible than is the case with whole organisms. This *in vitro* approach also satisfies ethical demands to reduce the number of animals sacrificed with the ultimate aim of replacing whole organisms for toxicity assays (Segner, 1998b).

The use of cells or tissues can produce different results, however, compared to working with whole organisms as a result, at least in part, of differing toxicokinetics between whole organisms and cells or tissues. Harmful effects observed in *in vitro* tests conclusively demonstrate susceptibility to xenobiotics or harmful species, and enable elucidation of the toxic pathways contributing to the death of the organism. Furthermore, use of cell lines has the advantage of allowing better control of experimental conditions by reducing unavoidable stress responses (Lee et al., 2009). According to Segner (1998b), most studies report a high correlation between *in vitro* cytotoxicity data from fish cell lines and *in vivo* fish lethality data. Segner (1998b) also reports that the best correlation between *in vitro*/*in vivo* tests is observed when comparing the effect of heterogeneous chemicals. Likewise, toxicity of complex effluent samples agreed well between these tests (Rusche and Kohlpoth, 1993). Issues that avoid obtaining a good correlation between *in vitro*/*in vivo* tests can be overcome by selecting suitable endpoints, and by establishing cell culture models with functions typical of their origin (Schirmer, 2006). For instance, a recent study where the present assay was used (Mooney et al., 2011) demonstrated that RTgill-W1 cells are more sensitive than larvae of sheepshead minnow to fatty acids produced by toxic phytoplankton. Fish larvae were apparently affected but no mortality was observed. By contrast, gill cells showed significant loss of viability, suggesting that gills are a potential target organ for ichthyotoxic compounds from harmful microalgae.

In aquatic ecosystems, gills of fish are a key organ because they are the breathing apparatus of fish where direct contact between water and fish occurs.

Additionally, gills are the site of several interconnected vital processes, including gas exchange, osmoregulation and ionic diffusion, pH regulation and nitrogen balance. Thus gills represent ideal target organs against which to evaluate the effect of toxicants or pathogens in aquatic toxicology studies (Evans et al., 2005) since they are susceptible to a broad range of bioactive compounds contained in the water. Indeed, gill cell lines are becoming popular alternatives to *in vivo* fish tests (Segner, 1998b; Sandbacka et al., 1999; Dayeh et al., 2005a). The cell line RTgill-W1 has been widely used for toxicity assays, including examining impacts from metals, wastewater products, industrial pollutants and engineered nanoparticles (Schirmer et al., 1997; Dayeh et al., 2005a; b; Kühnel et al., 2009). It has also been used as a host to support the growth and ultimately elucidation of the genome of the ASPV virus (Kvellestad et al., 2003).

Fish gills are also susceptible to active compounds produced by phytoplankton. The suggested damages are epithelial necrosis, loss of secondary lamellae, cellular hypertrophy and lysis of epithelial and chloride cells (Hiroishi et al., 2005; Deeds et al., 2006). Additionally, other studies have found negative effects caused by ichthyotoxic microalgae that affect gill function, including mucus excretion and vasodilation (Marshall et al., 2003), decrease or induction of enzymatic activities, osmotic distress caused by an augmentation of branchial chloride cells (Endo et al., 1985; Tang et al., 2007) and changes in the cardiorespiratory system (Lee et al., 2003).

The aim of this chapter was to develop a novel application of an *in vitro* assay for toxic assessment of the red tide phytoplankton *Chattonella marina* by using the gill cell line RTgill-W1 as a model.

2. Methods

2.1. Cell line: origin, maintenance and culturing

The permanent epithelial cell line RTgill-W1 was obtained from the American Type Culture Collection (CRL-2523, ATCC). The primary culture of this

cell line was initiated from the gill filaments of rainbow trout *Oncorhynchus mykiss* by Bols et al. (1994). The cells were routinely grown in 25-cm^2 culture-treated flasks (3100-025, Iwaki) in Leibovitz's L-15 medium (L1518, Sigma), supplemented with 10% (v/v) fetal bovine serum (FBS, 12003C, Sigma), and an antibiotic-antimycotic solution (A5955, Sigma) containing penicillin (10,000 units mL^{-1}), streptomycin (10 mg mL^{-1}) and amphotericin B (25 µg mL^{-1}). The cells were incubated at 19ºC (±1ºC) in the dark. This cell line forms an adherent monolayer and grows attached to the bottom of the flask. Detachment of cells for routine subculturing was achieved using 0.25% trypsin-0.02% EDTA in Hank's balanced salt solution (59428C, Sigma). Trypsinasation was achieved within 5-10 min. Confluent gill cells were normally subcultured at a ratio of 1:3, and medium renewal was carried out twice per week to provide more nutrients for good cell growth.

This gill cell line was used for all *in vitro* assays all along this thesis and usually only referred as gill cells.

2.2. Cell viability assays

Cell viability was determined with the indicator dye alamarBlue (DAL1025, Invitrogen) (Pagé et al., 1993). Once exposure was completed, the medium was discarded and the gill cells rinsed with phosphate buffer saline (PBS). A modified version of the L-15 medium, L-15/ex (Schirmer et al., 1997) without FBS and containing 5% alamarBlue (v/v), was added to all wells and cells were incubated for a period from 1 to 4 hrs (commonly 2 hrs, see section 2.3.1) at 19°C in the dark (Dayeh et al., 2005c). The oxidized form of alamarBlue (resazurin) is a non fluorescent substrate blue in colour that when taken into cells, the dye becomes reduced and turns into a fluorescent pink/red colour (resorufin). The extent of this conversion reflects cell viability, which is quantified with fluorescence detection (Pagé et al., 1993; Nakayama et al., 1997). The fluorescence of alamarBlue was detected using excitation and emission filters of 540 and 590 nm, respectively, in a microplate reader (FLUOstar OPTIMA, BMG Labtech, 413-3350). The viability results are expressed as percentage of the readings compared to the controls (% of

control). The controls in all tests represent gill cells exposed to the same media that experimental gill cells were exposed to but without the toxic component (i.e. algae, fatty acids). Exposure time and handling was always the same for both control and gill cells exposed to the different experimental treatments.

2.3. Assay for testing toxicity of algal fatty acids using conventional multiwell microplates

2.3.1. Influence of gill cell seeding concentration and alamarBlue incubation period

2.3.1.1. Fluorescence signal upon exposure to L-15 with 5% alamarBlue

Since the literature reports different cell seeding concentrations in 96-well microplates and this also depends on the size of the cells, five concentrations of RTgill-W1 cells were tested to determine the influence in fluorescence signal, and ultimately how this can affect the final calculus of cell viability as compared to the control (% of control).

Flasks with confluent gill cells were treated with trypsin-EDTA solution for detachment, then cells were counted using a haemocytometer, adjusted to concentrations of 1, 1.5, 2, 2.5 and 3×10^5 cells mL^{-1} and seeded in quadruplicate in 96-well flat bottom microplates (3860-096, Iwaki, Japan) using a volume of 100 µL per well. This procedure was carried out 48 hrs before the experiments in order to achieve a confluent monolayer and better attachment of the cells to the bottom of the plate (Dayeh et al., 2005b). Cells were rinsed and new L-15 medium was added to each well, then incubated for periods of 1, 2, 3, 4 and 5 hrs. After each period of time, the L-15 was discarded and L-15/ex medium containing 5% alamarBlue was added to the wells. The microplates were incubated for 1 and 4 hrs to monitor fluorescence signal at these two different times using the same fluorometer gain value of 1000.

2.3.1.2. Viability (% of control) calculation after exposure to seawater-based media

This procedure was also carried out to test the effect of the seawater-based media f/2 (Andersen et al., 2005) and GSe (Blackburn et al., 1989) on the RTgill-W1 cells. These media are widely used for the culture of marine microalgae, and the main purpose was to determine if direct exposure experiments with algae in conventional microplates were possible. Gill cells were seeded as described above, rinsed with PBS and then exposed to seawater (35 of salinity), GSe and f/2 media for 6 hrs at intervals of 1 hr (using a different plate for each). Cell viability was measured at each period of time, allowing incubations of 1 and 4 hrs in 5% alamarBlue to find out whether there were any differences in the final viability calculation.

2.3.2. L-15/ex medium at different times with fetal bovine serum

Fetal bovine serum (FBS) offers protection to cell lines against reactive oxygen species as it contains hormones, proteins and enzymes for stability and detoxification (Schirmer et al., 1997; Gstraunthaler, 2003). Additionally, some components of the conventional L-15 medium (i.e. riboflavin, tryptophan, and tyrosine) produce toxic compounds when exposed to daylight fluorescent light (Wang and Nixon, 1978). For this reason, L-15/ex medium was tested at different concentrations of FBS in order to replace the basal medium L-15 when investigating the effect of algal extracts on the gill cells. L-15/ex medium was prepared with inorganic salts, galactose and sodium pyruvate (Schirmer et al., 1997) in Milli-Q water. The pH was adjusted to 7.6 and then filter-sterilized through a 0.2 μm membrane (4632, VacuCap Filter Unit, Pall Corporation). Different volumes of FBS were added in order to achieve final concentrations of 0, 1, 2, 4, and 8 %.

Gill cells were detached as specified in 2.3.1.1, then seeded at a concentration of 2×10^5 cells mL^{-1} in quadruplicate in 96-well flat bottom microplates using a volume of 100 μL per well. Thirty six hrs after seeding the cells, the L-15 medium was discarded, and replaced with L-15/ex without FBS. After a further 12 hrs, the

gill cells were washed with PBS and subjected to the L-15/ex at different FBS concentrations. Viability was assessed at 12-hr intervals for 96 hrs at 19°C in the dark. A different plate was used for each 12-hr viability measurement.

2.3.3. Effect of L-15/ex medium with methanol on cell viability

The gill cells were exposed to methanol (MeOH) concentrations of 1, 2, 4 and 12 % (final concentration) in L-15/ex without FBS because some algal extracts are lipophilic and need to be dissolved in organic solvents before being mixed with aqueous media. L-15/ex media at different MeOH concentrations were adjusted to a pH of 7.6, and then filter-sterilized as stated above. The gill cells were seeded as described in the previous step, then washed with PBS and exposed to the experimental media. The exposure was carried out for 60 hrs at 19°C in the dark. Viability was assessed at 12-hr intervals using different plates for each experimental condition and time.

2.3.4. Exposure to palmitic acid

Different ichthyotoxic microalgae, such as dinoflagellates and raphidophytes, produce large amounts of fatty acids, especially those with large carbon chains and double bonds, such as polyunsaturated fatty acids (PUFAs). However, palmitic acid (for chemical structure refer to chapter 6) has also been detected in some toxic phytoplankton species at high concentrations (Marshall et al., 2002b; Mooney et al., 2007; Giner et al., 2008). Commercial palmitic acid (P0500, Sigma, Australia) was dissolved in MeOH and mixed with L-15/ex with a final MeOH concentration of 1%. The gill cells were seeded as described in 2.3.2 and exposed to palmitic acid concentrations of 20, 50, 80, 110 and 140 mg L^{-1} for 60 hrs at 19°C in the dark. Viability was measured every 12 hrs.

2.4. Cytotoxicity assay with living marine algae using permeable inserts in multiwell microplates

2.4.1. L-15/ex different salinity resistance of RTgill-W1

In addition to studies of the viability of the gill cells in seawater-based media in conventional microplates, their viability was also tested using permeable support membranes (pore size 0.4 μm) at the base of microplate 96-well inserts (Fig. 2.1). The advantage of this arrangement is twofold: i) gill cells can be seeded in L-15/ex medium and subsequently exposed to organisms growing in overlying seawater-based media, and ii) contamination of the seawater medium by the underlying L-15/ex medium is minimized as a result of the limited diffusion between the two compartments (with the extent of diffusion decreased by increasing the salinity of the L-15/ex medium), and thus allowing the survival of gill cells by uptake of nutrients from the lower compartment containing L-15/ex medium through the microporous membrane. L-15/ex (normal salinity ~9) was tested at salinities of 15, 20, 25, 30 and 35, by adding varying amounts of NaCl (S9888, Sigma). The different media were adjusted to pH 7.6 and then filter-sterilized. The gill cells were trypsinized and adjusted to 9×10^4 cells mL^{-1} in L-15 medium. A volume of 180 μL of L-15 without cells was added to each well (lower compartment) of the 96-well microplate, the Transwell® Permeable Support (CLS3381, Sigma) then set up and 80 μL of the detached cells in L-15 added to each insert in quadruplicate. The cells were allowed to attach and achieve a confluent monolayer for 48 hrs, but the L-15 medium from both compartments was replaced by L-15/ex during the last 12 hrs. Following this 12-hr period, the wells and inserts containing the gill cells were washed with PBS, then 180 μL of L-15/ex at each salinity placed in each well of the receiver plate (lower compartment) and 80 μL of the same experimental media added to the insert (upper compartment). The viability of the cells was then measured for 6 hrs at 1-hr intervals.

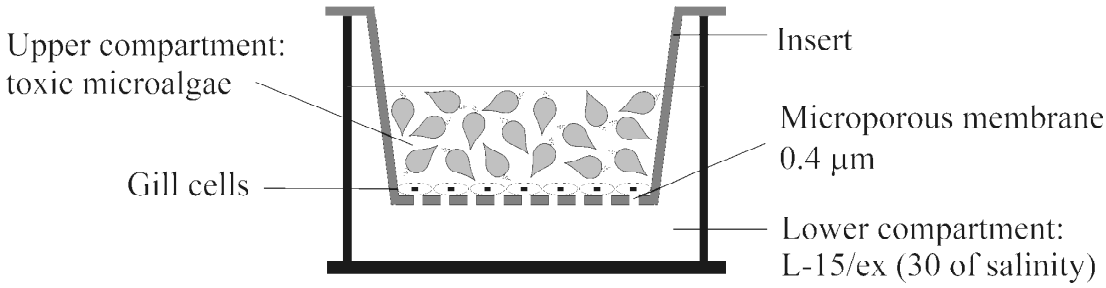

Figure 2.1. Transwell® Permeable Support for microplates. It is composed of an array of wells with permeable inserts connected by a rigid tray, so that all inserts can be handled as a single unit. Each insert of this unit is placed in a well of the microplate, producing two different compartments separated by a 0.4 µm pore size membrane. Gill cells are seeded on the membrane and remain attached since this membrane is tissue culture treated.

2.4.2. Resistance of RTgill-W1 to seawater

The gill cells were seeded and treated as specified previously. Once a confluent monolayer was achieved (48 hrs), the cells were washed with PBS, 180 µL of L-15/ex at 30 of salinity was added to each well of the 96-well microplate, then 80 µL of seawater, GSe or f/2 medium was added to each insert where the gill cells were attached. The gill cells were exposed to these seawater-based media for 6 hrs. Cell viability was assessed every hour using different plates for each exposure time.

2.4.3. Exposure to *Chattonella marina* and *Karlodinium veneficum*

The toxic microalgae *Chattonella marina* strain CMPL01 (originally collected from Port Lincoln, South Australia in April 1996 and isolated by J. Marshall) and *K. veneficum* strain KVSR01 (collected from the Swan River, Western Australia in March 2001 and isolated by M. de Salas) were obtained from the Harmful Algal Culture Collection of the University of Tasmania. Algae were grown in GSe medium at 35 of salinity, 19°C and at 150 µmol photons m^{-2} s^{-1} (cool white fluorescent lamps) under a 12/12-hr light/dark cycle. Two different algal suspensions, intact cells and ruptured cells, at five different concentrations ranging

from 7×10^2 to 7×10^6 cells L^{-1} for *C. marina*, and 1×10^4 to 1×10^8 cells L^{-1} for *K. veneficum*, were prepared from a culture in the exponential growth phase. The intact cell suspension was taken directly from the culture and diluted as needed. The ruptured cell suspension was prepared by sonication of the cultures for 1 min in a bath-type sonicator (Soniclean 120T, pulse swept power 60W) at 19°C.

The gill cells were seeded and treated as described in 2.4.1. Once the gill cells were washed with PBS, a volume of 180 µL of L-15/ex medium at 30 of salinity was added to each well of the 96-well microplate, and 80 µL of the different experimental algal concentrations and suspensions was added to the inserts where the gill cells were attached (Fig. 2.1). As a preliminary experiment, the exposure was carried out for 6 hrs and the viability of the cells was assessed every hour in order to determine the ideal time for the exposure. As the gill cells were 100% viable during the first 3 hrs, an exposure period of 2 hrs was subsequently used when conducting assays involving living algae. After adding the *C. marina* or *K. veneficum* aliquots, the gill cells were incubated at 19°C under 100 µmol photons m^{-2} s^{-1}. The gill cells were also exposed to the nontoxic green alga *Tetraselmis suecica*.

2.5. Scanning Electron Microscopy

Cells were seeded in 6-well conventional cell culture plates (Greiner bio-one, 657160) at 6×10^4 cells mL^{-1}, 48 hrs previous to the experiments. Gill cells were exposed to palmitic acid at a concentration of 140 mg L^{-1} for 60 hrs in the dark at 19°C. Another batch of cells was also seeded in 6-well plates with inserts (Corning, CLS3450) at 5×10^4 cells mL^{-1}, and exposed to *Chattonella marina* CMPL01 at 7×10^6 cells L^{-1} for 2 hrs under 100 µmol photons m^{-2} s^{-1} at 19°C. Cells were scraped off from the wells and inserts with a cell scraper (Corning, CLS3010) and centrifuged at 700 × g for 3 min. The supernatant was discarded and the cell pellet resuspended in Karnovsky's fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde. The cells were fixed for 4 hrs, then washed with PBS and stored at 4°C in PBS until processed by electron microscopy.

Cells were post-fixed with 4% osmium tetroxide for 30 min, and dehydrated in a graduated series of ethanol, including 100% dry acetone as final step. Critical point drying with CO_2 was carried out using Balzers CPD 020. Coating was performed under a high resolution platinum palladium coater (Cressington 208HRD) with a thickness of 5 nm. Samples were analyzed under an electron microscope JEOL JSM-6701F.

2.6. Data analysis

The data were subjected to analysis of variance (ANOVA). Normality of the data was tested with the Kolmogorov-Smirnov method and homogeneity of variances was assessed with Hartley's F_{max} tests. When multiple comparisons were required, the *a posteriori* Tukey test was performed in order to determine any difference among treatments. The significance level considered in all statistical tests was 95% (α=0.05). The software Statistica 8.0 was used for all statistical analyses.

3. Results

3.1. Assays in conventional microplates

3.1.1. Influence of gill cell seeding concentration and alamarBlue incubation time

3.1.1.1. Fluorescence signal after exposure to L-15

Fluorescence increased with gill cell seeding concentration, and also with incubation time in 5% alamarBlue (aB, 1 and 4 hrs). There was a slight but not statistically significant increase in the signal when comparing the incubation times in L-15 (1, 2 and 4 hrs) after reading at either 1 or 4 hrs in alamarBlue. Linear regressions show a good adjustment and so do coefficients of determination (R^2 ranged between 0.9731 and 0.9868) (Fig. 2.2), which coincides with the information provided by the supplier (DAL1025, Invitrogen). However, this information by the

supplier is only applicable to mammalian cells, incubated at 37°C whereas gill cells were incubated at 19°C, and uptake of alamarBlue is influenced by temperature.

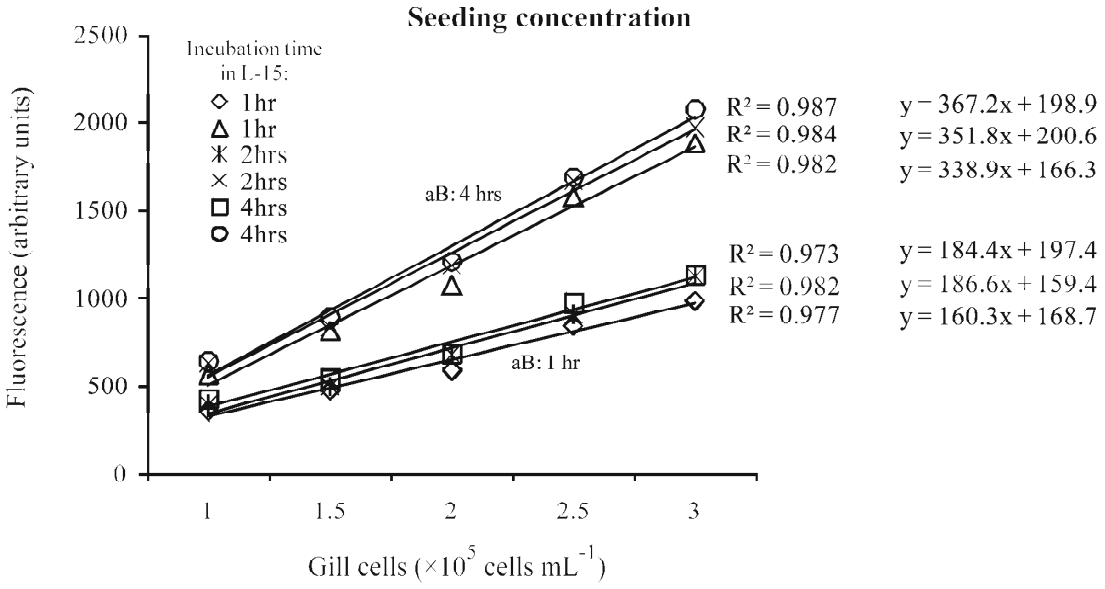

Figure 2.2. Fluorescence signal influenced by gill cell seeding concentration. Diamonds, asterisks and squares represent incubation times in L-15 medium for 1, 2 and 4 hrs, respectively, and fluorescence readings after 1 hr of incubation in 5% alamarBlue (aB). Triangles, crosses and circles represent incubation times in L-15 medium for 1, 2 and 4 hrs, respectively, and fluorescence readings after 4 hrs of incubation in 5% alamarBlue. Lines represent linear regressions for each treatment showing the coefficient of determination (R^2) and equation for each.

3.1.1.2. Viability after exposure to seawater-based media

Apart from the correlation between gill cell concentration and fluorescence intensity, it was necessary to calculate the final viability from these fluorescence values in order to detect any differences and therefore find out the best incubation time in alamarBlue. Figure 2.3 shows that GSe medium affected gill cells over time using conventional microplates, but still these results were useful to calculate viability from fluorescence. Viability calculations did not show any significant differences when comparing the incubation times in alamarBlue of 1 and 4 hrs, and they were also independent from gill cell concentration. Therefore, a seeding

concentration of 2×10^5 cells mL^{-1} was chosen for all experiments in conventional microplates, and 9×10^4 cells mL^{-1} for the inserts with membrane supports since they have a smaller area. Additionally, in order to save time, an incubation period of 2 hrs in 5% alamarBlue was commonly used for viability assessments, although according to our results fluorescence reading can be made anytime between 1 and 4 hrs of incubation in 5% alamarBlue.

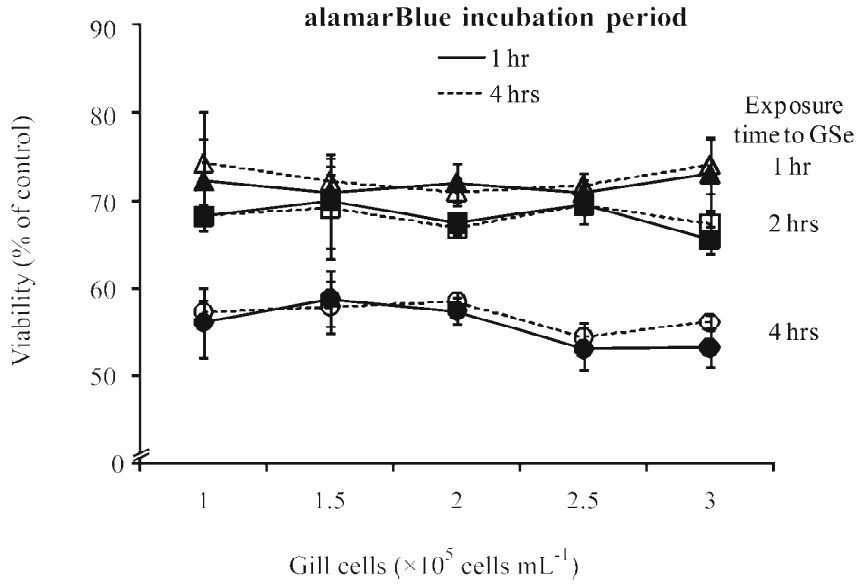

Figure 2.3. Comparison of gill cell viabilities after exposure to GSe medium using five gill cell concentrations and different alamarBlue incubation times. Open and closed triangles represent an exposure time to GSe of 1 hr, open and closed squares represent an exposure of 2 hrs, and open and closed circles represent an exposure of 4 hrs. Solid and dashed lines represent 1-hr and 4-hr incubation times, respectively, to 5% alamarBlue.

3.1.2. Effect of varying concentrations of fetal bovine serum on gill cells

RTgill-W1 cells remained 100% viable after 96 hrs when exposed to 8% FBS. However, gill cell viability decreased after 84 hrs of exposure to 4% FBS, with 10% loss of viability at 96 hrs. The cells were 100% viable during the first 72 hrs after exposure to FBS concentrations of 0, 1 and 2%, after which viability decreased significantly by 30, 27 and 15%, respectively, at 96 hrs (Fig. 2.4). These results

demonstrate that RTgill-W1 have an obligatory requirement for FBS, but are able to retain 100% viability for up to 72 hrs even in the complete absence of FBS.

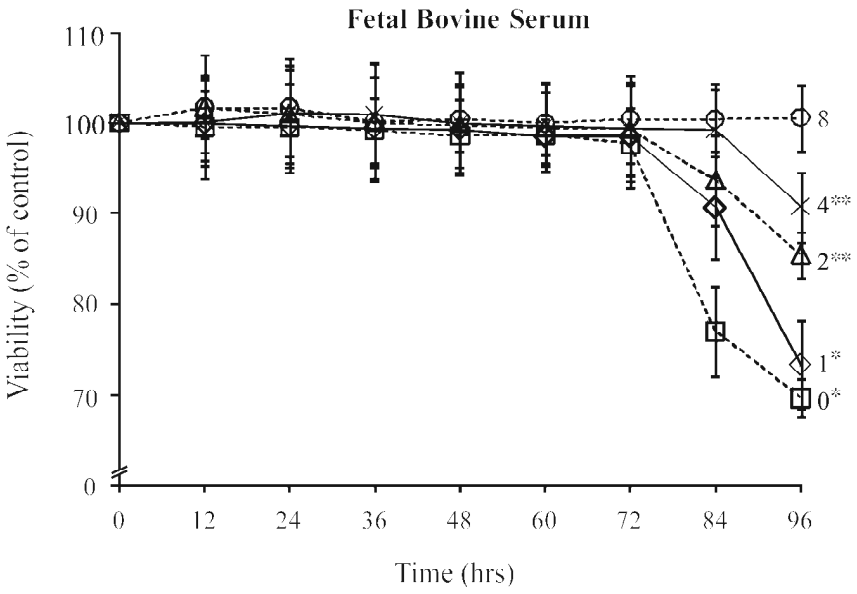

Figure 2.4. Effect of fetal bovine serum (FBS) concentration on the viability of the RTgill-W1 cells. Symbols represent the mean and error bars the standard deviation of cell viability from quadruplicate incubations containing 0% (squares with dashed line), 1% (diamonds with solid line), 2% (triangles with dashed line), 4% (crosses with solid line) and 8% (circles with dashed line) fetal bovine serum. Asterisks show significant differences among the treatments at 96 hrs.

3.1.3. Effect of methanol on cell viability

Methanol (MeOH) had no effect on gill cell viability at a concentration of 1% during the 60-hr exposure period. Similarly, there was no significant difference in viability at 2% MeOH during the first 36 hrs, but a 10% loss of viability was observed at 48 and 60 hrs at this same concentration. Gill cells remained 100% viable during the first 12 hrs of exposure to 4% MeOH, but suffered a decrease in viability of about 8% after 24 hrs, while viability was affected after only 12 hrs when exposed to 12% MeOH. The loss of viability at 12% MeOH was time dependent, showing the highest loss (23%) at the end of the exposure (Fig. 2.5). These findings

together with the concentration of FBS at which gill cells can remain viable, tell us that exposure of RTgill-W1 cells to fatty acids can be carried out using L-15/ex without FBS and 1% MeOH (containing the fatty acid) for a period no longer than 60 hrs.

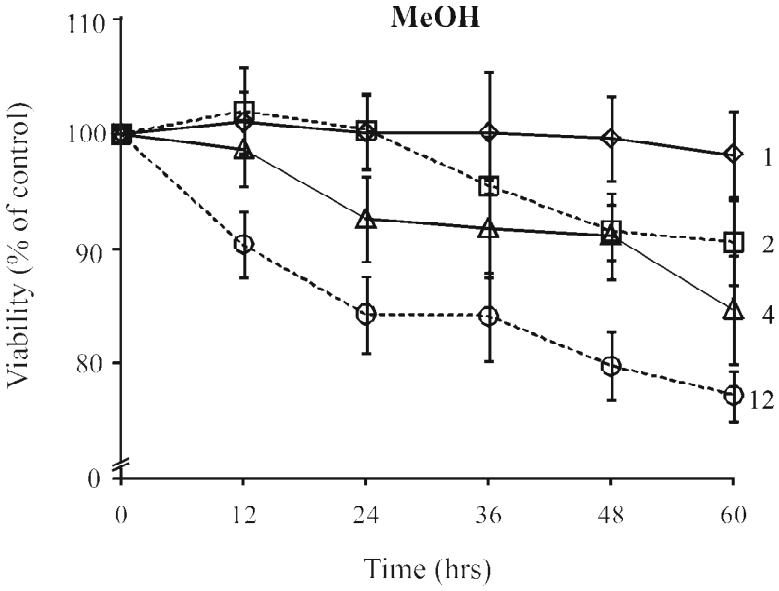

Figure 2.5. Viability of the gill cells after exposure to four concentrations of methanol (MeOH). Symbols represent the mean and error bars the standard deviation of cell viability from quadruplicate incubations containing 1% (diamonds with solid line), 2% (squares with dashed line), 4% (triangles with solid line) and 12% (circles with dashed line) methanol.

3.1.4. Exposure to palmitic acid

Palmitic acid had a comparable effect on the viability of the gill cells at all concentrations examined (20 to 140 mg L^{-1}) during the first 12 hrs, exhibiting a decrease of viability of ~10%. The gill cell viability ranged from 63 to 68% at 20 – 110 mg L^{-1} palmitic acid after 60 hrs, but the difference between the treatments was not statistically significant. However, the cells did show a significant and gradual loss of viability when exposed to 140 mg L^{-1} palmitic acid, with a viability of <50% resulting after 48 and 60 hrs of exposure (Fig. 2.6). Gill cells suffered membrane

damage after exposure to palmitic acid. Microvilli-like protrusions were almost completely lost, and loss of membrane in parts of the cell was evident (Fig. 2.10).

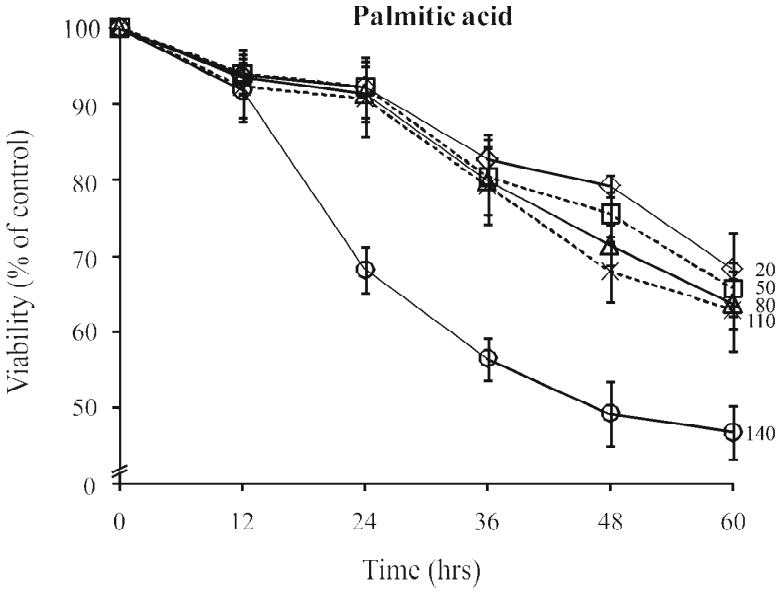

Figure 2.6. Effect of varying concentrations of palmitic acid on gill cell viability. Symbols represent the mean and error bars the standard deviation of cell viability from quadruplicate incubations containing 20 mg L^{-1} (diamonds with solid line), 50 mg L^{-1} (squares with dashed line), 80 mg L^{-1} (triangles with solid line), 110 mg L^{-1} (crosses with dashed line) and 140 mg L^{-1} (circles with solid line) palmitic acid.

3.2. Assays in microplates with inserts

3.2.1. Influence of L-15/ex at different salinities

Gill cells showed a wide tolerance to salinity in L-15/ex, with no significant loss of viability during the first 3 hrs of exposure. However, cell viability decreased to 84% at 35 of salinity after 6 hrs of exposure. A change in salinity in the L-15/ex medium from 9 (basal salinity) to 15, 20, 25 or 30 resulted in no statistically significant loss of viability of the gill cells after 6 hrs (Fig. 2.7).

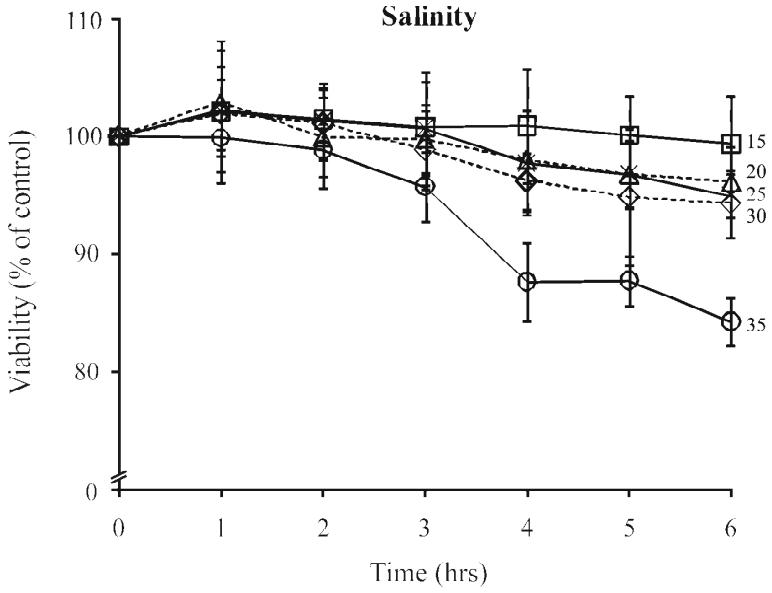

Figure 2.7. Influence of salinity on the viability of gill cells. Symbols represent the mean and error bars the standard deviation of cell viability from quadruplicate incubations containing 15 (squares with solid line), 20 (triangles with dashed line), 25 (crosses with solid line), 30 (diamonds with dashed line) and 35 (circles with solid line) of salinity.

3.2.2. Viability after exposure to seawater culture media

The viability of gill cells after exposure to f/2 and GSe media was similar, and was comparable to their viability as when exposed to L-15/ex at 35 of salinity. Gill cells did not exhibit any significant loss of viability during the first 3 hrs of exposure, but significant decreases were observed after 4 hrs, with only 73, 78 and 82% viability remaining after 6 hrs in gill cells exposed to seawater, f/2 and GSe media, respectively. At the end of the experiment, the highest and most statistically significant loss of viability of 27% occurred in gill cells exposed to seawater (Fig. 2.8).

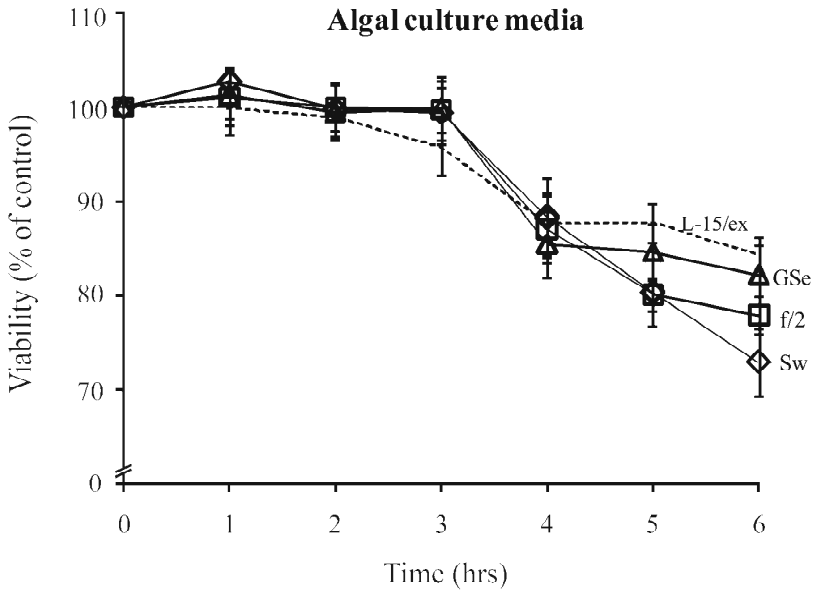

Figure 2.8. Tolerance of gill cells to algal culture media f/2 and GSe, natural seawater (Sw), and L-15/ex medium at 35 of salinity. Symbols represent the mean and error bars the standard deviation of cell viability from quadruplicate incubations in L-15/ex (horizontal bars with dashed line), GSe (triangles with solid line), f/2 (squares with solid line) and natural seawater (diamonds with solid line).

3.2.3. Exposure to *Chattonella marina* and *Karlodinium veneficum* cultures

Chattonella marina exhibited significant toxicity toward gill cells, as expressed by a decrease in viability of 22 and 16% after exposure to either intact or ruptured cells, respectively, at an initial algal concentration of 7×10^2 cells L^{-1}. Similar non-significant losses in viability were observed after exposure to 7×10^3 cells L^{-1} for both the intact and ruptured algal cells, with viability further decreasing in a concentration dependent manner. A 55% loss of viability was registered after 2 hrs of exposure to the highest concentration of the intact cell suspension, while the ruptured cell suspension showed still higher toxicity, with viability loss of 71% (Fig. 2.9a). In contrast, the dinoflagellate *K. veneficum* exhibited lesser toxicity towards the RTgill-W1 cells than *C. marina*. However, similar to *C. marina*, the intact cell cultures were less toxic than the ruptured cell preparations. The highest losses of viability observed in the gill cells were 24 and 38% for the intact and ruptured *Karlodinium veneficum* cells, respectively (Fig. 2.9b).

No loss of viability was observed when gill cells were exposed to the nontoxic green alga *Tetraselmis suecica* (Fig. 2.9c). Similar to the effects observed after exposure to palmitic acid, gill cells also suffered loss of membrane integrity when exposed to *C. marina* (Fig. 2.10).

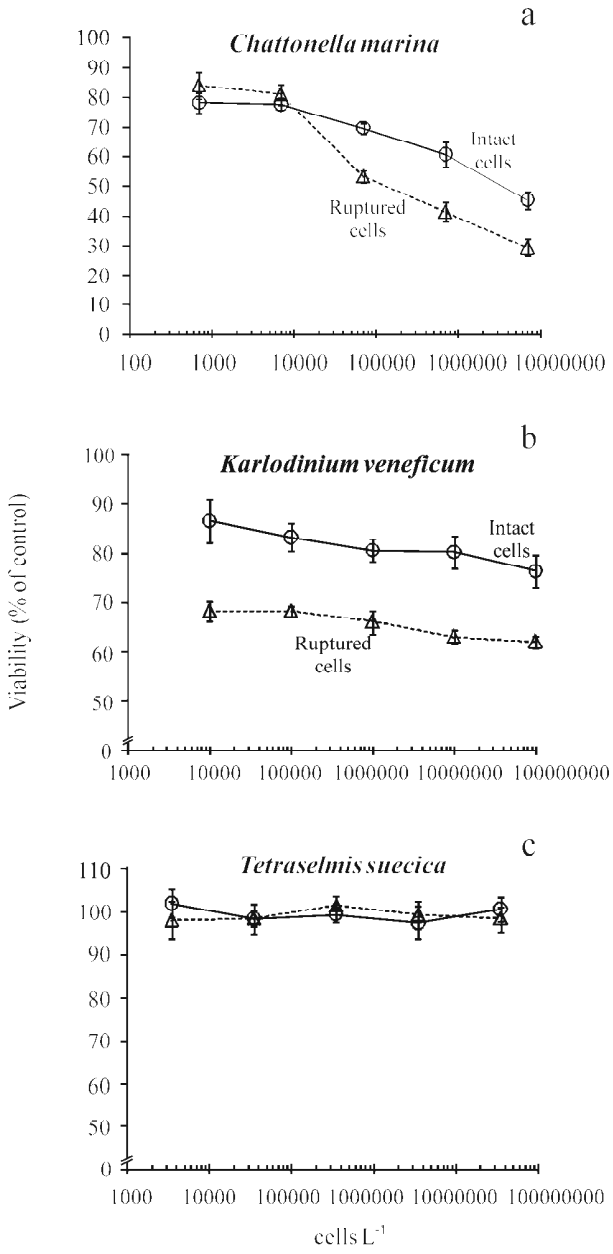

Figure 2.9. Concentration dependent effect of intact (circles with solid line) and ruptured (triangles with dashed line) *Chattonella marina* (a), *Karlodinium veneficum* (b) and *Tetraselmis suecica* (c) cells towards the cell line RTgill-W1. Symbols represent the mean and error bars the standard deviation of cell viability from quadruplicate incubations.

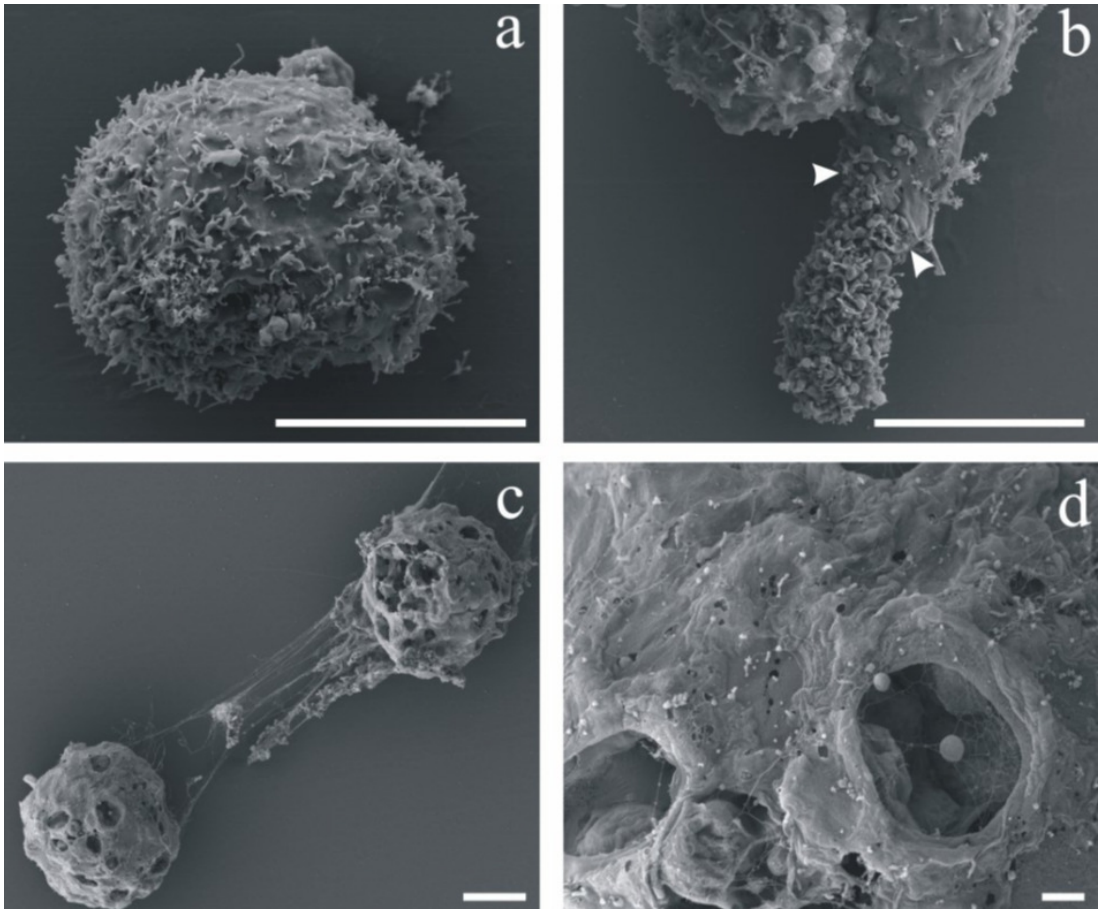

Figure 2.10. SEM micrographs of fish gill cells. RTgill-W1 cells were exposed to: a) L-15/ex with 1% methanol representing the control (control in GSe showed no damages either); b) 140 mg L^{-1} palmitic acid, arrows indicate the area where membrane damage occurred, showing a complete loss in that part of the gill cell; c) and d) exposed to ruptured cells of *Chattonella marina* CMPL01 at 7×10^{6} cells L^{-1}. Scale bars for a, b, c: 10 μm; d: 1 μm.

3.3. Recommendations for assay procedures

Table 2.1 summarizes our test results and outlines recommended assay procedures for: a) algal extracts, such as fatty acids, and b) marine phytoplankton cells.

Table 2.1. Protocol for assessment of toxicity of harmful marine phytoplankton to the fish gill cell line RTgill-W1.

Procedure	**Fatty acids**	**Living algae**
1) Microplate type	96-well flat bottom conventional microplate	96-well microplate with Transwell® Permeable Support, pore size 0.4 μm
2) Gill cell seeding concentration (cells mL^{-1})	2×10^5	9×10^4
3) Gill cell seeding volume (μL)	100	180 in lower compartment (only L-15); 80 in upper compartment (L-15 with gill cells)
4) Time for replacement of L-15 by L-15/ex without FBS	36 hrs after seeding (or 12 hrs before the experiments)	
5) Rinse with Phosphate Buffer Saline		
6) Volume of media (μL)	100 of L-15/ex without FBS	180 in lower compartment (L-15/ex without FBS at 30 of salinity); 80 in upper compartment of f/2 or GSe with algae
7) Maximum time of exposure	60 hrs if using only L-15/ex without FBS; 60 hrs for 1% MeOH; 36 hrs for 2% MeOH; 12 hrs for 4% MeOH; All in darkness	2 hrs at 19°C under 100 μmol photons m^{-2} s^{-1} light
8) Rinse with Phosphate Buffer Saline		
9) Media for viability assay	5% alamarBlue in L-15/ex without FBS	
10) Conditions for viability assay	2-hr incubation period in the dark at 19°C Measure alamarBlue fluorescence: 540 – 570 nm excitation and 580 – 610 nm emission filters	

The control treatments contain exactly the same as the experimental treatments except for the toxic component. For example, for exposure to fatty acids, gill cells are exposed to L-15/ex without FBS and with MeOH (1, 2 or 4%) (no fatty acids); for exposure to living algae, the lower compartment contains L15/ex without FBS at 30 of salinity and in the upper compartment only f/2 or GSe (no algae).

4. Discussion

It was demonstrated that bioassays with the gill cell line RTgill-W1 can provide a powerful tool both for the assessment of chemical toxicants as well as impact from harmful microalgae. Testing the effect of algal extracts, such as palmitic acid, was achieved in conventional microplates, whereas testing whole algal cells involved the use of microplates with inserts containing a membrane support. Algal extracts can be dissolved in either a modified version of the gill cell basal medium L-15/ex if they are hydrophylic, or in organic solvents mixed with the L-15/ex medium if they are lipophilic. Exposures as long as 60 hrs for testing toxicity under these conditions were successfully achieved. In contrast, exposure of the gill cells to living algae had to be carried out in a seawater-based medium. As the RTgill-W1 cells were not resistant to a short-term seawater exposure in conventional microplates, use of the membrane support with inserts was necessary to achieve survival for at least 3 hrs with a lower compartment containing L-15/ex medium and the upper compartment holding the algae in seawater medium (Fig. 2.1).

Two step-by-step detailed protocols for conducting routine culturing, maintenance, toxicity tests (using individual chemicals) and viability assays with the cell line RTgill-W1 were previously published by Ganassin et al. (2000) and Dayeh et al. (2005c). The present study provides a modified protocol for toxicity assays of algal extracts and living marine algae. A previous study showed the lack of tolerance of the mammalian cell lines HeLa, XC, CHO, L929 and MDCK to the seawater-based medium ESM in conventional microplates. However, the cell line Vero (African green monkey kidney) was able to survive for up to 3 hrs, allowing this assay to be used for the assessment of the toxic activity of the dinoflagellates *Heterocapsa circularisquama*, *Alexandrium fraterculus, A. tamiyavanichii, A. tamarense* and *A. affine*. Additionally, Vero and mouse neuroblastoma cell lines were used for the detection of cytotoxic substances produced by *Alexandrium tamarense* (Katsuo et al., 2007). An inconvenience of working with mammalian cell lines is that they must be incubated and maintained at 37ºC in a 5%-CO_2 atmosphere. By contrast, fish cell lines can usually be maintained between 18 and 22ºC in a normal atmosphere (Dayeh et al., 2005c), which is compatible with the temperatures

for growth of a wide range of toxic phytoplankton. The present study offers the advantage of using a fish cell line, representing a more ecologically realistic approach for ichthyotoxic evaluation of harmful phytoplankton.

The use of transwell membrane chambers for cytotoxicity assays using the RTgill-W1 cells had previously demonstrated the high tolerance of this cell line not only to hyper-osmotic but also to hypo-osmotic conditions (Lee et al., 2009). However, the use of different seawater algal culture media called for modifications since they are enriched with different components compared to L-15 medium; in particular, microalgae need to be supplemented with metals and compounds such as silica and selenium not present in L-15. Although some metals contained in f/2 and GSe media, such as iron, zinc and copper, are present in much lower concentrations than levels reported to be toxic for RTgill-W1 cells (Dayeh et al., 2005b), the lack of essential aminoacids and other nutrients might be the reason why the gill cells did not survive in conventional microplates. Diffusion of L-15/ex from the lower compartment of the microplate well through the membrane where the gill cells were seeded and remained attached was crucial for them to survive for a short period of time (3 hrs) when using the support with inserts.

A 2-hr exposure of the gill cells to *Chattonella marina* and *Karlodinium veneficum* was adequate to demonstrate acute impact on the gill cells. The two different algal culture preparations (intact cells and ruptured cells) showed a different impact on the viability of the gill cells, with the sonicated preparation exhibiting the highest toxicity for both species.

These results show that *C. marina* is toxic in a concentration-dependent manner, with an initial harmful effect at a concentration as low as 7×10^2 cells L^{-1}. The exposure was carried out during the exponential growth phase, and because the sonicated suspension showed a higher toxicity towards the gill cells, it appears that there is release of toxic compounds into the medium. The same happened with *K. veneficum*, which has been previously demonstrated to produce karlotoxins that impact fish gills (Deeds et al., 2006; Mooney et al., 2010). However, since *C. marina* does not seem to produce any distinct chemical toxins (strains used in this thesis were all negative for brevetoxins) and this species was more toxic to gill cells than *K.*

veneficum, reactive oxygen species (ROS) and fatty acids, produced in high amounts by *C. marina* (Marshall et al., 2005) may well have a greater impact on RTgill-W1 cells than karlotoxins. Additionally, *K. veneficum* also produces ROS (although not as much as *C. marina*) and fatty acids (Marshall et al., 2005; Mooney et al., 2007) which may form part of their fish killing mechanism.

Placing the fish gill cell line in context, if the experiments had been carried out using whole fish, five aquariums would have been required for each algal suspension, plus one for the control group. Furthermore whole fish assays tend to use small samples (5 – 7 fish per treatment) with no replicates. By contrast, the gill cell assay for *C. marina* required one 96-well microplate with inserts and one 25-cm^2 flask with a confluent monolayer of RTgill-W1 to be detached and seeded in the inserts. Comparing both assays, a major saving of time and facilities was achieved when using *in vitro* rather than *in vivo* assays. The cell line assay not only has a high sensitivity, it also possesses very good precision, with errors of 1.5 – 6%.

There are some issues to consider when applying this *in vitro* assay. First, the user needs to have the skills to work with microscopic cells in order to perform the cell counts properly, as well as using multichannel pipettes for media handling to assure that the same volumes and cell concentrations are pipetted to all the wells of the microplate. Finally, once the exposure period has been accomplished, the user needs to make sure that no algal cells become stuck in the microporous membrane of the inserts when rinsing all wells to remove the experimental media.

It has been suggested that sensitivity of fish cell lines tends to be lower than that of intact fish (Segner, 1998b). However, a published work (Mooney et al., 2011, part of chapter 6) has demonstrated that the fish cell line RTgill-W1 was more sensitive than the larvae of sheepshead minnow (*Cyprinodon variegans*) upon exposure to fatty acids produced by toxic dinoflagellates. No whole fish mortalities were observed after exposure to the fatty acids, although they were apparently affected, showing erratic swimming and gulping at the surface. In contrast, the gill cells exhibited a significant decrease in viability after exposure to the same fatty acids.

In conclusion, two assays that represent a valuable alternative to whole fish experimentation were developed. They save time, money, laboratory facilities, and ethics approval procedures. These assays are easy, reproducible, and sensitive. Additionally, they are a good tool for the assessment of the toxicity of algal extracts and living red tide phytoplankton. Applying *in vitro* techniques, such as using the fish gill cell line RTgill-W1, provides a new and better approach for the study of ichthyotoxic phytoplankton, allowing for the assessment of cellular toxicity and screening for specific toxic mechanisms.

Chapter 3

Toxicity of different geographic strains of *Chattonella marina* towards gill cells

Abstract

Toxicity of six different geographic strains of *Chattonella marina* was assessed using the fish gill cell line RTgill-W1. Three different preparations of *C. marina* at varying concentrations were used: intact cells, cell-free supernatant, and ruptured cells. Ruptured cells of concentrated cultures ($\geq 7\times10^5$ cells L^{-1}) of the Australian strain CMPL01 were the most toxic, together with the Japanese strain N-118, with losses of gill cell viabilities of 65 – 71%. *Chattonella marina* was also toxic under normal culture conditions (intact cell cultures), sometimes as toxic as ruptured cell preparations. The Mexican strain CMCV-1 was always the least toxic at high concentrations in all three preparations. Highest toxicity of this strain was found in intact and ruptured cell cultures, where gill cells lost viability by 35%. Physical damage observed by Scanning Electron Microscopy revealed membrane disruption on gill cells upon exposure to *C. marina*. This raphidophyte was toxic under no stress conditions (intact cell preparation) and when the supernatant of centrifuged cultures was tested. Additionally, its toxicity increased after cell rupture, suggesting that this raphidophyte possesses certain compounds with cytotoxic properties that are released upon cell lysis and increased its toxicity.

1. Introduction

The term "strain" applied to the study of phytoplankton refers to a unialgal culture of defined origin which is kept as a distinct, discrete lineage by serial transfer or continual culture (Lakeman et al., 2009). The use of these microalgal isolates that are kept in control conditions for optimum growth and continual supply represent a great advantage when studying particular issues, especially when aiming to determine any possible differences between strains of the same species. Interstrain differences may be reflected in growth capacity, cell size, genetics, tolerance to varying physicochemical parameters such as temperature, salinity and irradiance, toxin production and composition, and toxicity as commonly assessed with the use of bioassays.

It has been discussed that strains can undergo changes when kept in laboratory conditions. For instance, it was found that the dinoflagellate *Alexandrium minutum* (=*lusitanicum*) lost its capacity to produce saxitoxins after being maintained in continual culture for more than 30 years (Martins et al., 2004). In natural conditions algal blooms are not always toxic due to suspected variable environmental factors that control expression of toxicity, as usually happens with the raphidophyte *Heterosigma akashiwo* (O'Halloran et al., 2006). Similar results have been found in laboratory conditions, when different strains of this raphidophyte showed varying or zero toxicity (Fredrickson et al., 2011) and differing growth capacity due to environmental factors (Martínez et al., 2010). Different geographic strains of the dinoflagellate *Karlodinium veneficum* have been shown to contain different types of karlotoxins, either KmTx1 or KmTx2, as well as different content and potency (Bachvaroff et al., 2009; Mooney et al., 2009).

Differences among *Chattonella marina* strains have been found when studying their lipid profiles, salinity, temperature and irradiance tolerance, pigments, and production of superoxide anion (Marshall and Hallegraeff, 1999; Marshall and Newman, 2002; Marshall et al., 2002b; 2005). But varying toxicity among different strains has not been documented in the literature.

The present chapter aimed to prove toxicity of six different geographic strains of *Chattonella marina* under three different preparation conditions towards the gill cell line RTgill-W1.

2. Methods

2.1. Cell line culturing and viability assays

Maintenance and culturing of RTgill-W1 cells was performed as described in chapter 2, section. 2.1. Additionally, cell viability was assessed as indicated in section 2.2, chapter 2.

2.2. Stock cultures of *Chattonella marina*

Different non-axenic clonal strains of the raphidophyte *Chattonella marina* were used in the present study. Four Australian strains were obtained from the Harmful Algal Culture Collection of the University of Tasmania, a Japanese strain was obtained from the National Institute of Environmental Studies (NIES), Japan, and a Mexican strain was obtained from the Marine Dinoflagellate Strain Collection of The Northwestern Centre for Biological Research, Mexico (Table 3.1). The cultures were grown in GSe medium at 35 of salinity, 19°C and 150 µmol photons m^{-2} s^{-1} (cool white fluorescent lamps) under a 12/12-hr light/dark cycle.

Table 3.1. Details of *Chattonella marina* strains used in the present study. Four Australian, one Japanese and one Mexican strain were investigated focusing on their toxicity toward the fish cell line RTgill-W1.

Strain code	Origin	Isolator	Year of isolation	Source
CMCV-1	Bahia Concepcion, B.C.S., Mex.	C. Band-Schmidt	2000	The Northwestern Centre for Biological Research, B.C.S., Mex.
CMPL01	Port Lincoln, S.A., Aus.	J. A. Marshall	1996	University of Tasmania, Aus.
CMPB	Parson Bay, Tas., Aus.	M. de Salas	2003	University of Tasmania, Aus.
CMDE	Derwent River, Tas., Aus.	M. de Salas	2002	University of Tasmania, Aus.
CMPL04	Port Lincoln, S.A., Aus.	M. de Salas	2001	University of Tasmania, Aus.
N-118	Seto Inland Sea, Japan	S. Yoshimatsu	1983	National Institute of Environmental Studies, Japan

2.3. Preparation of algal suspensions

Three different suspensions of the algal cultures in GSe were assessed for the toxicity exposure: 1) Intact cells, 2) Cell-free supernatant and 3) Ruptured cell preparation. The intact cell suspension was taken directly from the cultures containing living algae; the cell-free medium or supernatant was prepared by gentle agitation of the culture, followed by centrifugation at 3000 × g for 1 min at room temperature (Kim et al., 2001). And finally, the ruptured cell suspension was prepared by sonication of the cultures for 1 min in a bath-type sonicator (Soniclean 120T, pulse swept power 60W) at 19°C. Each cell suspension was prepared at five different logarithmic concentrations, ranging from 7×10^2 to 7×10^6 cells L^{-1}, and sampled during the exponential growth phase.

2.4. Exposure of gill cells to *Chattonella marina*

Gill cells were trypsinized and adjusted to 9×10^4 cells mL^{-1} in L-15 medium. A volume of 180 µL of L-15 without cells was added to each well (lower compartment) of the 96-well microplate, then the Transwell® Permeable Support (CLS3381, Sigma) was set up and 80 µL of the detached cell suspension in L-15 was added to each insert by quadruplicate (upper compartment). Cells were allowed to attach and achieve a confluent monolayer for 48 hrs, but the L-15 medium from both compartments was replaced by L-15/ex in the last 12 hrs. Wells and inserts with attached cells were washed with PBS, each well of the receiver plate (lower compartment) was rinsed and filled up with 180 µL of L-15/ex at 30 of salinity. The membrane support with inserts was set up and 80 µL of the different experimental algal suspensions was added to the inserts where the cells were attached (Dorantes-Aranda et al., 2011). The gill cells were incubated at 19°C under 100 µmol photons m^{-2} s^{-1} for 2 hrs, and then viability of the gill cells was measured.

2.5. Scanning and Transmission Electron Microscopy

Gill cells were seeded in 6-well plates with inserts (Corning, CLS3450) at 5×10^4 cells mL^{-1}, and exposed to *Chattonella marina* at 7×10^6 cells L^{-1} for 2 hrs under 100 µmol photons m^{-2} s^{-1} at 19°C. Cells were scraped off from the inserts (*C. marina*) with a cell scraper (Corning, CLS3010) and centrifuged at 700 × g for 3 min. The supernatant was discarded and the cell pellet resuspended in Karnovsky's fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde. Cells were fixed for 4 hrs, then washed with PBS and stored at 4°C in PBS until processed by electron microscopy.

Cells were post-fixed with 4% osmium tetroxide for 30 min, and dehydrated in a graduated series of ethanol, including 100% dry acetone as final step. For SEM, critical point drying with CO_2 was carried out using Balzers CPD 020. Coating was performed under a high resolution platinum palladium coater (Cressington 208HRD) with a thickness of 5 nm. Samples were analyzed under an electron microscope JEOL JSM-6701F. For TEM, gill cells were fixed in agar and resin, then sectioned

with a diamond knife (acknowledgements to Jenny Penschow for performing this sectioning). Samples were stained in uranyl acetate for 5 min and then in lead citrate for 10 min. Samples were analyzed under a TEM microscope Philips CM100 operating with a LaB6 filament and imaging at 80 kV.

2.6. Data analysis

Data were subjected to analysis of variance (ANOVA). Normality of the data was tested with the Kolmogorov-Smirnov method and the homogeneity of variances was assessed with Hartley's F_{max} tests. When multiple comparisons were required, the *a posteriori* Tukey test was performed in order to determine differences among treatments. The significance level considered in all statistical tests was 95% (α=0.05). The software Statistica 8.0 was used for these statistical analyses.

3. Results

3.1. Toxicity of *Chattonella marina*

Chattonella marina exhibited different toxic effects among the six strains tested. The Mexican strain CMCV-1 had a similar toxic effect at the five different algal concentrations of each suspension. Viability of gill cells at the two lowest concentrations of 7×10^2 and 7×10^3 cells L^{-1} was not significantly different (α=0.05) when comparing either the intact cell, cell-free supernatant or ruptured cell suspension. However, gill cell viability decreased after exposure to 7×10^4 cells L^{-1}, and then it remained the same at higher concentrations. Nevertheless, the cell-free supernatant showed the lowest toxic effect compared to the intact cells and ruptured cell suspension at 7×10^5 and 7×10^6 cells L^{-1}, with gill cell losses of viability of up to 26% when exposed to the cell-free supernatant suspension and 35% corresponding to the intact and ruptured cell preparations (Fig. 3.1a). Additionally, among all ruptured cell preparations of *C. marina* strains, CMCV-1 showed the lowest toxicity at the two highest algal concentrations (Fig. 3.3b).

Similar to Mexican strain, the Japanese *C. marina* N-118 showed the highest toxicity when exposing the gill cells to the intact cell and ruptured cell suspensions, with no significant differences in gill cell viability between these two preparations. Gill cells showed loss of viability after exposure to different algal concentrations of N-118. However, this gradual loss of viability was not as high as observed in other *C. marina* strains. For instance, gill cells viability decreased from 66.6 to 50.3% after exposure to the cell-free supernatant from a culture with 7×10^2 and 7×10^6 cells L^{-1}, or from 54.5 to 35.1%, respectively, of the ruptured cell suspension (Fig. 3.1b). This Japanese strain however, showed the highest toxicity among all strains at the two lowest algal concentrations of 7×10^2 and 7×10^3 cells L^{-1}, with losses of viability of 45.5 and 51%, respectively. Thus, Japanese N-118 was the most toxic at low concentrations of the ruptured cell preparation and at all concentrations of intact cell cultures (Fig. 3.3).

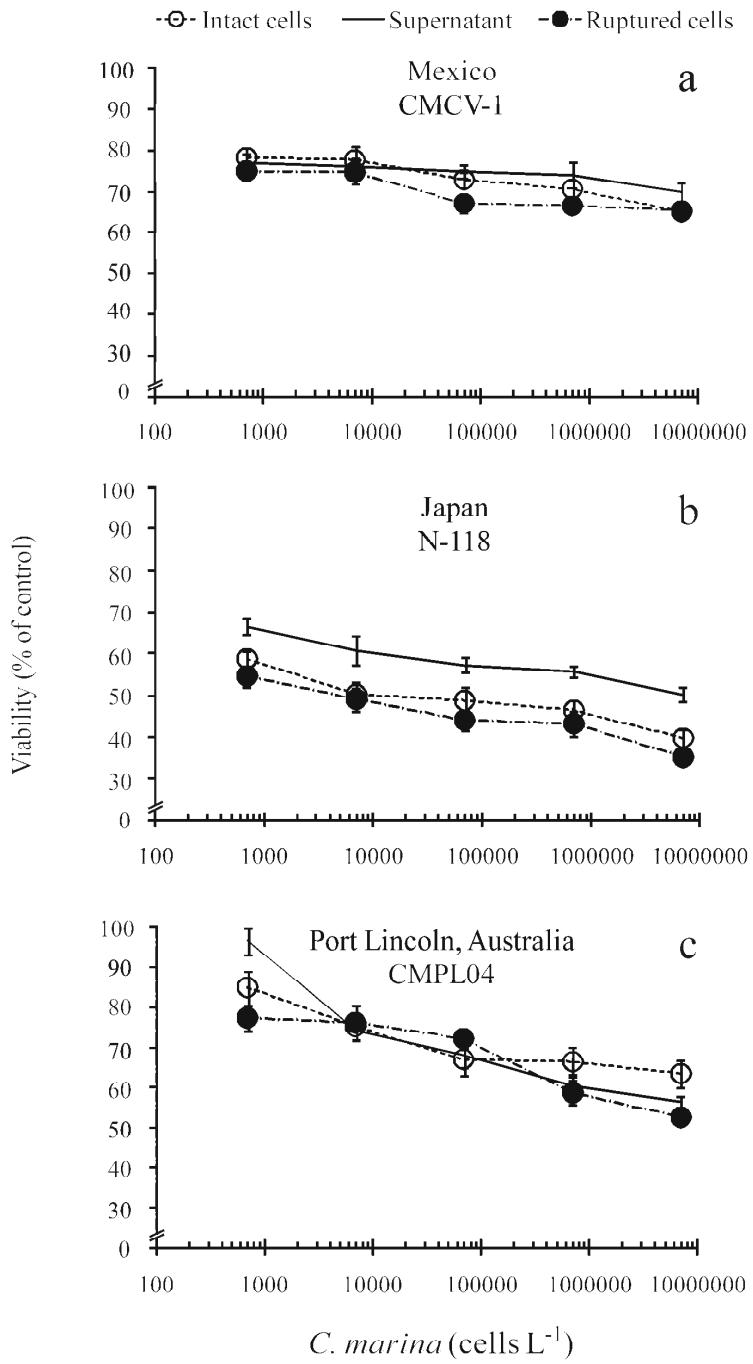

Figure 3.1. Toxicity of *Chatttonella marina* to RTgill-W1 cells. a) Mexican strain CMCV-1, b) Japanese strain N-118, and c) Australian strain CMPL04. Mexican CMCV-1 was the least toxic at high concentrations, whereas Japanese N-118 was the most toxic at low algal concentrations in the three different preparations.

Cell-free supernatant of the Australian CMPL04 did not show a toxic effect towards the gill cells at the lowest concentration; but a loss of viability was observed at 7×10^3 cells L^{-1}, which was as toxic as 7×10^4 cells L^{-1} for this and the other two algal preparations. Intact cell suspension was the least toxic at the highest concentration by 37% loss of gill cell viability, whereas viability losses of 44 – 48% was observed after exposure to cell-free supernatant and ruptured cell preparations (Fig. 3.1c).

The Australian strain CMDE showed toxic effects at low algal concentrations (23 – 29% loss of viability), then viability decreased with increasing *C. marina* concentrations of up to 7×10^4 cells L^{-1}. However, no significant differences were observed in gill cell viabilities after exposure to algal concentrations between 7×10^4 and 7×10^6 cells L^{-1}, which ranged from 47 to 54%. Additionally, no significant differences were found in any of the three different preparations, thus the same pattern was observed in all suspensions (Fig. 3.2a).

Cell-free supernatant of the Australian strains CMPB and CMPL01 had the same effect on gill cells, with toxicity increasing together with algal concentration. Initial loss of gill cell viability of 11% was observed at 7×10^2 cells L^{-1} of *C. marina*, but it increased to 53 – 55% at 7×10^6 cells L^{-1}. Intact cell and ruptured cell suspensions showed different toxicity towards RTgill-W1 cells. CMPB had the same toxicity (46% loss of viability) at 7×10^5 and 7×10^6 cells L^{-1} in intact cell suspensions. Whereas, a higher toxic effect (~60% loss of viability) was observed in the ruptured cell preparation from algal cultures containing 7×10^4 to 7×10^6 cells L^{-1} (Fig. 3.2b).

Ruptured cells of CMPL01 had the strongest effect on gill cells among all *C. marina* strains studied. It was the least toxic at low concentrations, with losses of gill cell viability of 16 and 19% at 7×10^2 and 7×10^3 cells L^{-1}, respectively. However, toxicity was concentration-dependent, and CMPL01 was the most toxic at high algal concentrations, together with the Japanese N-118 (65 – 71% loss of cell viability) in which no significant differences were observed (Figs. 3.3b and 3.4). Ruptured cell preparations of Australian CMPL01 were more toxic than intact cells and cell-free supernatant at concentrations higher than 7×10^3 cells L^{-1}. These two preparations

showed the same impact on gill cell viability, and statistical comparisons showed no significant differences (Fig. 3.2c).

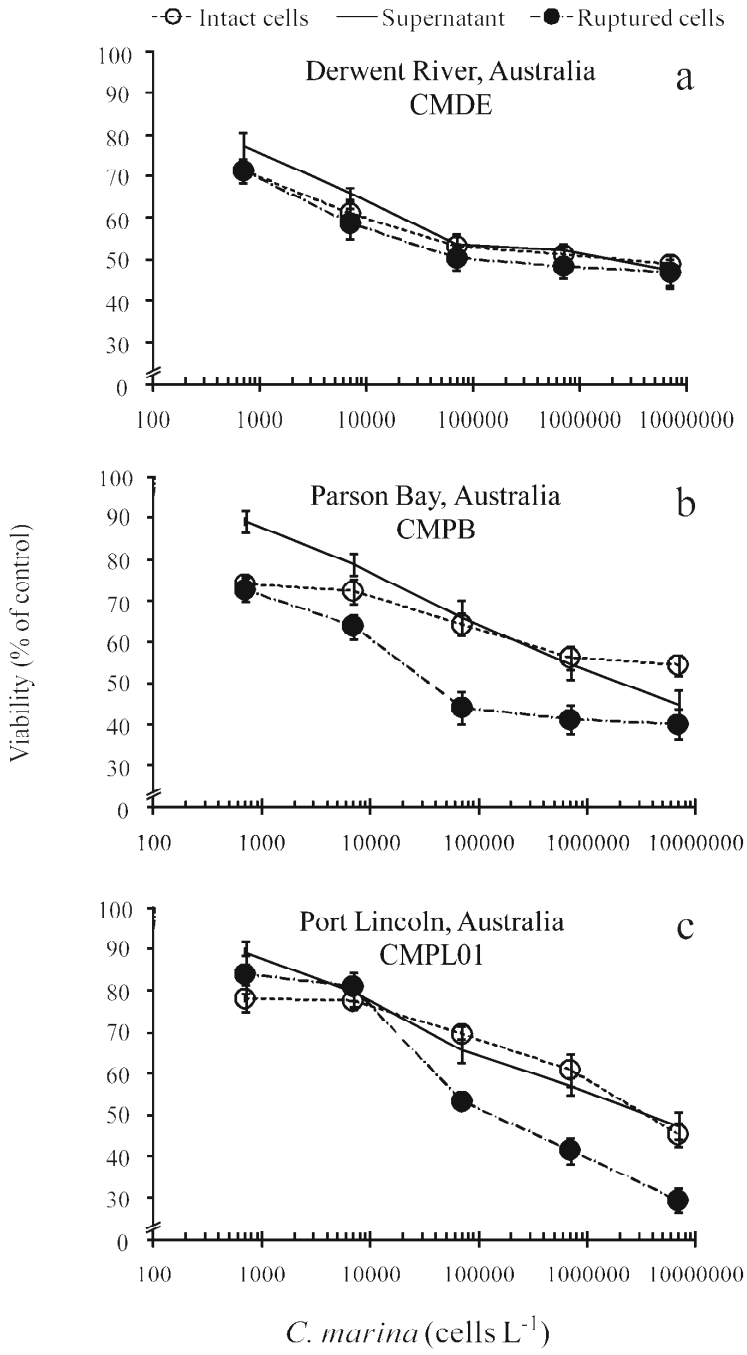

Figure 3.2. Toxicity of Australian *Chatttonella marina* strains to RTgill-W1 cells. a) CMDE, b) CMPB, c) CMPL01. The three different preparations of CMDE had the same toxicity, and the same occurred with intact cell cultures and cell-free supernatant of CMPL01. Cell-free supernatant of CMPB and CMPL01 had the same effect on gill cells, with toxicity increasing together with algal concentration.

Among Australian *C. marina* strains, CMPL04 from Port Lincoln, S.A. (2001) was the least toxic, followed by CMDE from the Derwent River, Tas., then CMPB from Parson Bay, Tas., and finally the most toxic was CMPL01 from Port Lincoln (1996), S.A., especially when considering the effect of the ruptured cell suspensions at high algal concentrations (Fig. 3.3b).

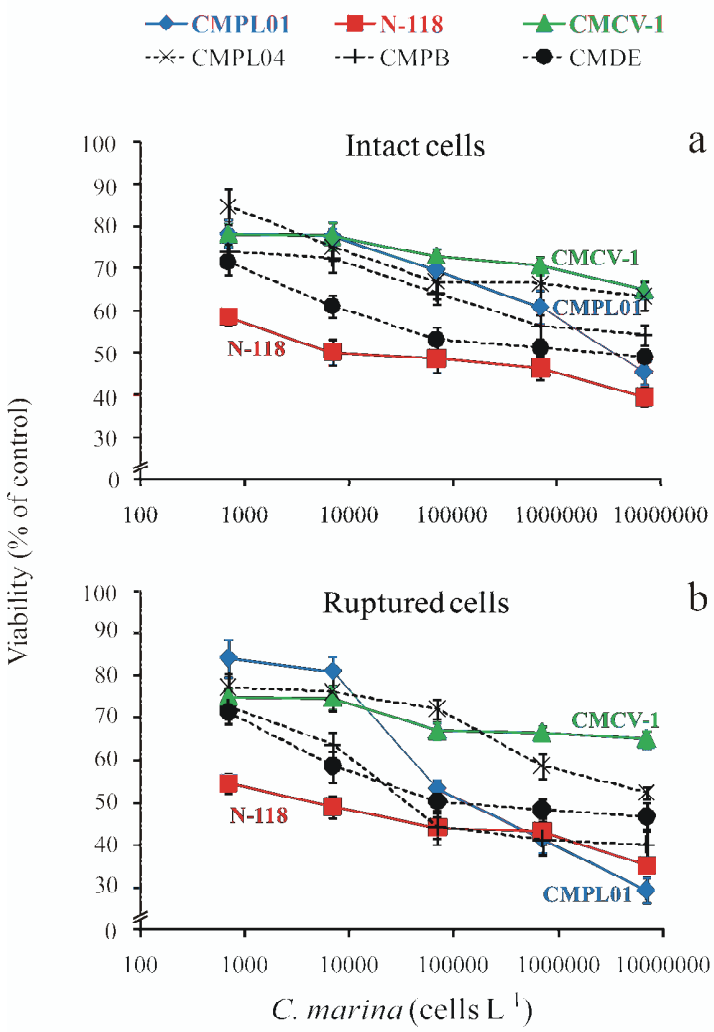

Figure 3.3. Toxicity of two preparations of *Chatttonella marina* toward gill cells: a) Intact cell medium, b) Ruptured cell suspension. The Mexican CMCV-1 was usually the least toxic, particularly at the two highest algal concentrations of ruptured cell preparations. The Japanese N-118 was the most toxic at low concentrations, whereas the Australian CMPL01 was the least toxic at low algal concentrations but the most toxic, together with N-118, at high concentrations of ruptured cell preparations.

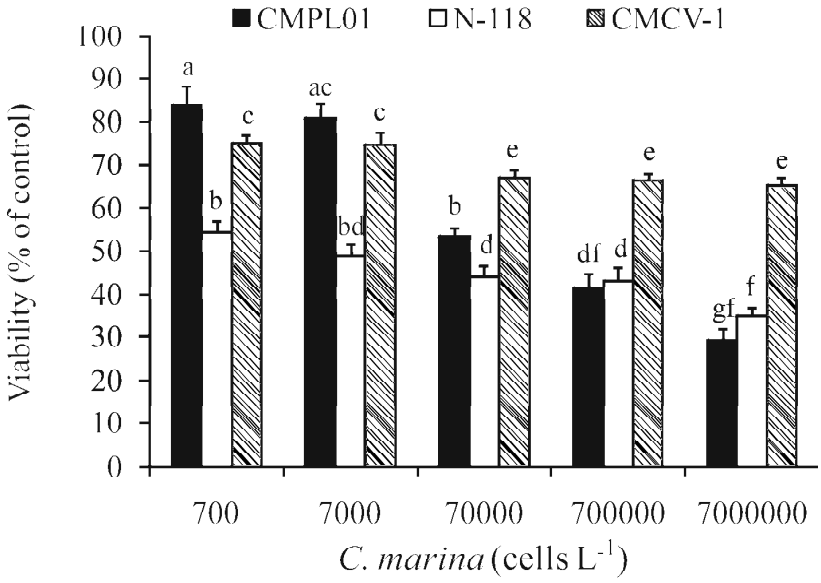

Figure 3.4. Comparison of the toxicity of Australian CMPL01, Japanese N-118 and Mexican CMCV-1 strains of ruptured cell preparations of *C. marina* towards the fish cell line RTgill-W1. Lowest toxicity was observed for CMCV-1 and CMPL01 strains at low concentrations, whereas N-118 and CMPL01 were the most toxic at high algal concentrations.

SEM micrographs showed occurrence of membrane disruption in gill cells after exposure to *Chattonella marina*. Rupture of membrane and holes all over the gill cell surface were observed, particularly after exposure to Japanese N-118 and Australian CMPL01. Loss of microvilli-like protrusions was also another effect found in rounded gill cells (Fig. 3.5). Additionally, loss of membrane integrity was confirmed with TEM micrographs, showing membrane breakage (Fig. 3.6).

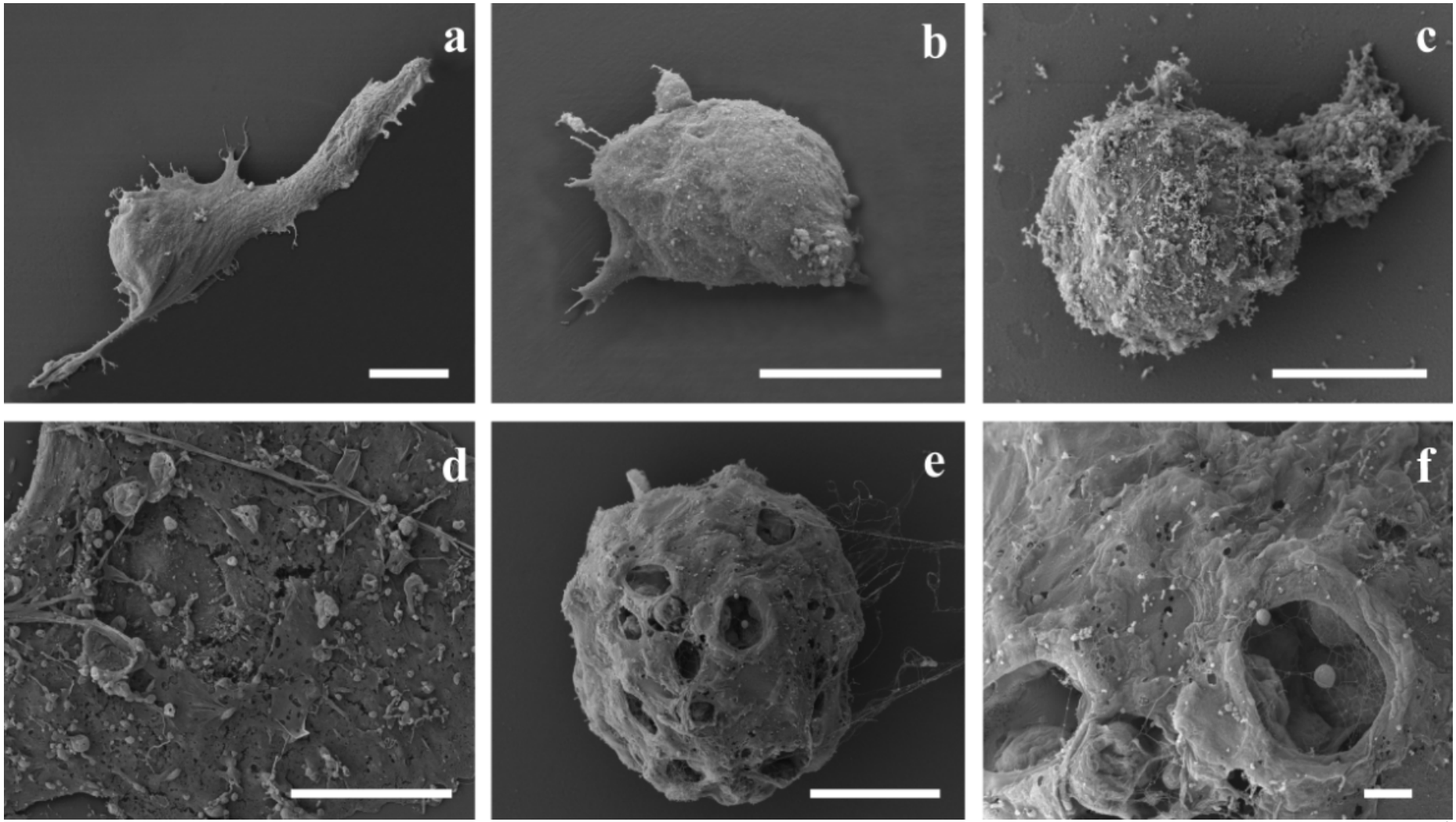
a
b
c
d
e
f

Figure 3.5. Gill cells observed by SEM after exposure to *Chattonella marina*. Micrographs of undamaged control cells (a, b, c) show sequential phases after cell detachment. Flattened and elongated cells (a) start taking a round shape (b) within minutes after detachment, having a size reduction until they become completely rounded and having microvilli-like protrusions covering the cell membrane (c). RTgill-W1 cells showed membrane disruption after exposure to intact *C. marina* N-118 cells (d). Despite being able to change from a spread to round shape after detachment, gill cells also showed membrane loss when exposed to ruptured cells of *C. marina* CMPL01 (e) and (f). Scale bars for a, b, c, d, e: 10 μm; f: 1 μm.

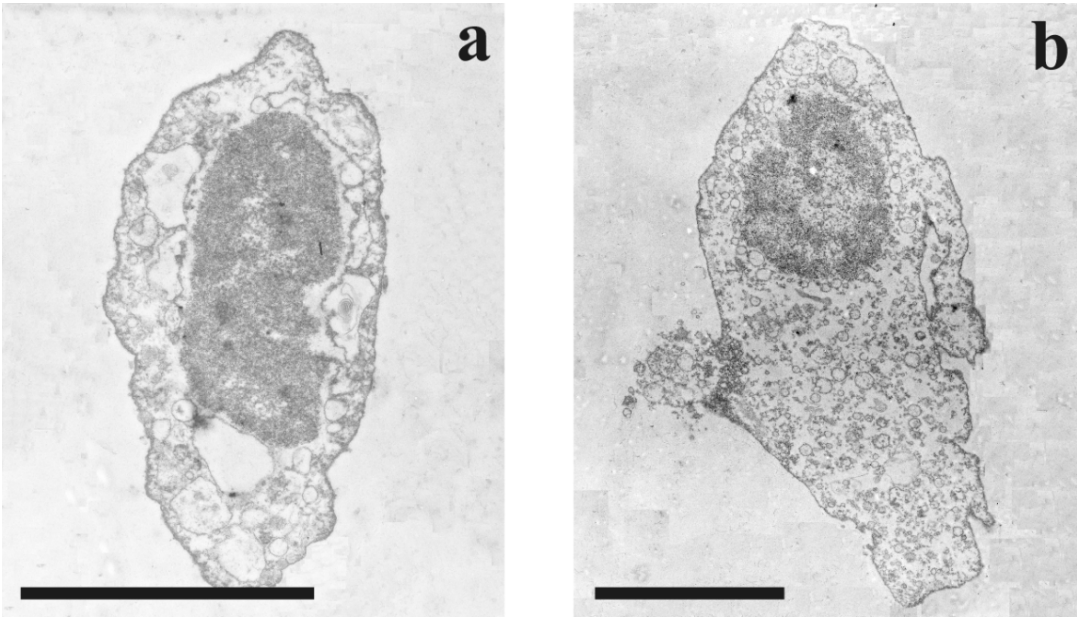

Figure 3.6. TEM micrographs of RTgill-W1 cells. Control cells exposed to GSe showing normal cells (a), and cells confirming membrane disruption upon exposure to *Chattonella marina* CMPL01 (b). Scale bars: 5 μm.

4. Discussion

The three algal preparations (intact, supernatant and ruptured cells) of *Chattonella marina* showed different toxicity towards RTgill-W1 cells. In some cases the highest toxicity was observed in ruptured cell preparations, suggesting that certain toxic compounds were released under stress conditions, when *Chattonella marina* lysed. This raphidophyte was also toxic under normal culture conditions as intact cells, sometimes were as toxic as ruptured cell preparations, especially for Japanese N-118 and Australian CMDE strains. Cytotoxicity of *C. marina* has been previously found after exposing several mammalian cell lines to a hemolytic fraction derived from this raphidophyte, showing a concentration dependent impact (Kuroda et al., 2005).

Marshall et al. (2005) found that the Japanese *C. marina* N-118 was the highest superoxide producer, followed by the Australian CMPL04 and CMPL01 strains. This may explain why N-118 was always the most toxic at low algal

concentrations ($\leq 7\times10^4$ cells L^{-1}) in the intact and ruptured cell preparations. However, CMPL01 was as toxic as N-118 at high concentrations ($\geq 7\times10^5$ cells L^{-1}), thus there may be other factors apart from superoxide that contribute to toxicity by *C. marina*. The *C. marina* strains CMCV, CMPL01 and N-118 were analysed by Paul McNabb from Cawthron Institute in New Zealand and none of the samples showed presence of brevetoxins.

Kim et al. (2001) suggested that a polysaccharide-coating structure on the cell surface known as glycocalyx is the toxic component in *C. marina*. They presumed that the glycocalyx is discharged from algal cells after gentle agitation followed by centrifugation at 3000 × g for 1 min. The cell-free supernatant was prepared under the same conditions here, but the cell-free supernatant usually showed lower toxic effects on gill cells compared to ruptured cell preparations or even intact cell cultures. According to these findings *C. marina* is more toxic either when cells are present and direct contact with gill cells occurs, or when algal cells are ruptured and release certain harmful compounds than when cells are not directly present (i.e. after centrifugation). Thus it appears that the glycocalyx was released upon sonication but not after centrifugation of algal cells, which may be due to varying fragility among strains since the Mexican CMCV-1 strain is slightly larger (less fragile), and showed lesser toxicity. Additionally, more potent effects of ultrasonic ruptured cells have been previously found after hemolytic activity was detected only in intact cell suspensions of *C. marina* but not in the supernatant (Kuroda et al., 2005).

Kim et al. (2006; 2008a) have suggested the production of nitric oxide (NO) as part of the toxic mechanism in *C. marina*. NO is useful to make insoluble iron available for chloroplasts in plants (Graziano et al., 2006). It also works as a protective agent against ROS by capturing superoxide anions (Beligni et al., 2002). *Chattonella marina* is the highest ROS producer among phytoplankton species (Marshall et al., 2005), thus, production of NO may be a self defense against their own ROS. Apart from these suggested toxic compounds, production of fatty acids by red tide microalgae seems to play a main role in ichthyotoxicity (Sola et al., 1999; Fu et al., 2004).

In conclusion, these results documented cytotoxicity of *Chattonella marina* using a fish gill cell line. This raphidophyte was toxic under no stress conditions in intact cell cultures, as well as in its cell-free supernatant. Additionally, toxicity increased after ultrasonic rupture of *C. marina* cells, suggesting that this raphidophyte produces certain compounds with cytotoxic properties that are released upon lysis.

Chapter 4*

Comparative toxicity to gill cells by eleven species of raphidophytes, dinophytes, haptophytes and green algae

Abstract

Toxicity of eleven phytoplankton species was tested with the *in vitro* assay using fish gill cells. Production of superoxide anion was also measured in algae using a chemiluminescence assay. The nontoxic green algal species *Tetraselmis suecica* and *Dunaliella tertiolecta* showed no effect on gill cells and very low production of superoxide. A low production of O_2^- (≤0.924 pmol cell^{-1} hr^{-1}) was found by all dinoflagellate species despite some of them, such as *Karenia mikimotoi*, *K. brevis*, *Gymnodinium catenatum* and *Karlodinium veneficum*, showing toxicity towards gill cells (≤57% loss of viability). In a similar way, the haptophyte *Prymnesium parvum* produced very low levels of O_2^- (≤0.003 pmol cell^{-1} hr^{-1}), but it was the most toxic species (≤73% loss of gill cell viability) together with two strains of the raphidophyte *Chattonella marina*. Six strains of this raphidophyte showed variability in toxicity to gill cells. The raphidophytes *Heterosigma akashiwo* and *Fibrocapsa japonica*, were also toxic towards gill cells (≤35 and ≤53% loss of viability, respectively) but had low production of superoxide anion (≤0.036 and ≤0.245 pmol cell^{-1} hr^{-1}, respectively). In contrast, *Chattonella marina* was the greatest producer of O_2^- (up to 19.0 pmol cell^{-1} hr^{-1}), especially after cell rupture, and showed high toxicity (up to 70% loss of viability). Variations in toxicity were observed between intact and ruptured algal cells. Ruptured cells of *K. veneficum*, *C. marina* and *P. parvum* were

*Published in part as Mooney, B.D., Dorantes-Aranda, J.J., Place, A.R. and Hallegraeff, G.M. 2011. Ichthyotoxicity of gymnodinioid dinoflagellates: PUFA and superoxide effects in sheepshead minnow larvae and rainbow trout gill cells. Marine Ecology Progress Series 426, 213-224.

more toxic than intact cells, but intact cells of *H. akashiwo* and *K. mikimotoi* had higher toxicity than ruptured cells. Production of superoxide anion was only associated with toxicity caused by *C. marina* but not with other red tide species including dinoflagellates, haptophytes and other raphidophytes.

1. Introduction

The species with the highest impacts on marine finfish farming include *Chattonella antiqua*, *C. marina*, *Heterosigma akashiwo* (Raphidophyceae)*, Karenia mikimotoi*, *Cochlodinium polykrikoides* (Dinophyceae), and *Prymnesium parvum* (Prymnesiophyceae) (Landsberg, 2002). Without exception all these species are extremely fragile, and most affect finfish via gill damage. This may vary among microalgal groups, and sometimes among species; however, microalgae with highest impacts on mariculture seem to share similar harmful compounds, including reactive oxygen species (ROS) and fatty acids (Lee, 1996; Mooney et al., 2011). One species that differs from these ichthyotoxic algae is the dinoflagellate *Karlodinium veneficum*, which is known to produce the potent toxins karlotoxins, but still causes damage to fish gills since it has been shown that these organs are the main target of these toxins (Deeds et al., 2006).

Measurements of ROS (generally as superoxide anion) have been previously reported for some of these ichthyotoxic species using a chemilumninescence assay based on the reaction of MCLA (2-methyl-6-(4-methoxyphemyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3(7H)-one) with either superoxide (O_2^-) or dioxygen ($^1O_2$). However, most workers report their results only as Total or Cellular Chemiluminescence Units (TCU or CCU, respectively) from cultures grown under normal conditions (Oda et al., 1997; Marshall et al., 2005; Mooney et al., 2007; Portune et al., 2010). A more recent application of this technique (Godrant et al., 2009) allows measurement of superoxide production rates, and thus results are expressed as concentration of superoxide per cell per hour (e.g. pmol O_2^- $cell^{-1}$ hr^{-1}). Additionally since this assay includes longer periods to monitor the signal and known steady-state concentrations of superoxide using a calibration curve with the

enzymatic system xanthine/xanthine oxidase (Garg et al., 2007; Rose et al., 2008), it represents a more sensitive and precise technique.

The *in vitro* assay described in chapter 2 and the more precise technique to measure superoxide production by microalgae were used to determine whether there exist any association between toxicity and superoxide anion production by marine phytoplankton.

2. Methods

2.1. Algal cultures

A total of 20 strains, corresponding to 11 species, were obtained from algal collections as specified on Table 4.1. All microalgae were grown in GSe medium (Blackburn et al., 1989) at 35 of salinity, and maintained at 19°C with a light intensity of 200 μmol photons m^{-2} s^{-1} (cool white fluorescent lamps) under a 12/12-hr light/dark cycle. All cultures were used for experiments in the exponential growth phase (day 8).

2.2. Exposure of gill cells to microalgae

The seeding procedure of gill cells was exactly the same as in chapter 2, section 2.4.1. Exposure assays of gill cells to all microalgae were carried out as specified in chapter 2, section 2.4.3. Algal concentrations used for the exposure assays are presented on table 4.2. Finally, viability assays after exposure to microalgae were performed as in chapter 2, section 2.2.

2.3. Production of superoxide anion by phytoplankton

Intact-cell and ruptured-cell cultures of all algal strains were analyzed for production of superoxide anion using the method described by Godrant et al. (2009). The intact cell suspension was taken directly from the cultures containing the algae, and the ruptured cell suspension was prepared by sonication of the cultures for 5 min

in a bath-type sonicator (Soniclean 120T, pulse swept power 60W) at 19°C. Algal cell concentrations used for superoxide production measurements were the same as for the toxicity bioassays, which are specified on table 4.2.

A volume of 3 µL of xanthine (X7375, Sigma) at 5 mM was added to a 96-well microplate (CLS3917, Sigma), then 270 µL of the algal culture was added. Superoxide dismutase (S7571, Sigma) at 5 kU mL^{-1} (3 µL) was used for blank correction, and a standard curve was performed using three standard solutions of xanthine oxidase (X1875, Sigma) at 0.1, 0.7 and 1.5 U L^{-1} (3 µL). Immediately after adding algal cultures, 5 µL of MCLA (87787, Sigma) at 125 µM was injected to all wells and luminescence was monitored for 20 min in a microplate reader (FLUOstar OPTIMA, BMG Labtech, 413-3350, Germany).

2.4. Exposure of gill cells to superoxide

The seeding procedure of gill cells was performed as in chapter 2, section 2.3.2.

Superoxide was produced synthetically with the enzymatic system xanthine/xanthine oxidase according to Marshall et al. (2003) who found that a mixture 5 µM xanthine with 10 – 30 U L^{-1} of xanthine oxidase results in similar superoxide levels to those produced by *C. marina.* A mix of 5 to 25 µM xanthine with 30 U L^{-1} of xanthine oxidase was prepared in L-15/ex medium and immediately added to the microplate wells containing the gill cells. This mixture was renewed every 15 min during a 2-hr exposure period. Once this time was achieved, gill cells were washed with PBS and viability was measured as described in chapter 2, section 2.2 using 5% alamarBlue in L-15/ex medium.

2.5. Data analysis

The data were subjected to analysis of variance (ANOVA). Normality of the data was tested with the Kolmogorov-Smirnov method and homogeneity of variances was assessed with Hartley's F_{max} tests. The Tukey multiple comparison test was

performed to determine any difference among treatments. The significance level considered in all statistical tests was 95% (α=0.05). The software Statistica 8.0 was used for statistical analysis.

Table 4.1. Phytoplankton species used for *in vitro* toxicity and production of superoxide anion. *Tetraselmis suecica* and *Dunaliella tertiolecta* are nontoxic species and were used for control purposes.

Class	Species	Strain code	Origin	Isolator (year)	Original source
Prasinophyceae	*Tetraselmis suecica*	TSCS187	Brest, France	A. Dodson	CSIRO Microalgal Supply (original collection CCMP), Aus.
Chlorophyceae	*Dunaliella tertiolecta*	DTCS175	unknown	unknown	CSIRO Microalgal Supply (original collection CCMP), Aus.
Prymnesiophyceae	*Prymnesium parvum*	PPCWP10	Flade So Thy, Denmark	T. Christensen (1984)	Cawthron Institute, N.Z. (original collection University of Copenhagen)
		PPSR01	Serpentine River, W.A., Aus.	J. A. Marshall	University of Tasmania, Aus.
Dinophyceae	*Karenia mikimotoi*	KMCWD63	Waimangu, N.Z.	A. Haywood (1994)	Cawthron Institute, N.Z.
		KMWL01	West Lakes, S.A., Aus.	M. de Salas (2006)	University of Tasmania, Aus.
	Karenia brevis	KBCCMP718	Florida, USA	W. Wilson (1958)	CSIRO Microalgal Supply (original collection CCMP), Aus.
	Karlodinium veneficum	KVSH01	Sydney Harbour, N.S.W., Aus.	M. de Salas (2002)	University of Tasmania, Aus.
		KVSR01	Swan River, W.A., Aus.	M. de Salas (2001)	University of Tasmania, Aus.
	Cochlodinium polykrikoides	CPNU01	Nubeena, Tas., Aus.	M. de Salas	University of Tasmania, Aus.
	Gymnodinium catenatum	GCGB02	Georges Bay, Tas., Aus.	M. de Salas	University of Tasmania, Aus.
Raphidophyceae	*Heterosigma akashiwo*	HACS169	West Lakes, S.A., Aus.	J. L. Stauber (1983)	CSIRO Microalgal Supply (original collection CCMP), Aus.
		HAGB01	Georges Bay, Tas., Aus.	M. de Salas	University of Tasmania, Aus.
	Fibrocapsa japonica	FJCS332	Tsuda Bay Kagawa, Japan	K. Yuki (1978)	CSIRO Microalgal Supply (original collection CCMP), Aus.
	Chattonella marina	CMCV-1	Bahia Concepcion, B.C.S., Mex.	C. Band-Schmidt (2000)	The Northwestern Centre for Biological Research, B.C.S., Mex.
		CMPL01	Port Lincoln, S.A., Aus.	J. A. Marshall (1996)	University of Tasmania, Aus.
		CMPB	Parson Bay, Tas., Aus.	M. de Salas (2003)	University of Tasmania, Aus.
		CMDE	Derwent River, Tas., Aus.	M. de Salas (2002)	University of Tasmania, Aus.

	CMPL04	Port Lincoln, S.A., Aus.	M. de Salas (2001)	University of Tasmania, Aus.
	N-118	Seto Inland Sea, Japan	S. Yoshimatsu (1983)	National Institute of Environmental Studies, Japan

3. Results

3.1. Toxicity of phytoplankton species

Viability of gill cells was variable upon exposure to the different algal species and strains tested. The control nontoxic green algal species *Tetraselmis suecica* (TSCS187) and *Dunaliella tertiolecta* (DTCS175) showed no effect on cell viability. Intact and ruptured cells of the dinoflagellate *Karenia mikimotoi* strain KMCWD63 did not show any toxicity towards gill cells; however, the more recently isolated strain KMWL01 of the same species affected gill cell viability, showing losses of 57 and 46% after exposure to intact and ruptured cells, respectively, with intact cells more toxic than ruptured cells. The same effect was observed for *Karlodinium veneficum.* The strain KVSH01 had no effect on gill cells; in contrast, the strain KVSR01 had an impact on cell viability, especially after cell lysis, decreasing gill cell viability up to 38%. Other dinoflagellates that were also tested included *Cochlodinium polykrikoides* CPNU 01 (no toxicity observed), the brevetoxin producing *Karenia brevis* KBCCMP718 (≤45% loss of gill cell viability) and the saxitoxin producing *Gymnodinium catenatum* GCGB02 (25% of cell viability loss); no statistically significant differences were observed between intact and ruptured cells of *K. brevis* and *G. catenatum* (Fig. 4.1).

For the group of the raphidophytes, the two strains of *Heterosigma akashiwo* were toxic to gill cells; HACS169 was slightly less toxic than HAGB01 (16 – 18% vs 24 – 35%, respectively), and no significant differences were observed between intact and ruptured cells. All strains of *Chattonella marina* were toxic, affecting gill cell viability between 35 and 65%, with ruptured cells being generally more toxic than intact cells. Mexican strain CMCV-1 was the least toxic, and Australian CMPL01 and Japanese N-118 were the most toxic among this species. The only strain of *Fibrocapsa japonica* FJCS332 also had a high impact on cell viability (53% loss of viability), intact and ruptured cells were equally toxic (Fig. 4.1).

Ruptured cells of two strains of the haptophyte *Prymnesium parvum* (PPCWP10 from Denmark and PPSR01 from Australia) were as toxic as *C. marina* strains CMPL01 and N-118. Intact cells of *P. parvum* PPCWP10 were less toxic than

ruptured cells, whereas the strain PPSR01 did not show significant differences between intact and ruptured cells (Fig. 4.1).

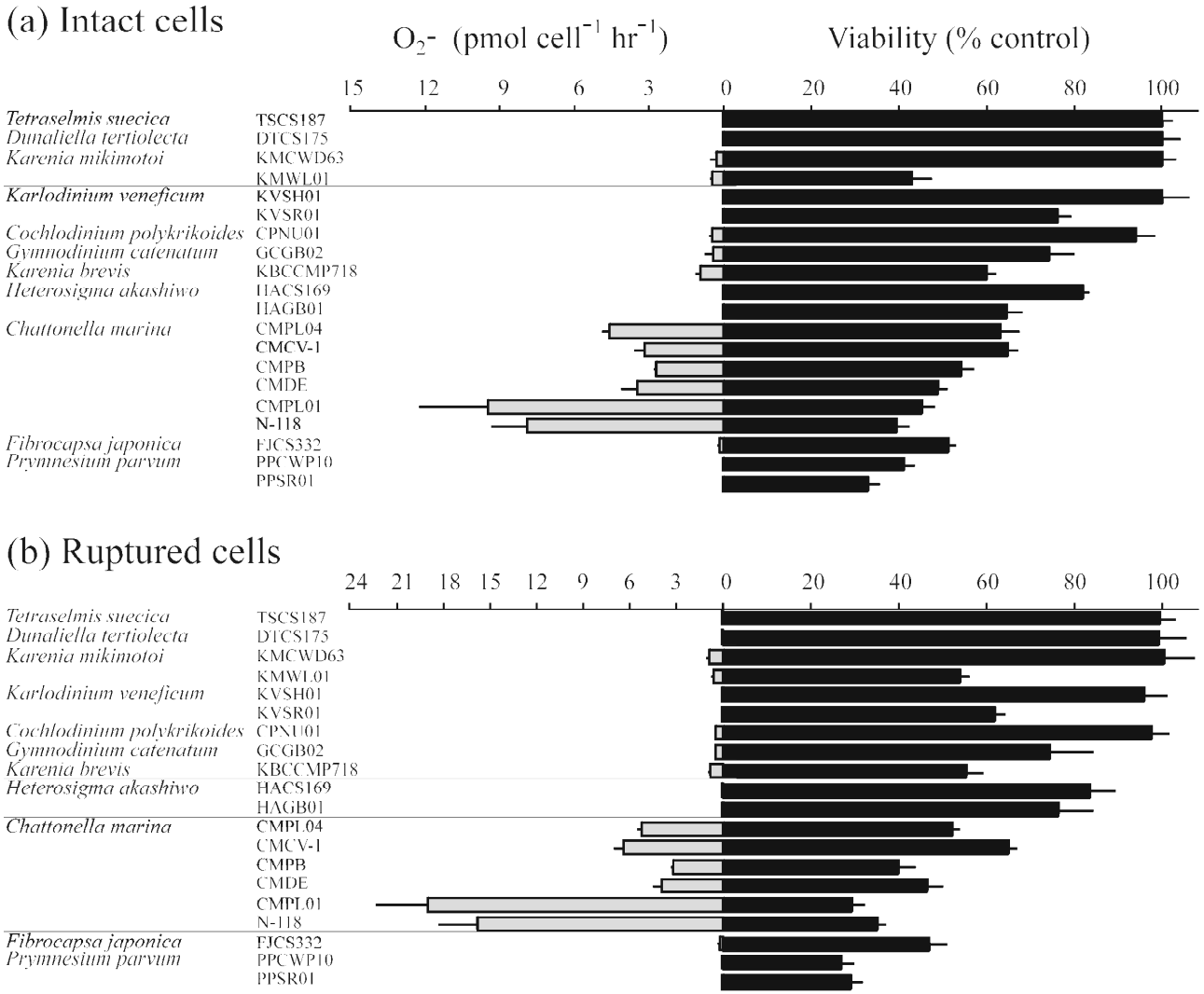
(a) Intact cells
O2- (pmol cell-1 hr-1)
Viability (% control)
15 12 9 6 3 0 20 40 60 80 100
Tetraselmis suecica TSCS187
Dunaliella tertiolecta DTCS175
Karenia mikimotoi KMCWD63
KMWL01
Karlodinium veneficum KVSH01
KVSR01
Cochlodinium polykrikoides CPNU01
Gymnodinium catenatum GCGB02
Karenia brevis KBCCMP718
Heterosigma akashiwo HACS169
HAGB01
Chattonella marina CMPL04
CMCV-1
CMPB
CMDE
CMPL01
N-118
Fibrocapsa japonica FJCS332
Prymnesium parvum PPCWP10
PPSR01
(b) Ruptured cells
24 21 18 15 12 9 6 3 0 20 40 60 80 100
Tetraselmis suecica TSCS187
Dunaliella tertiolecta DTCS175
Karenia mikimotoi KMCWD63
KMWL01
Karlodinium veneficum KVSH01
KVSR01
Cochlodinium polykrikoides CPNU01
Gymnodinium catenatum GCGB02
Karenia brevis KBCCMP718
Heterosigma akashiwo HACS169
HAGB01
Chattonella marina CMPL04
CMCV-1
CMPB
CMDE
CMPL01
N-118
Fibrocapsa japonica FJCS332
Prymnesium parvum PPCWP10
PPSR01

Figure 4.1. Viability of gill cells (black columns) after exposure to intact (a) and ruptured (b) cells of 20 phytoplankton strains. Superoxide production levels (grey columns) are presented to compare gill cell viability. Superoxide production values are presented on Table 4.2. Bars represent standard deviations of measurements on triplicate wells.

3.2. Production of superoxide anion by phytoplankton

Production of superoxide anion was very low in the nontoxic species *Tetraselmis suecica* and *Dunaliella tertiolecta*, for both intact and ruptured cells ranging from 0 to 0.01 pmol O_2^- $cell^{-1}$ hr^{-1}. Both strains of *Prymnesium parvum* PPCWP10 and PPSR01, and the strain KVSR01 of *Karlodinium veneficum* also showed low production of superoxide anion (≤0.003 pmol O_2^- $cell^{-1}$ hr^{-1}); however, *K. veneficum* KVSH01 had a slightly higher production of 0.01 pmol O_2^- $cell^{-1}$ hr^{-1}. For the remaining dinoflagellates, superoxide production was between 0.27 and 0.92 pmol O_2^- $cell^{-1}$ hr^{-1}, and almost all ruptured cell cultures of all strains produced higher superoxide levels, except for *Karenia brevis* KBCCMP718 that produced more O_2^- when cells were intact. The latter species had the highest production of superoxide anion among all dinoflagellates.

The two strains of *Heterosigma akashiwo*, HACS169 and HAGB01, had the lowest production of O_2^- among all raphidophytes, with levels of 0.02 – 0.04 pmol $cell^{-1}$ hr^{-1}. *Fibrocapsa japonica* FJCS332 also showed low superoxide production (0.16 – 0.25 pmol $cell^{-1}$ hr^{-1}). In contrast, *Chattonella marina* was the greatest producer of O_2^- among all the species screened, with a significant variability within the six strains. The Australian strain CMPB had the lowest levels of O_2^- (2.73 – 3.19 pmol $cell^{-1}$ hr^{-1}), followed by Mexican CMCV-1 and Australian strains CMDE and CMPL04 (3.20 – 6.41 pmol O_2^- $cell^{-1}$ hr^{-1}). Finally, the Japanese N-118 and Australian CMPL01 strains had the highest superoxide production of 7.886 and 9.49 pmol $cell^{-1}$ hr^{-1} corresponding to intact cells, respectively, with no significant differences observed. Production of O_2^- by ruptured cells of these two strains was almost twofold compared to intact cells, with levels of 15.8 and 19.0 for N-118 and CMPL01, respectively. Additionally, comparable results were obtained with those reported in the literature (Table 4.2).

Table 4.2. Values of superoxide anion (O_2^-) production by 20 strains of phytoplankton. *Tetraselmis suecica* and *Dunaliella tertiolecta* are nontoxic species and represent control references. Values presented on this table are plotted in figures 4.1, 4.2 and 4.3.

Class	Species	Strain code	cells L^{-1}	pmol O_2^- $cell^{-1}$ hr^{-1}		Marshall et al. (2005)		Mooney et al. (2011)	Portune et al. (2010)		Oda et al. (1997)
				Intact cells	Ruptured cells	TCU ($\times 10^4$)	CCU	CCU	TCU ($\times 10^4$)	CCU	pmol µg cell $protein^{-1}$ min^{-1}
Prasinophyceae	*Tetraselmis suecica*	TSCS187	5.5×10^8	0.006[a]	0.006[a]	0.49	0.05	0			
Chlorophyceae	*Dunaliella tertiolecta*	DTCS175	3.3×10^9	0.001[a]	0.004[a]	0	0.01	0			
Prymnesiophyceae	*Prymnesium parvum*	PPCWP10	4.7×10^9	0.001[a]	0.001[a]	3					
		PPSR01	4.5×10^9	0.001[a]	0.003[a]	1.22	0.06				
Dinophyceae	*Karenia mikimotoi*	KMCWD63	3.3×10^7	0.27±0.1	0.87±0.2						
		KMWL01	3.5×10^7	0.48±0.0	0.61±0.1						
	Karenia brevis	KBCCMP718	1.0×10^7	0.92±0.2	0.80±0.1	4.16[b]	28.1[b]	7.13			
	Karlodinium veneficum	KVSH01	2.8×10^8	0.01[a]	0.01[a]	2.29[b]	0.02[b]	0.2[b]			
		KVSR01	3.1×10^8	0.003[a]	0.003[a]	2.19[b]	0.03[b]	0.16			
	Cochlodinium polykrikoides	CPNU01	4.3×10^6	0.47±0.1	0.51±0.0						
	Gymnodinium catenatum	GCGB02	3.3×10^6	0.43±0.1	0.52±0.0	0.64[b]	115.5[b]				
Raphidophyceae	*Heterosigma akashiwo*	HACS169	4.5×10^8	0.04[a]	0.03[a]	5.74	0.38	4.26[b]	97.67[b]	26.5[b]	19[b]
		HAGB01	4.3×10^8	0.02[a]	0.03[a]	2.38	0.12				
	Fibrocapsa japonica	FJCS332	1.8×10^7	0.16±0.1	0.25±0.1	3.47	13.8			0.097[b]	17[b]
	Chattonella marina	CMCV-1	1.3×10^7	3.20±0.4	6.41±0.6	79.5[b]	517[b]				
		CMPL01	1.6×10^7	9.49±1.7	19.0±3.3	149	169	20.16[b]	65.12[b]	214.7[b]	
		CMPB	1.7×10^7	2.73±0.0	3.19±0.2	194[b]	255[b]				

	CMDE	2.0×10^{7}	3.50±0.6	3.98±0.5			19.67	
	CMPL04	2.0×10^{7}	4.59±0.3	5.25±0.2	167	403		
	N-118	1.7×10^{7}	7.89±1.5	15.8±1.5	189	215		41[b]

TCU = Total chemiluminescence units; CCU = Cellular chemiluminescence units.

[a] Standard deviations were lower than 0.003 pmol O_2^- $cell^{-1}$ hr^{-1}.

[b] Correspond to the same species but different geographic strains.

3.3. Relation between superoxide production and *in vitro* toxicity

A very low production of superoxide anion by nontoxic microalgae (*Tetraselmis suecica* and *Dunaliella tertiolecta*) was related to zero loss of viability by gill cells. Similar low levels of superoxide anion were produced by both strains of *Prymnesium parvum* but these two strains, either intact or ruptured cells, were among the most toxic towards gill cells. There was no relation between superoxide production and toxicity for the dinoflagellates (Fig. 4.2). Loss of gill cell viability was variable (0 – 57%) after exposure to dinoflagellate species, and all strains showed low O_2^- production (Table 4.2). A general pattern of high production of O_2^- with high toxicity was observed for raphidophyte strains of *Chattonella marina*, especially for Japanese N-118 and Australian CMPL01 strains (Fig. 4.3), which showed the highest toxicity after cell rupture with the highest production of superoxide among all species and strains studied (Fig. 4.1). The other raphidophytes, *Heterosigma akashiwo* and *Fibrocapsa japonica*, affected gill cell viability but produced low levels of superoxide anion.

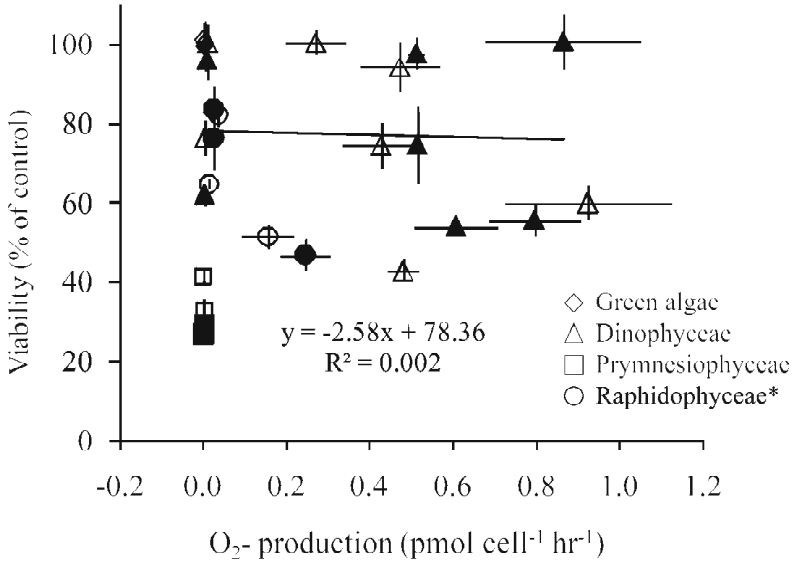

Figure 4.2. Viability of gill cells versus superoxide production of intact (solid symbols) and ruptured cells (open symbols) of 14 phytoplankton strains grouped into their taxonomic classes. Raphidophyceae* does not include strains of *Chattonella marina*. Bars represent standard deviations of measurements on triplicate wells for superoxide production in microalgae on the "x" axis, and viability measurements on quadruplicate wells for gill cell viability on the "y" axis.

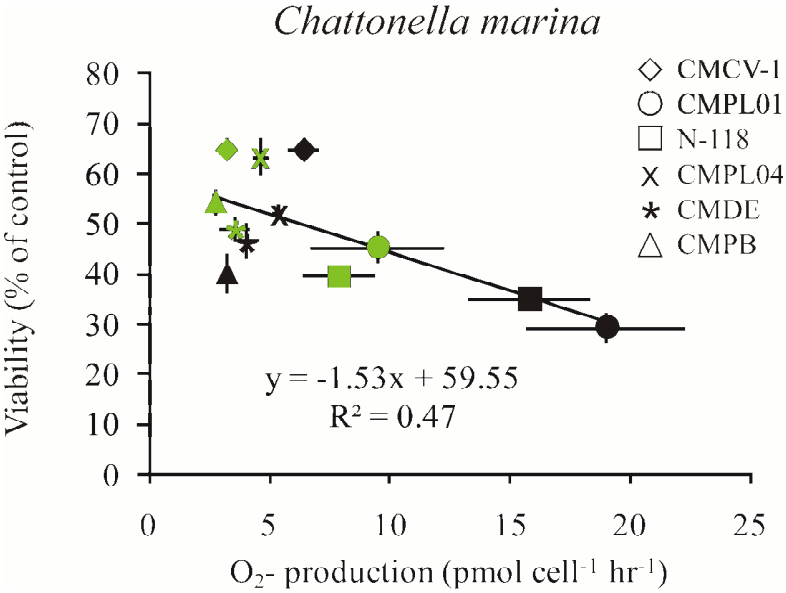

Figure 4.3. Relation between superoxide anion production by six strains of *Chattonella marina* (CMCV-1 Mexican, CMPL01 Australian 1, N-118 Japanese, CMPL04 Australian 2, CMDE Australian 3, CMPB Australian 4; see table 4.1 for all details) and their toxicity towards gill cells. Green symbols represent intact cells and black symbols ruptured cells. Bars represent standard deviations of measurements on triplicate wells for superoxide production in microalgae on the "x" axis, and viability measurements on quadruplicate wells for gill cell viability on the "y" axis.

3.4. Toxicity of synthetically-produced superoxide

Gill cells suffered a loss of viability of 10 – 14% during the 2-hr exposure period to superoxide produced by the enzymatic system xanthine/xanthine oxidase (Fig. 4.4).

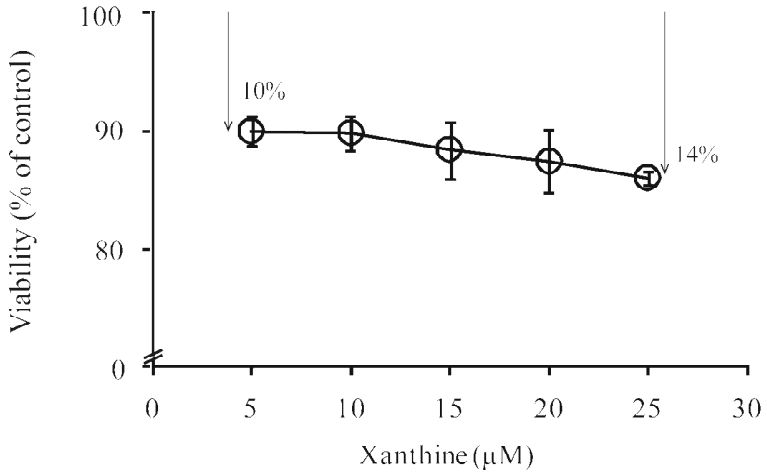

Figure 4.4. Toxicity of superoxide to gill cells. Superoxide was generated by mixing 5 to 25 μM xanthine with 30 U L^{-1} of xanthine oxidase, being equivalent to superoxide levels produced by *C. marina* (Marshall et al., 2003). Bars represent standard deviations of gill cell viability measurements on quadruplicate wells.

4. Discussion

As expected, very low levels of superoxide production together with zero toxicity was found for the nontoxic species *Tetraselmis suecica* and *Dunaliella tertiolecta*. However, *Prymnesium parvum* did not have a high production of O_2^- (≤0.003 pmol $cell^{-1}$ hr^{-1}) despite showing a high toxicity towards gill cells (≤73% loss of viability), indicating that this species produces other compounds different to superoxide that are responsible for fish kills, such as prymnesins or fatty acids (Henrikson et al., 2010; Valenti et al., 2010).

All dinoflagellates produced higher O_2^- levels than the green algal species and haptophytes, but never as high as raphidophytes, and since all species showed variable toxicity to gill cells this also suggests different toxic compounds produced by dinoflagellate species. Three of the species screened are known to produce potent toxins. *Karlodinium veneficum* produces karlotoxins that mainly damage fish gills (Deeds et al., 2006). Karlotoxin production varies among strains and ichthyotoxicity by *K. veneficum* has been particularly found after cell lysis (Mooney et al., 2010; Dorantes-Aranda et al., 2011). The strain KVSH01 was not toxic but the strain

KVSR01, which produces 2.1 pg KmTx 2-1 cell^{-1} (Mooney et al., 2009), was even more toxic after cell rupture. Superoxide production by *K. veneficum* was low (≤0.01 pmol cell^{-1} hr^{-1}) compared to the highest O_2^- producer *Chattonella marina* (up to 19 pmol cell^{-1} hr^{-1}) as has been previously reported by Marshall et al. (2005) and Mooney et al. (2011). *Karenia brevis* also showed toxicity (45% loss of viability) but low O_2^- production (≤0.9 pmol cell^{-1} hr^{-1}); it is well documented that this species produces brevetoxins (21 – 47 pg PbTx-3 eq cell-1 for the strain used here according to Haubois et al., 2007) with neurotoxic effects that are the main cause of its toxicity (Lekan and Tomas, 2010). However, it has been demonstrated that some strains of this species can stop producing brevetoxins overtime in culture conditions (McNabb et al., 2006). *Gymnodinium catenatum* is the other dinoflagellate that is a toxin producer, and saxitoxins are particularly responsible for its toxicity (Negri et al., 2007). These toxins are not known to cause gill damage but surprisingly *G. catenatum* showed toxicity towards gill cells (25% loss of viability), and superoxide anion does not seem to play a role since only low levels of O_2^- were found here (≤0.5 pmol cell^{-1} hr^{-1}). However, it is possible that apart from toxin production, polyunsaturated fatty acids may also contribute to toxicity by this dinoflagellate since it produces significant levels of eicosapentaenoic acid (EPA, 20:5ω3; 12 – 15%) and octadecapentaenoic acid (OPA, 18:5ω3; 2 – 22%) (Hallegraeff et al., 1991; Mansour et al., 2003), which have shown toxicity to gill cells and hepatocytes from rainbow trout, respectively (Mooney et al., 2011; Fossat et al., 1999).

The strain of *Cochlodinium polykrikoides* CPNU01 did not affect gill cell viability and neither did *Karenia mikimotoi* strain KMCWD63. However, another strain of *K. mikimotoi*, KMWL01, did decrease cell viability (≤57%) but comparable to the rest of the dinoflagellate species it produced low levels of superoxide anion (≤0.61 pmol cell^{-1} hr^{-1}). Toxicity of this species has been associated with polyunsaturated fatty acids (Gentien et al., 2007), and the polyether gymnocin, which only showed weak toxicity to fish cells (Satake et al., 2005). Any involvement of ROS has been discarded to explain its toxicity (Fossat et al., 1999). Toxicity of polyunsaturated fatty acids has not just been linked to some dinoflagellate species, but also to raphidophytes. *Fibrocapsa japonica* appears to produce high levels of fatty acids that have haemolytic properties (Fu et al., 2004; de Boer et al., 2009), which seem to be involved in fish kills rather than superoxide anion since low

production of O_2^- was observed (≤0.25 pmol cell^{-1} hr^{-1}). Other studies have reported comparable levels of superoxide anion between *F. japonica* and *C. marina* (Oda et al., 1997); whereas other workers, similar to this study, also reported low levels of O_2^- (Marshall et al., 2005; Portune et al., 2010). The same scenario has been found for *Heterosigma akashiwo* since some studies reported comparable production of superoxide anion (Oda et al., 1997; Portune et al., 2010; Mooney et al., 2011) but other workers observed low O_2^- production (Marshall et al., 2005) (Table 4.2). Twiner et al. (2001) showed that *H. akashiwo* produces the other ROS, hydrogen peroxide, but at a concentration much lower than required to observe mortality for vertebrate cell lines and the brine shrimp *Artemia salina*, indicating that toxicity of *H. akashiwo* is not mediated by ROS. *H. akashiwo* is the most variable red tide phytoplankton since blooms are not always toxic and can grow under a wide range of salinities (Kempton et al., 2008; O'Halloran et al., 2006; Rensel et al., 2010), which makes it more complicated to find its toxic compounds and mechanisms. However, production of extracellular organic compounds by *H. akashiwo* has been associated with induction of cell death *in vitro* (Twiner et al., 2005).

Contrary to what was observed for the rest of the phytoplankton species, superoxide anion does appear to be associated, at least in part, with toxicity caused by the raphidophyte *Chattonella marina* (Fig. 4.3), as it was observed in Japanese N-118 and Australian CMPL01 strains, since they produced the highest O_2^- levels after cell rupture (15.8 and 19.0 pmol cell^{-1} hr^{-1}, respectively), and showed the highest toxicity towards gill cells (65 and 70% loss of viability). Additionally, synthetically produced superoxide caused a loss of gill cell viability of 10 – 14%. Marshall et al. (2003) claimed that the levels of superoxide produced via this enzymatic system are the equivalent to those produced by *C. marina* under normal growth conditions (original recipe of GSe medium with 5 µM Fe(III) and grown under a 12/12-hr light/dark cycle). However, it was demonstrated here that *C. marina* is able to produce higher levels of superoxide under other circumstances, such as stress caused by cell disruption. Therefore, there might be other conditions where higher superoxide production by *C. marina* occurs, including nutrient limitation and dark adaptation. On the other hand, since low levels of superoxide were found for dinoflagellate species, but still toxicity was recorded, these results confirm

observations by Mooney et al. (2011) who found that superoxide anion does not seem to play a role on ichthyotoxicity caused by dinoflagellate species.

In conclusion, these results showed that *Prymnesium parvum* and *Chattonella marina* were the most toxic algae towards fish gill cells, especially after cell rupture. It was also confirmed that the raphidophyte *C. marina* is the greatest producer of superoxide anion, and that O_2^- is associated with toxicity caused by *C. marina* but not with other red tide species including dinoflagellates, haptophytes and the raphidophytes *Heterosigma akashiwo* and *Fibrocapsa japonica*.

Chapter 5

Production of superoxide anion by *Chattonella marina* as influenced by iron, growth phase and dark adaptation

Abstract

Production of superoxide anion by six different geographic strains of *Chattonella marina* was assessed at varying iron concentrations, growth phases and dark adaptation. Iron and growth phase affected superoxide generation, with highest production at 1 μM Fe(III) in GSe medium and in the exponential growth phase by Japanese N-118 and Australian CMPL01 strains with 21.4 and 25 pmol O_2^- $cell^{-1}$ hr^{-1}, respectively. The highest production of superoxide from all culture conditions was measured in algal cultures that were adapted for 2 days to complete darkness and quickly exposed to light, which was also found for Japanese and Australian strains (33.4 and 37.6 pmol O_2^- $cell^{-1}$ hr^{-1}, respectively).

1. Introduction

Reactive oxygen species (ROS) are commonly produced by aerobic organisms as respiration by-products. ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl (OH•) radical. A free radical is any species that has one or more unpaired electrons, like O_2^- and OH•, although H_2O_2 has no unpaired electrons and is not a radical but still is highly reactive and can produce O_2^- in the presence of Fe(III) (Halliwell and Gutteridge, 1984).

Mechanisms and causes of superoxide production by *Chattonella marina* are not completely clear yet. Photosynthesis has been linked to O_2^- production by *C. marina*, with a higher production during the exponential growth phase, related to a higher metabolic activity (Marshall et al., 2002a). High production of superoxide radical in the exponential phase was also found in the toxic dinoflagellate *Cochlodinium polykrikoides* (Kim et al., 1999). Additionally, light appears to influence O_2^- production since both *C. marina* and *C. polykrikoides* produced less superoxide radicals in darkness. However, since they were able to produce superoxide in the darkness, it appears that they also possess other mechanisms to generate O_2^- besides photosynthesis (Kim et al., 1999; Marshall et al., 2002a).

Production of ROS by higher plants represents a defense strategy against pathogens (Sagi and Fluhr, 2001). An increase in superoxide production has also been reported for the microalga *Chlorella vulgaris* after exposure to the mycoplasma *Acholeplasma laidlawii* (Loseva et al., 2003), suggesting that this "oxidative burst" for defense purposes may also occur in microalgae. An alternative reason for superoxide production, relates to iron acquisition, which has been suggested for *Chattonella marina* and the cyanobacterium *Lyngbya majuscula*, able to produce O_2^- as an electron shuttle to reduce ferric iron (Fe III) to ferrous iron (Fe II), and hence increasing its bioavailability (Rose et al., 2005, Garg et al., 2007).

This chapter aimed to investigate the effect of iron content in growth medium, growth phase and dark adaptation on production of superoxide anion by six different geographic strains of *Chattonella marina*.

2. Methods

2.1. Algal cultures

Six strains of *Chattonella marina* (refer to Table 3.1 in chapter 3) were grown in four different preparations of GSe medium (Blackburn et al., 1989) enriched with iron [Fe(III) as $FeCl_3 \cdot 6H_2O$] at 0.01, 0.1, 1 and 5 μM. Seawater used had a salinity of 35 and cultures were maintained at 19°C. Five different batches of cultures were used for superoxide production assays. Each batch was composed of 24 cultures: 6

strains × 4 Fe(III) in GSe. Two of these batches were grown under a 12/12-hr light/dark cycle with a light intensity of 200 μmol photons m^{-2} s^{-1} (cool white fluorescent lamps); one of them was used in the exponential phase (day 7 at 8–11×10^6 cells L^{-1}) and the other in the stationary phase (day 14 at 4–6×10^7 cells L^{-1}). Three other batches were grown under a 12/12-hr light/dark cycle (200 μmol photons m^{-2} s^{-1}) for 5 days and then adapted to complete darkness for 2 days (8–11×10^6 cells L^{-1}). One batch was used within the next 5 min after exposing the cells to light; another batch was used in the same conditions except that cultures were filtered (Whatman, 10463500 sterile filters, 0.2 μm pore size membrane) and only the filtered medium was assessed for O_2^- production, representing a control group to observe any possible photochemical production of O_2^-. Finally, the fifth batch that had been kept in the dark for 2 days was tested for superoxide production 2 hrs after exposure to light.

2.2. Assay for production of superoxide anion

Production of superoxide anion was measured using the method described by Godrant et al. (2009). A volume of 3 μL of xanthine (X7375, Sigma, Sydney, NSW, Australia) at 5 mM was added to a 96-well microplate (CLS3917, Sigma, Sydney, NSW, Australia), then 270 μL of the algal culture was added. Superoxide dismutase (S7571, Sigma, Sydney, NSW, Australia) at 5 kU mL^{-1} (3 μL) was used for blank correction, and a standard curve was performed using three standard solutions of xanthine oxidase (X1875, Sigma, Sydney, NSW, Australia) at 0.1, 0.7 and 1.5 U L^{-1} (3 μL). Immediately after adding algal cultures, 5 μL of MCLA (87787, Sigma, Sydney, NSW, Australia) at 125 μM was injected to all wells and luminescence was monitored for 20 min in a microplate reader (FLUOstar OPTIMA, BMG Labtech, 413-3350, Germany).

3. Results

3.1. Influence of iron and growth phase

Cultures from all strains produced less superoxide levels in the stationary than in exponential growth phase when grown under a 12/12-hr light/dark cycle. Mexican CMCV-1 generated the least with 3.2 – 4.9 pmol cell^{-1} hr^{-1} and 1.9 – 2.6 pmol cell^{-1} hr^{-1}, in the exponential and stationary phases, respectively. Among the Australian strains, CMPL01 produced the most (25 pmol cell^{-1} hr^{-1}) and CMPL04 produced the least (7.1 pmol cell^{-1} hr^{-1}) in the exponential phase [at 1 µM Fe(III)]. Japanese N-118 and Australian CMPL01 strains exhibited the lowest production at 0.01 µM, with a slight increase at 0.1 µM, and the highest production at 1 µM (25 and 21.4 pmol cell^{-1} hr^{-1}, respectively), levels then decreased at 5 µM to 9.5 and 7.9 pmol cell^{-1} hr^{-1}, respectively (Fig. 5.1).

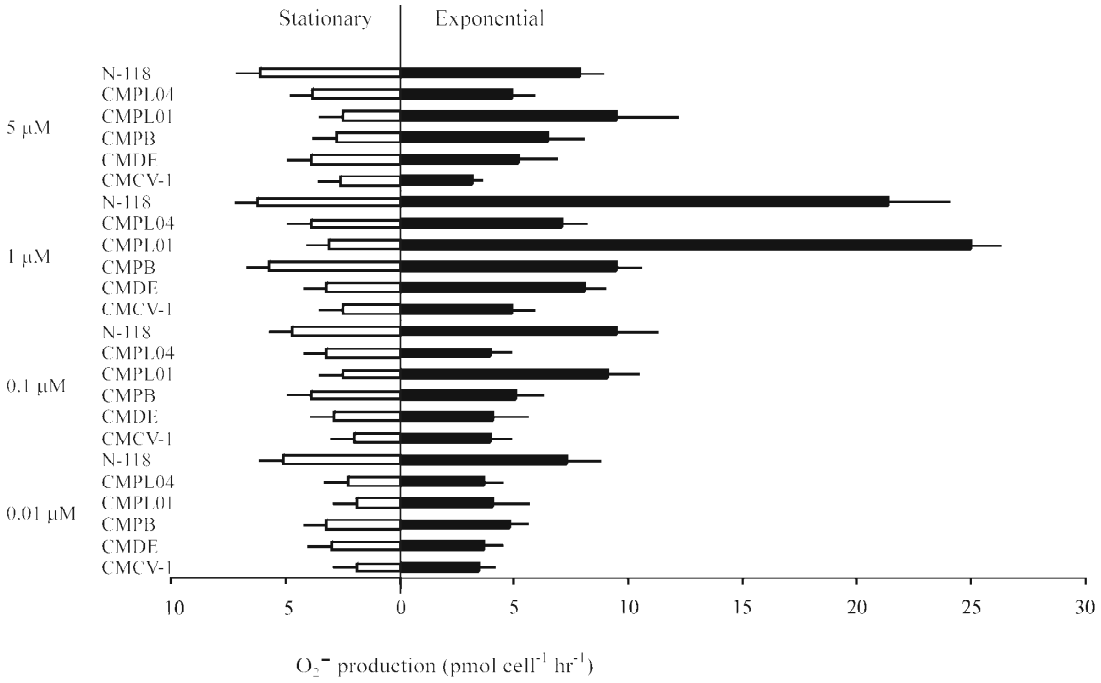

Figure 5.1. Production of superoxide by 6 different geographic strains of *Chattonella marina* as influenced by iron Fe(III) (0.01, 0.1, 1 and 5 µM) and growth phase (exponential and stationary). CMCV-1, Mexico; CMDE, CMPB, CMPL01, CMPL04, Australia; N-118, Japan. All strains were grown under a 12/12-hr light/dark cycle. Bars represent standard deviations of measurements on triplicate wells.

3.2. Influence of iron and dark adaptation

Controls (filtered GSe medium, without algal cells) showed negligible production of superoxide at levels of 0.1 – 1 pmol hr^{-1}. An increase in O_2^- production was observed from 0.01 to 1 µM Fe(III) for all strains, with a significant decrease at 5 µM. Dark adapted cultures that were assessed within 5 min of exposure to light showed significantly higher production of superoxide than cultures grown under a 12/12-hr light/dark cycle (e.g. Japanese N-118 at 5 µM produced 18.9 vs 7.9 pmol $cell^{-1}$ hr^{-1}, respectively). In contrast, dark adapted cultures tested after 2 hrs of exposure to light generated similar levels of O_2^- to cultures grown under a photoperiod cycle (e.g. Japanese N-118 at 5 µM had 7.7 vs 7.9 pmol $cell^{-1}$ hr^{-1}, respectively). Australian CMPL01 and Japanese N-118 strains exhibited the highest production when grown at 1 µM iron and 5 min of exposure to light, with 37.6 and 33.4 pmol $cell^{-1}$ hr^{-1}, respectively. O_2^- levels decreased at 5 µM for both CMPL01 and N-118 strains (26.1 and 18.9 pmol $cell^{-1}$ hr^{-1}, respectively) but were still higher than cultures grown at 0.01 and 0.1 µM, especially for CMPL01 (26.1 vs 7 – 7.9 pmol $cell^{-1}$ hr^{-1}) (Fig. 5.2).

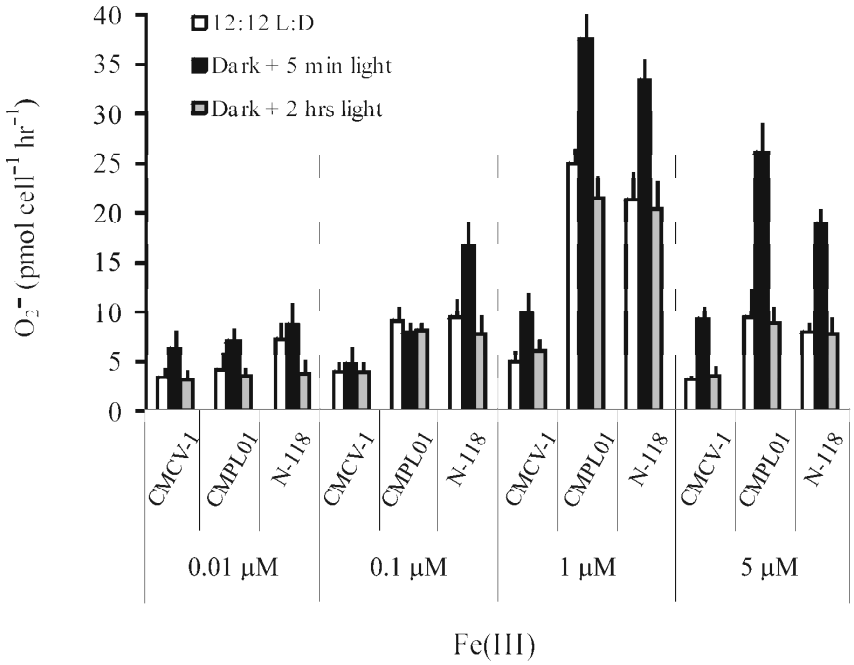

Figure 5.2. Production of superoxide by 3 strains of *Chattonella marina* as influenced by iron and dark adaptation. CMCV-1, Mexico; CMPL01, Australia; N-118, Japan. All strains were assessed in the exponential growth phase. Open columns

represent cultures grown under a 12/12-hr light/dark cycle. Black and grey columns represent cultures grown under a 12/12-hr light/dark cycle for 5 days, then adapted to complete darkness for 2 days and tested within 5 min (black) and after 2 hrs (grey) of returned to light. Bars represent standard deviations of measurements on triplicate wells.

4. Discussion

Production of superoxide by *Chattonella marina* was consistently lower in the stationary than in the exponential growth phase. Australian CMPL01 and Japanese N-118 strains showed up to 8 and 3.5 times more O_2^- production in the exponential phase, respectively. Production was generally lowest at 0.01 μM and highest at 1 μM Fe(III) for all conditions tested, including exponential and stationary growth phases, and dark adapted cultures. Dark adaptation had a major impact on production of superoxide, since O_2^- levels increased between 0.8 and 2 times compared to cultures grown on a photoperiod, for all strains at iron concentrations of 0.01, 0.1 and 1 μM. The highest increase in O_2^- production of 2.2 – 2.9 times was observed at 5 μM; Mexican CMCV-1 strain showed almost a threefold increase from 3.2 pmol O_2^- cell^{-1} hr^{-1} for cultures grown under a photoperiod to 9.2 pmol O_2^- cell^{-1} hr^{-1} after dark adaptation. The highest production of O_2^- was observed when CMPL01 and N-118, grown at 1 μM Fe(III), were suddenly returned to light after adaption to darkness for 2 days, showing a production of 37.6 and 33.4 pmol O_2^- cell^{-1} hr^{-1}, respectively, but then a decrease (21.6 and 20.5 pmol O_2^- cell^{-1} hr^{-1}, respectively) was observed after 2 hrs of maintaining the cultures in the light.

These findings have implications for the ecology of harmful algal blooms by *Chattonella marina* since a sudden exposure to light after dark adaptation results in a high production of superoxide anion radical, one of the compounds involved in ichthyotoxicity caused by this raphidophyte (Marshall et al., 2003). This scenario relates to vertical migration of *C. marina*, spending the night on the sediment surface to take up nutrients from the nutrient-rich lower layers and swimming to the surface during peak sunlight hours (Watanabe et al., 1995; Handy et al., 2005). This

compares with ROS production by *C. antiqua* with higher production of superoxide and hydrogen peroxide during the exponential growth phase and light hours than in stationary phase and dark conditions (Kim et al., 2004). Production of O_2^- has also been associated with photosynthesis in *C. marina*, at least partially since the glycocalyx material of this alga produced up to 20% of the total O_2^- production (Marshall et al., 2002a). Production of O_2^- in the glycocalyx has been associated with the enzyme NADPH oxidase (Kawano et al., 1996; Kim et al., 2001), which generates superoxide using NAD(P)H as an electron donor to reduce O_2 to O_2^-, since incubation of cultures with NADPH-oxidase inhibitors decreased O_2^- production (Marshall et al., 2002a; Garg et al., 2007). Thus it appears that the enzyme NADPH oxidase was stimulated when shifted from dark to light, as has been found in higher plants (i.e. *Arabidopsis thaliana*) that the gene RBOHD (respiratory burst oxidase homolog D), contained in NADPH oxidase, mediated a rapid systematic signal accompanied by accumulation of ROS as a result of external stimuli including high light intensity (Miller et al., 2009).

The role of iron in production of superoxide by *Chattonella marina* has been previously studied by Garg et al. (2007), who observed that superoxide production by *C. marina* can facilitate iron bioavailability when it is complexed only to weak but not to strong ligands. They used the strain CMDE (Australian), and the range of O_2^- production they found was 0.29 – 2.8 pmol cell^{-1} hr^{-1}, which was significantly lower than the production found here for the same strain (2.9 – 8.9 pmol cell^{-1} hr^{-1}) grown under similar conditions. However, CMDE produced up to 18.4 pmol cell^{-1} hr^{-1} when returned to light from a 2-day dark-adaptation period. These authors used the same growth medium used in this study, GSe, including a lower Fe(III) concentration (2.5 nM). In preliminary experiments it was observed that all *C. marina* strains were unable to grow at iron concentrations lower than 10 nM. This could be due to (i) a different source of seawater and soil extract for medium preparations influenced *C. marina* growth since Garg et al. (2007) achieved growth rates of 0.31 d^{-1} compared to 0.5 d^{-1} in this study; or (ii) strains adapted to high iron concentrations used in conventional GSe (5 µM) for routine maintenance since N-118 was isolated in 1983, and CMDE in 2003. It is noted that iron concentrations in the Seto Inland Sea and Gulf of California range between 3.9 – 10 nM (Nakamura, 1990) and up to 7.2 nM

(Segovia-Zavala et al., 2010), respectively, regions from were Japanese and Mexican strains were isolated. Algal blooms often occur after rain or strong wind periods (as it happened in South Australia from where the strain CMPL01 was isolated), mixing the water column and enriching upper layers with nutrients, such as iron, and thus stimulating algal growth (Alonso-Rodríguez and Páez-Osuna, 2003; Zheng and Tang, 2007), suggesting that iron is essential for development of algal blooms (Trick et al., 2010).

Despite using different iron concentrations in medium compared to Garg et al. (2007), a similar pattern in O_2^- production was observed, characterized by highest production in the exponential growth phase, and higher production with increasing iron concentration, except for the medium with 5 μM Fe(III), where O_2^- production decreased compared to 1 μM. It appears that iron limitation stressed *C. marina* cells significantly, especially at 1 μM, and this produced high levels of superoxide possibly to facilitate iron bioavailability. However, concentrations of 10 and 100 nM Fe(III) in growth medium did not trigger as high superoxide production as in 1 μM, supporting the claim that facilitation of iron acquisition is not the only purpose of superoxide production by *C. marina* (Garg et al., 2007).

In conclusion, production of superoxide by *Chattonella marina* as influenced by growth phase was confirmed, with highest production in the exponential phase, and by iron content in growth medium, with highest production at 1 μM Fe(III) and lowest at 0.01 μM. The most interesting finding was that dark adaptation had the highest impact on production of superoxide by *C. marina* since it produced highest superoxide levels after a sudden change from the dark to light. This discovery has a important relevance to vertical migration by *C. marina*, suggesting that adverse effects during blooms of this raphidophyte may occur during peak sunlight hours. Bloom mitigation potentially could be achieved by creating shading in the area where fish pens are located and adding strong ligands to bind iron.

Chapter 6*

In vitro toxicity of fatty acid fractions and karlotoxins from dinoflagellates

Abstract

Algal extracts containing fatty acid fractions from the toxic dinoflagellates *Amphidinium carterae* and *Karlodinium veneficum*, and the karlotoxin KmTx2 from the latter, were tested for toxicity on gill cells. Octadecapentaenoic acid from *K. veneficum* showed a positive effect on gill cells, showing an increase in viability of up to 17%. However, this fatty acid in combination with palmitic acid, showed a decrease in cell viability (44.6%). A commercial preparation of palmitic acid confirmed its toxicity. Fatty acid fractions from *A. carterae* also exhibited toxicity to gill cells. This was more evident with increasing concentrations of eicosapentaenoic acid, which was also confirmed with its commercial preparation (up to 99% loss of cell viability). The karlotoxin KmTx2 exhibited toxicity towards gill cells, with a LC_{50} of 203 ng mL^{-1} and 100% loss of cell viability at 1000 ng mL^{-1} during a 5-hr exposure. Fatty acids do appear to play a role in ichthyotoxicity by phytoplankton. Additionally, it is suggested that toxicity by *K. veneficum* is due to both karlotoxins and fatty acids.

* Published in part as Mooney, B.D., Dorantes-Aranda, J.J., Place, A.R. and Hallegraeff, G.M. 2011. Ichthyotoxicity of gymnodinioid dinoflagellates: PUFA and superoxide effects in sheepshead minnow larvae and rainbow trout gill cells. Marine Ecology Progress Series 426, 213-224.

1. Introduction

It has been demonstrated that dinoflagellates do not produce high levels of superoxide (as shown in chapter 4), thus the search for other toxic compounds has focused on polyunsaturated fatty acids (PUFA) and/or toxins.

All marine phytoplankton species, including toxic and non-toxic, produce large amounts of fatty acids. Some of the major fatty acids are similar among different microalgal groups. For instance, it has been reported that major fatty acids in some diatoms are palmitoleic (16:1ω7, 18-33%), eicosapentaenoic (EPA, 20:5ω3, 10-30%), palmitic (PA, 16:0, 5-29%), myristic (14:0, 4-22%) and hexadecatrienoic (16:3ω4, 4-11%) acids; some cryptophytes have α-linolenic (ALA, 18:3ω3, 26-36%), PA (11-30%), octadecatetraenoic (OTA, 18:4ω3, 8-20%), EPA (3-18%) and docosahexaenoic (DHA, 22:6ω3, 5-9%) acids (Mansour et al., 2005). On the other hand, fatty acids content in some dinoflagellates is characterized by high amounts of octadecapentaenoic (OPA, 18:5ω3, 7-35%), PA (14-34%), OTA (9-34%), DHA (8-26%), EPA (23-24%) and myristic (4-21%) acids (Hallegraeff et al., 1991; Mansour et al., 2005; Mooney et al., 2007). Finally, raphidophytes generally possess OTA (12-27%), PA (8-26%), EPA (12-25%), myristic (5-20%), oleic (18:1ω9c, 4-16%) and palmitoleic (8-10%), acids (Nichols et al., 1987; Marshall et al., 2002b).

The PUFA octadecatetraenoic, eicosapentaenoic and arachidonic (AA, 20:4ω6) acids extracted from the raphidophyte *Fibrocapsa japonica* exhibited hemolytic activity (de Boer et al., 2009). Similar PUFA have been found in toxic dinoflagellates, but generally palmitic, octadecapentaenoic and docosahexaenoic acids are the most abundant in *Cochlodinium polykrikoides* (Dorantes-Aranda et al., 2009), *Karenia brevis* (brevetoxin producer)*, K. mikimotoi, Karlodinium veneficum* (karlotoxin producer) (Mooney et al., 2007), and *Amphidinium carterae* (Řezanka et al., 2008)

It is well known that *Karlodinium veneficum* produces potent karlotoxins with high impacts on fish gills (Deeds et al., 2006) and hemolytic activity (Mooney et al., 2009). Additionally, toxicity of live cultures has been demonstrated on the gill cell line RTgill-W1 (Dorantes-Aranda et al., 2011; chapter 2). Another toxic dinoflagellate *Amphidinium carterae* produces amphidinols, with antifungal and

hemolytic properties (Echigoya et al., 2005). However, since these dinoflagellates also produce some of the PUFA mentioned above, they may also contribute to ichthyotoxicity.

The aim of this chapter was to test the effect of different fatty acid fractions from *A. carterae* and *K. veneficum*, as well as karlotoxins from the latter.

2. Methods

2.1. Exposure assays for toxicity of fatty acid fractions

Fatty acids extracted from the dinoflagellates *Amphidinium carterae* and *Karlodonium veneficum* were kindly provided by Ben Mooney (see Mooney et al., 2011). All fatty acid fractions were evaporated to dryness under a stream of nitrogen and a small volume of chloroform was added and then immediately stored at -20°C in the dark until used to avoid oxidation (1 – 2 min). The vials containing the fatty acids were evaporated to dryness again using nitrogen and a precise volume of methanol was added to achieve the desired concentration ~1 – 2 min).

The gill cells were exposed to different fatty acid fractions containing OPA-pure MGDG (monogalactosyl diglycerides) and OPA-rich MGDG from *A. carterae*; OTA-rich MGDG from *K. veneficum*; and to the commercial preparations of palmitic (PA) and eicosapentaenoic (EPA) acids (Table 6.1, see Fig. 7.1 in chapter 7 for chemical structures).

Table 6.1. Composition of the fatty acid fractions extracted from *Karlodinium veneficum* and *Amphidinium carterae* that were tested on RTgill-W1 cells for toxicity.

Fatty acid designation	**Components of mixture (purity)**	**Nomenclature (lipid numbers)**	**Origin**
OPA	OPA (100%)	18:5ω3	*Karlodinium veneficum*
OPA-rich	OPA (73%) PA (26%)	16:0	*K. veneficum*
	OPA (43%) OTA (43%)	18:4ω3	*Amphidinium carterae*
OTA-rich	OTA (51%) OPA (23%) EPA (26%)	20:5ω3	*A. carterae*
	OTA (49%) EPA (49%) DHA (2%)	22:6ω3	*A. carterae*
	OTA (37%) EPA (5%) ALA (36%)	18:3ω3	*A. carterae*
EPA	99%	20:5ω3 (all *cis*-5,8,11,14,17)	Commercial preparation. Natural source: fish oil. (Matreya, USA, Cat. # 1167)
PA	≥99%	16:0	Commercial preparation (Sigma, Aus., Cat. # P0500)

Abbreviations OPA, octadecapentaenoic acid; OTA, octatetraenoic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic; ALA, α-linolenic acid; MGDG, monogalactosyl diglyceride; PA, palmitic acid.

Gill cells were seeded as described in chapter 2, section 2.3.2. The fatty acids (pure or in cocktail as specified on table 6.1) were dissolved in MeOH and mixed with L-15/ex medium (Schirmer et al., 1997) for the exposure assays. The final concentration of MeOH was of 1%. The gill cells were washed with PBS and exposed to the fatty acids at concentrations of 0.02, 0.2, 2, 20 and 120 mg L^{-1} for 60 hrs at 19°C in the dark. Viability was measured every 12 hrs (as per chapter 2, section 2.2).

2.2. Exposure assays for karlotoxin

Karlotoxin (KmTx2, Fig. 6.1) extracted from the dinoflagellate *Karlodinium veneficum* (strain CCMP 2778 isolated in 2005 from Sarasota, FL, USA) was kindly provided by Allen Place. Karlotoxin was dissolved in methanol to achieve a stock solution of 100 μg mL^{-1}. Gill cells were seeded as described in chapter 2, section 2.3.2 and exposed to concentrations of 0.1, 1, 10, 100 and 1000 ng mL^{-1} (dissolved in L-15/ex medium at a final MeOH concentration of 1%) and at five different times (1, 2, 3, 4 and 5 hrs) at 19°C. Gill cells were washed with PBS before and after the exposure. Viability was measured after each exposure time (as per chapter 2, section 2.2).

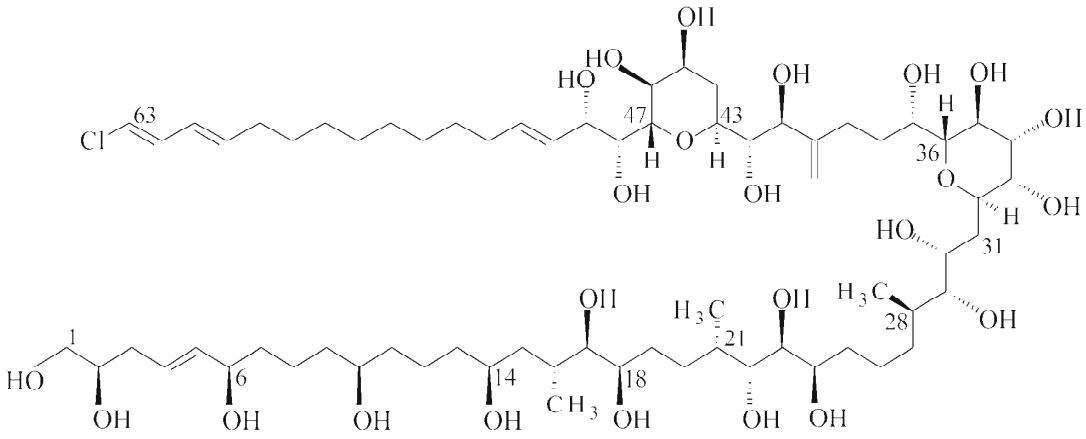

Figure 6.1. Chemical structure of karlotoxin (KmTx2) (Peng et al., 2010).

2.3. Scanning Electron Microscopy

Gill cells were seeded in 6-well conventional cell culture plates at 6×10^4 cells mL^{-1}, 48 hrs previous to the experiments. Gill cells were exposed to eicosapentaenoic acid at a concentration of 120 mg L^{-1} for 12 hrs in the dark at 19°C. Cells were scraped off from the wells with a cell scraper and centrifuged at 700 × g for 3 min. The supernatant was discarded and the cell pellet resuspended in Karnovsky's fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde. Cells were fixed

for 4 hrs, then washed with PBS and stored at 4°C in PBS until processed by electron microscopy.

Cells were post-fixed with 4% osmium tetroxide for 30 min, and dehydrated in a graduated series of ethanol, including 100% dry acetone as final step. Critical point drying with CO_2 was carried out using Balzers CPD 020. Coating was performed under a high resolution platinum palladium coater with a thickness of 5 nm. Samples were analyzed under an electron microscope JEOL JSM-6701F.

3. Results

3.1. Toxicity of fatty acids

Most of the fatty acids from *K. veneficum* exhibited toxicity towards gill cells, except for OPA-pure MGDG which unexpectedly had a positive effect on the gill cells. Viability increased by 110 and 117% during the first 24 hrs when exposed to 20 and 120 mg L^{-1}, respectively (Fig. 6.2a). However, the combination of OPA (73%) and palmitic acid (PA, 26%) exhibited a significant toxic effect but only at a concentration of 120 mg L^{-1} (α=0.05). This toxic effect was time dependent with highest toxicity after 60 hrs and a loss of cell viability of 44.6% (Fig. 6.2b). A commercial preparation of palmitic free fatty acid had a similar impact at 120 mg L^{-1} but with a more gradual effect, and with cell toxicity increased with concentration and time of exposure (Fig. 6.2c).

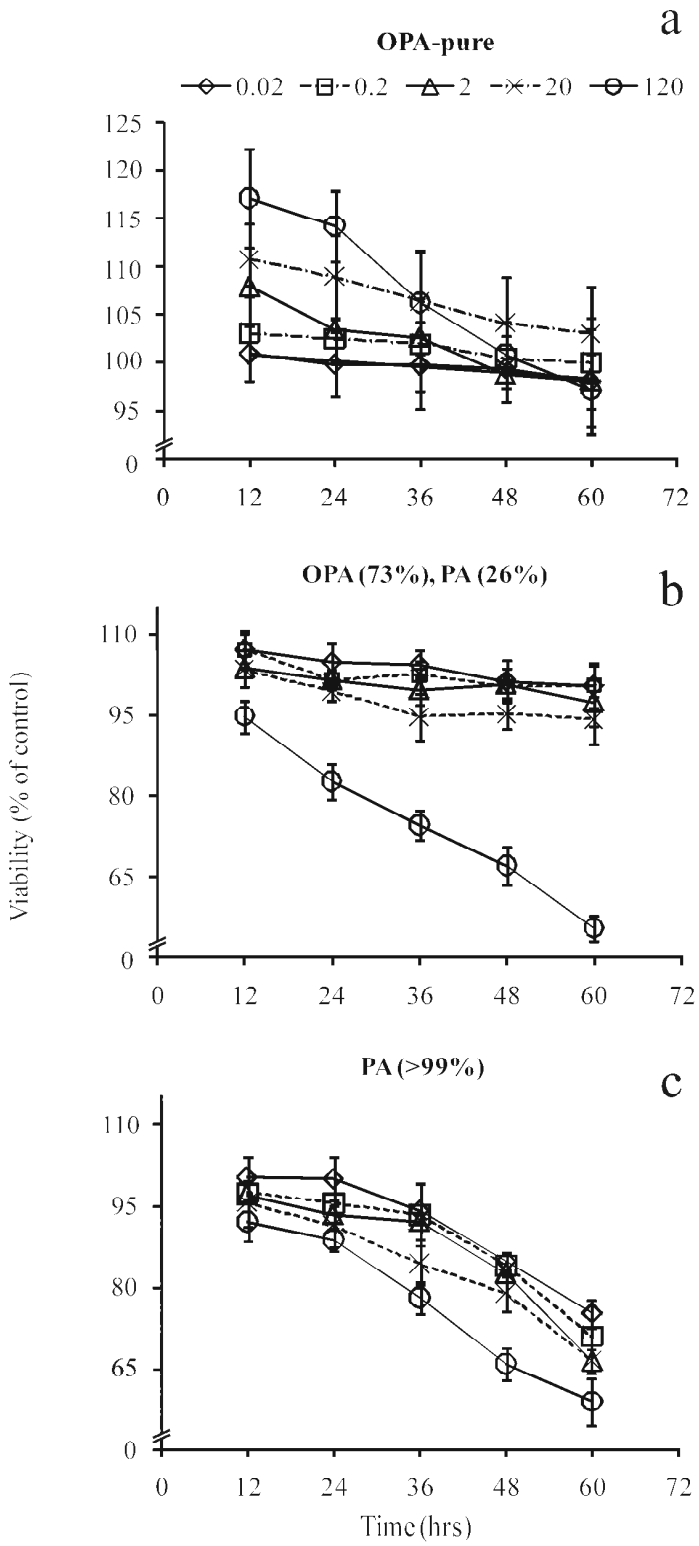

Figure 6.2. Toxicity of fatty acid fractions from *Karlodinium veneficum* containing OPA 100% (a), and a mixture of OPA and PA (b). Toxicity of PA was confirmed with the commercial preparation (c).

OTA-rich MGDG from *A. carterae* showed both a time and concentration-dependent effect. The highest and fastest toxicity was observed in combinations where EPA was present, especially at highest concentrations. The fatty acid fraction containing 5% of EPA did not show a high toxicity, since gill cells only lost 10% of viability during a 60-hr exposure at 120 mg L^{-1} (Fig. 6.3a). In the mixture containing 26% of EPA, gill cells were 99.8% viable when exposed to 120 mg L^{-1} during the first 12 hrs (Fig. 6.3b), but viability decreased to 19.9% in combination with 49% EPA (Fig. 6.3c). Toxicity of EPA (99%) was confirmed with the commercial preparation, which produced the highest loss of gill cell viability observed, decreasing from ~40 to 1.5% when exposed to 120 mg L^{-1} at 12 and 60 hrs (α=0.05), respectively (Fig. 6.3d).

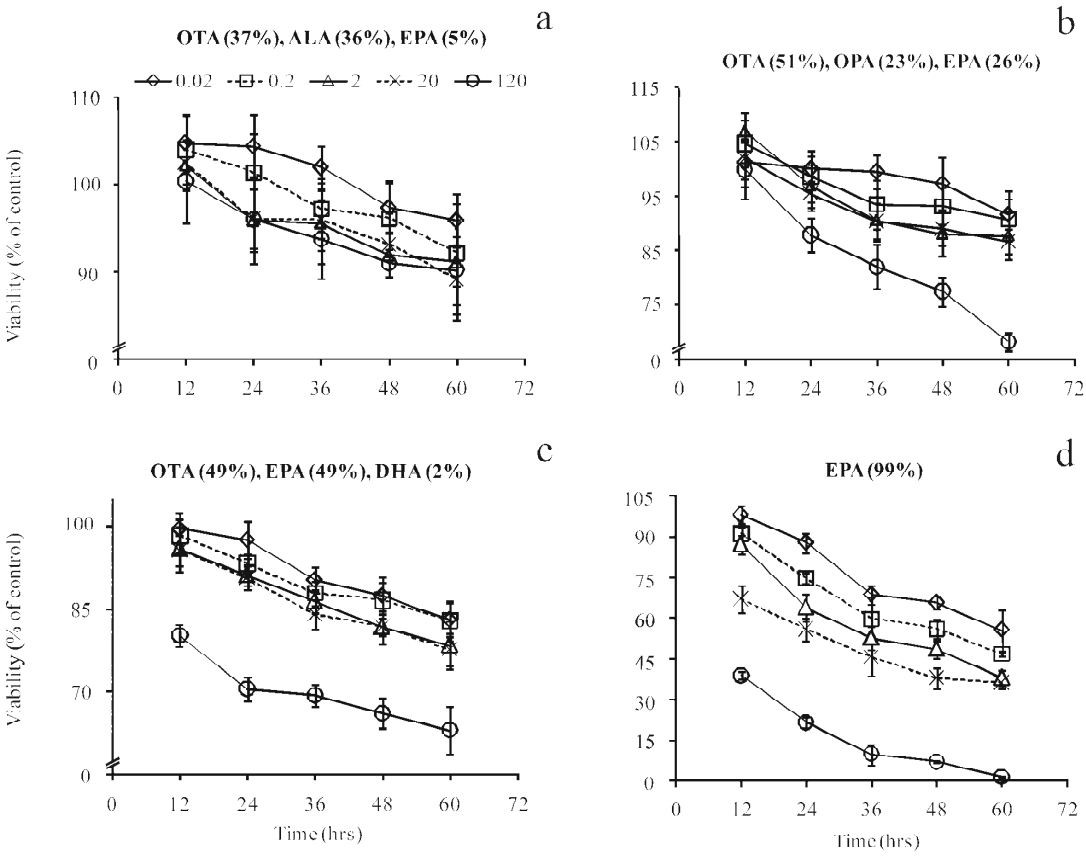

Figure 6.3. Toxicity of fatty acid fractions extracted from *Amphidinium carterae* containing varying concentrations of EPA: 5% (a), 26% (b), 49% (c). Toxicity of EPA was confirmed using a commercial preparation (d).

The evident physical damage of EPA towards gill cells was membrane disruption, especially at the highest concentration. Gill cells suffered loss of membrane, with holes observed by SEM on the surface of cells (Figs. 6.4 and 6.5). Gill cells have an elongated shape when are attached and gradually become spherical after trypsination for detachment. These two different shapes were observed when fixed for SEM observations.

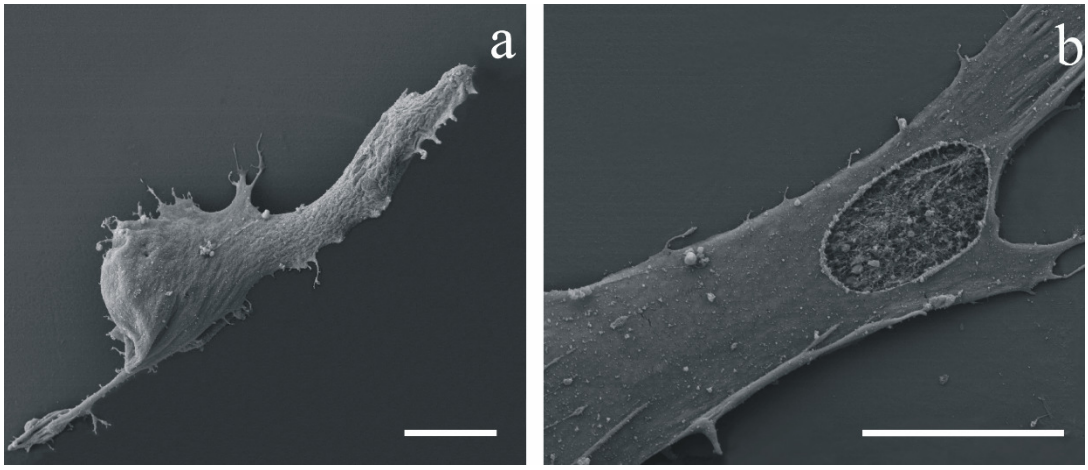

Figure 6.4. SEM micrographs of gill cells (elongated shape) after exposure to L-15/ex with 1% MeOH representing the control treatment (a), and EPA at 120 mg L^{-1} for 12 hrs (b). Scale bars 10 μm.

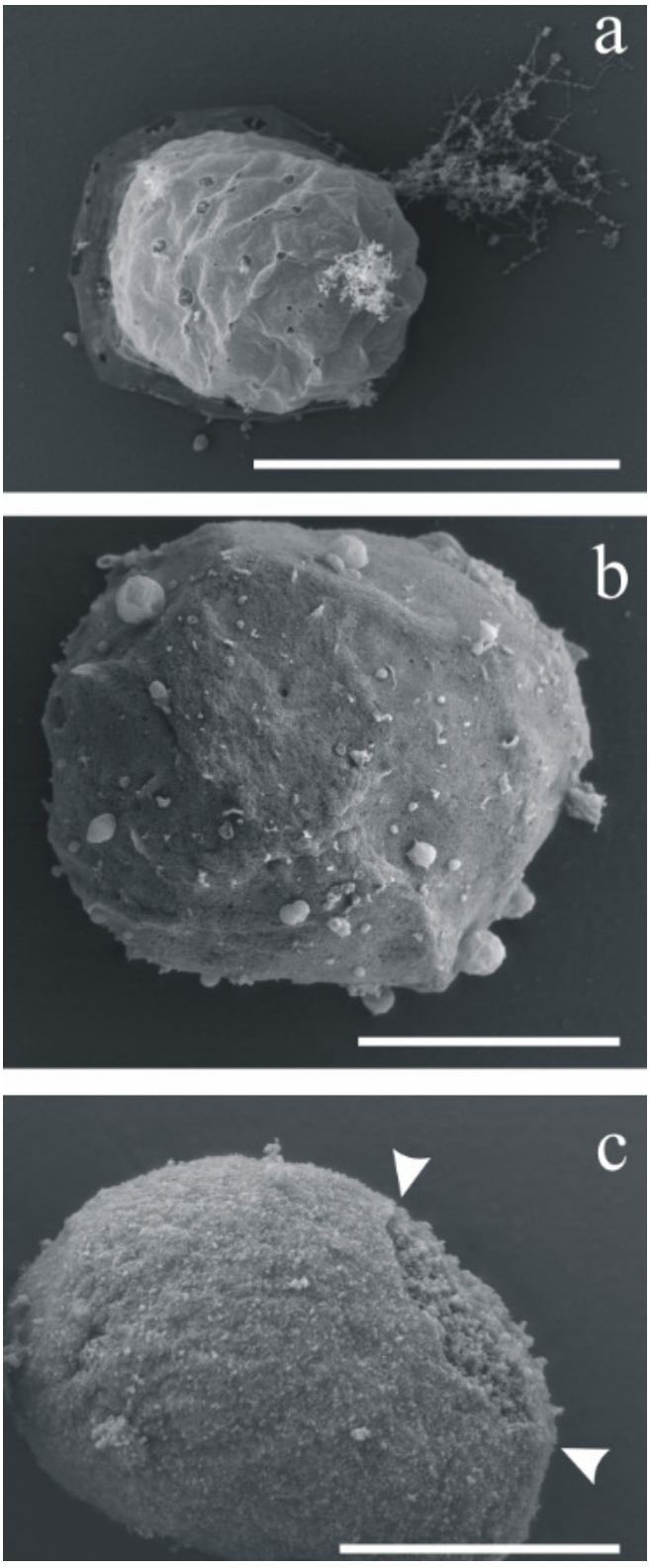

Figure 6.5. SEM micrographs of gill cells (spherical shape) after exposure to L-15/ex with 1% MeOH representing the control treatment (a, b), and EPA at 120 mg L^{-1} for 12 hrs (c). Arrows in figure c indicate where membrane disruption occurred. Scale bars 5 µm.

3.2. Toxicity of karlotoxin

No loss of gill cell viability occurred at karlotoxin concentrations of 0.1 and 1 ng mL^{-1}. Viability decreased slightly at 10 ng mL^{-1} but only at an exposure time of 5 hrs, with a loss of viability of 11%. Higher concentrations of KmTx2 had a higher toxic effect on gill cells. They were still 100% viable after 1-hr exposure to 100 ng mL^{-1}, but it decreased by 29% at 5 hrs. The highest effect was observed at 1000 ng mL^{-1}, where losses of viability ranged from 10 to 99% after 1 and 5 hrs of exposure, respectively (Fig. 6.6).

Considering the curve obtained from the exposure to KmTx2 at 1000 ng mL^{-1}, a median lethal time (LT_{50}) of 2.5 hrs was obtained. Indicating that half of the gill cell population losses viability at 2.5 hrs after exposure to 1000 ng mL^{-1} (Fig. 6.6). In a similar way, median lethal concentrations (LC_{50}) were calculated but only at exposure times of 3, 4 and 5 hrs, with losses of gill cell viability higher than 50%. LC_{50} values were 380, 293 and 203 ng mL^{-1} for 3, 4 and 5 hrs exposure times, respectively (Fig. 6.7).

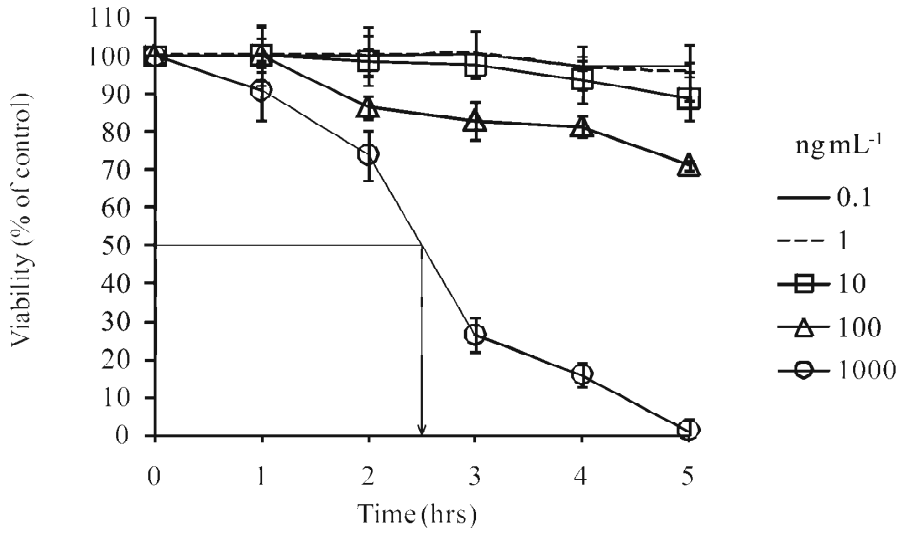

Figure 6.6. Toxicity of karlotoxin (KmTx2) towards gill cells at varying concentrations and exposure times. A median lethal time (LT_{50}, as indicated with the arrow) of 2.5 hrs was obtained for the highest concentration of KmTx2 of 1000 ng mL^{-1}. Bars represent standard deviations of quadruplicate measurements.

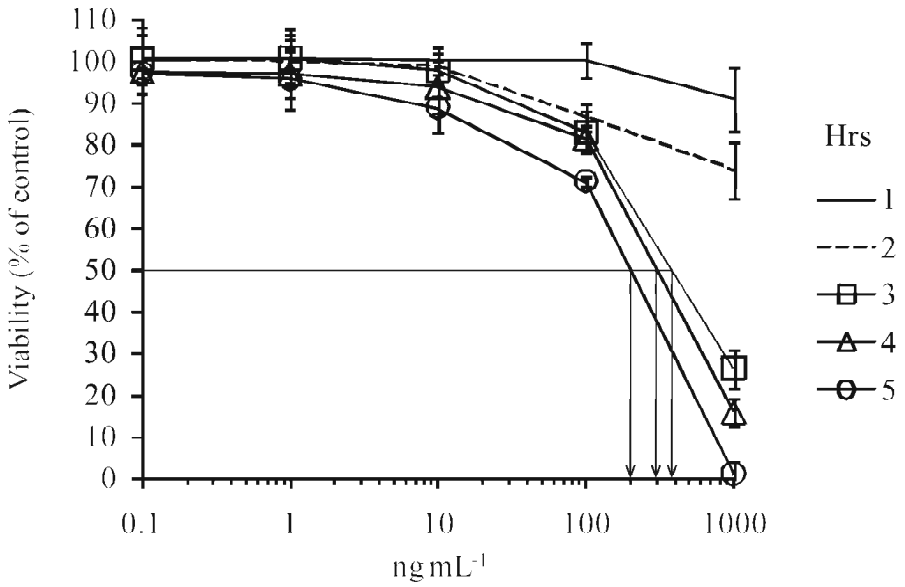

Figure 6.7. Toxicity of karlotoxin (KmTx2) towards gill cells at varying concentrations and exposure times. Median lethal concentrations (LC_{50}, as indicated with the arrows) of 380, 293 and 203 ng mL^{-1} were obtained for times of 3, 4 and 5 hrs (which exhibited losses of viability >50%). Bars represent standard deviations of quadruplicate measurements.

The effect of karlotoxin (KmTx2) was compared to toxicity (at 2 hrs) of intact and ruptured cells of *Karlodinium veneficum* KVSR01 (from the Swan River, Western Australia; data reported in chapter 2) as equivalent to KmTx2 content assuming that it contains 2.1 pg $cell^{-1}$ as reported by Mooney et al. (2009) for this strain. Therefore cell concentrations of *K. veneficum* ranging between 1×10^4 and 1×10^8 cells L^{-1} were equivalent to KmTx2 concentrations of 0.02 and 210 ng mL^{-1}, respectively. Cultures of *K. veneficum*, especially when cells were ruptured, exhibited a higher effect on gill cell viability than karlotoxin itself. KmTx2 had no effect on gill cells at concentrations of ≤10 ng mL^{-1}. However, cell viability decreased significantly at higher concentrations (up to 26% loss of viability at 1000 ng mL^{-1}). In contrast, algal cultures showed toxicity (13 – 32% loss of viability) at a low concentration of 1×10^4 cells L^{-1} equivalent to 0.021 ng mL^{-1} of KmTx2; and up to 38% loss of viability after exposure to ruptured algal cells at 1×10^8 cells L^{-1} equivalent to 210 ng mL^{-1} of karlotoxin (Fig. 6.8).

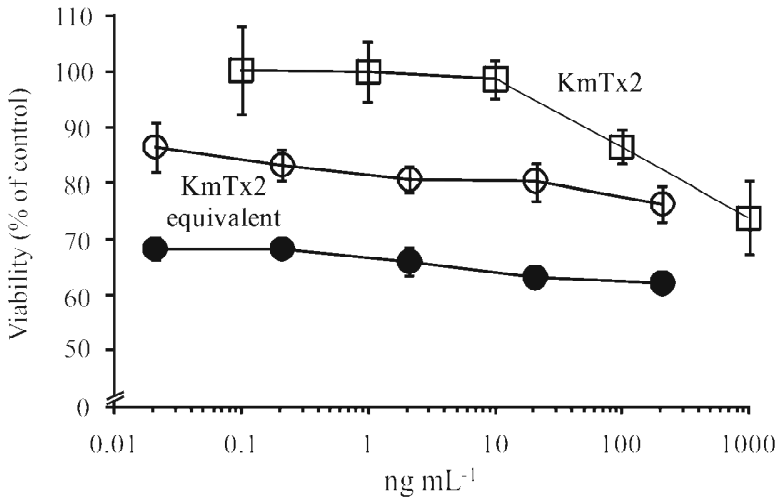

Figure 6.8. Toxicity of karlotoxin (KmTx2, squares), intact cells (open circles) and ruptured cells (solid circles) of *Karlodinium veneficum* KVSR01 (as in chapter 2) as equivalent to KmTx2 content according to Mooney et al. (2009) who reported a content of 2.1 pg cell^{-1} for this strain. Toxin concentrations of 0.02 and 210 ng mL^{-1} are equivalent to algal concentrations of 1×10^4 and 1×10^8 cells L^{-1}, respectively. All data were from exposures of 2 hrs. Bars represent standard deviations of quadruplicate measurements.

4. Discussion

The gill cell line RTgill-W1 was sensitive to the fatty acids produced by the toxic dinoflagellates *Karlodinium veneficum* and *Amphidinium carterae*. The positive effect (increase of viability) of pure OPA on the gill cells might be due to the ability of the gill cells to uptake certain fatty acids, as it has been previously reported that cultured fish cells are able to uptake OPA (Ghioni et al., 2001). Since we found this viability increase in the extract with pure OPA and a loss of viability in the extract with 72%-OPA and 26%-PA acid, we relate this negative effect to the latter, as it was also confirmed with the commercial preparation. Palmitic acid has caused cell death to some human melanoma cell lines via apoptosis and necrosis and it also decreased the number of viable cells of some other cell lines (de Sousa et al., 2005). The apparent toxic mechanism is the loss of membrane integrity and/or DNA fragmentation, as has been also found in hamster lung cells when exposed to palmitic acid and an inhibition of growth was observed. A large amount of triacylglycerol

accumulated in the cells caused large cytoplasmic clefts due to the cylindrical-solid deposits of triacylglycerol, indicating that the accumulation of solid lipid may perturb cell functions and injure cells. Thus the toxic effects of the saturated fatty acids were related to their physical properties (Urade and Kito, 1982).

On the other hand, *Amphidinium carterae* produced EPA together with OTA, OPA and ALA acids. OTA and EPA have been previously associated with harmful properties, including haemolysis (Fu et al., 2004; de Boer et al., 2009) and lipid peroxidation. Additionally, it has been suggested the occurrence of a synergistic effect between EPA (free fatty acid form) and the reactive oxygen species superoxide anion (O_2^-), causing a chain reaction and producing toxic byproducts through lipid peroxidation, and a faster mortality of fish was observed than when EPA on its own was used (Marshall et al., 2003). A loss of viability was observed in the four different combinations of fatty acids from *A. carterae* containing OTA, EPA or both. In the combinations where they occurred together, a higher viability loss was observed (up to 37%), suggesting a magnified toxic effect by the presence of both acids together. These results support the hypothesis of the harmful effect of the fatty acids OTA and EPA, which were extracted from *A. carterae* and the palmitic acid from *K. veneficum*. Besides, the commercial formula of EPA and the palmitic acid (free acids) helped to confirm their toxicity on the gill cells. It is important to consider that toxicity of fatty acids appears to be higher when they occur as free acids (Mooney et al., 2011, Marshall et al., 2003), which can be quickly produced upon cell disruption and their toxicity may be also due to oxidative degradation products (Jüttner 2001; 2005; chapter 7).

An exposure of sheepshead minnow larvae to the same fatty acid fractions used here showed that fish were affected, exhibiting sluggish swimming, gulping at the surface, inability to maintain an upright position in the water column and delayed movements; however, no fish mortalities occurred (Mooney et al., 2011). Thus since the gill cells RTgill-W1 did show loss of viability upon exposure to these fatty acid fractions, it indicates that they were more sensitive than whole fish. A possible cause of the different sensitivity may be due to the lack of well developed gills in larval fish. This is because gills are the main organ where a direct exchange between the external and internal sites of fish occurs; also, because gills of juvenile and adult fish

are much more specialized and perform more physiological functions (i.e. ion exchange, acid-base balance) than gills of larval fish (Rombough, 1999).

Karlotoxin, KmTx2 extracted from *Karlodinium veneficum*, also showed toxicity towards gill cells at concentrations of ≥100 ng mL^{-1} for exposures longer than 2 hrs. Median lethal concentrations (LC_{50}) of 380, 293 and 203 ng mL^{-1} were obtained for exposures of 3, 4 and 5 hrs, respectively. Finding that gill cells lost almost 100% of viability at 1000 ng KmTx2 mL^{-1} in 5 hrs. Mooney et al. (2010) found LC_{50} (at 2 hrs) of 508.2 – 563.2 ng mL^{-1} in sheepshead minnow larvae after exposure to KmTx2. In contrast, Deeds et al. (2006) found LC_{50} of ~760 (at 24 hrs) and ~310 ng mL^{-1} (at 6 hrs) in larvae and juveniles of zebrafish, respectively. Indicating that juveniles were much more sensitive than larvae since they died at ≥500 ng mL^{-1} in just 1 hr, whereas larvae did not die at this concentration but exhibited 80 – 100% mortality at ≥1000 ng mL^{-1} in 24 hrs. This suggests that the gill cell line RTgill-W1 appears to be more sensitive than these two fish species, and supporting the previous statement that fish need to have developed gills in order to be affected by KmTx2 as well, since this toxin damaged fish gills as shown in juvenile zebrafish by Deeds et al. (2006). On the other hand, karlotoxin KmTx2 was not as potent as cultures (as intact and ruptured cells) of *K. veneficum* were on gill cells; suggesting two possibilities (i) that apart from this toxin, this dinoflagellate possesses other toxic compounds, such as fatty acids, as found here as well; or (ii) there is a difference in potency between strains since the karlotoxin used here came from a different strain (CCMP 2778 from Florida, USA, 2005) than the one used for the exposure of whole cultures (KVSR01, from the Swan River, Western Australia, 2001). Or possibly and more likely, a combination of both may have occurred since it was shown here that fatty acids from this dinoflagellate exhibited toxicity, and also it has been demonstrated that different strains of *K. veneficum* showed varying toxicity (chapter 4), toxin content (4.3 vs 2.1 pg $cell^{-1}$ for CCMP 2778 and KVSR01, respectively) (Bachvaroff et al., 2009; Mooney et al., 2009), and since the strain CCMP 2778 contains twice more karlotoxin than KVSR01, it is probable that toxin potency is different as has been previously measured by hemolytic activity (Mooney et al., 2009).

In conclusion, fatty acid fractions from the two toxic dinoflagellates *Amphidinium cartarae* and *Karlodinium veneficum* showed significant toxicity towards gill cells, with evident membrane disruption and possible culprits being palmitic and eicosapentaenoic acids. The karlotoxin KmTx2 also exhibited toxicity towards gill cells, with a LC_{50} of 203 ng mL^{-1} at a 5-hr exposure, and 100% loss of cell viability at 1000 ng mL^{-1} at the same time. It appears that toxicity by *K. veneficum* is due to production of karlotoxins and fatty acids (mainly eicosapentaenoic acid in its free form).

Chapter 7*

Strain variability in fatty acid composition of *Chattonella marina* and its relation to differing toxicity

Abstract

Lipid profiles of three different geographic strains (Mexico, Australia, Japan) of *Chattonella marina* were studied under varying growth (phosphate, light and growth phase) and harvest (intact and ruptured cells) conditions. Triacylglycerol levels were always <2%, sterols <7%, free fatty acids varied between 2 and 33%, and polar lipids were the most abundant lipid class (>51% of total lipids). The major fatty acids in *C. marina* were palmitic (16:0), eicosapentaenoic (20:5ω3), octadecatetraenoic (18:4ω3), myristic (14:0) and palmitoleic (16:1ω7c) acids. Higher levels of EPA were found in ruptured cells (21.4 – 29.4%) compared to intact cells (8.5 – 25.3%). In general, Japanese N-118 *C. marina* was the highest producer of EPA (14.3 – 29.4%), and Mexican CMCV-1 the lowest producer (7.9 – 27.1%). Free fatty acids from *C. marina*, and the two aldehydes 2*E*,4*E*-decadienal and 2*E*,4*E*-heptadienal (suspected fatty acids-derived products) were tested against fish gill cells. Free fatty acid fractions, obtained by base saponification of total lipids from *C. marina* showed a potent toxicity towards gill cells (LC_{50} (at 1 hr) of 0.44 μg mL^{-1} in light conditions, with a complete loss of viability at >3.2 μg mL^{-1}). The aldehydes 2*E*,4*E*-decadienal and 2*E*,4*E*-heptadienal also showed high impact on gill cell viability, with LC_{50} (at 1 hr) of 0.34 and 0.36 μg mL^{-1}, respectively. These results indicate that *C. marina* is

*Submitted for publication to the Journal of Phycology as Dorantes-Aranda, J.J., Nichols, P.D., Waite, T.D. and Hallegraeff, G.M. Strain variability in fatty acid composition of *Chattonella marina* (Raphidophyceae) and its relation to differing ichthyotoxicity towards rainbow trout gill cells.

more toxic after cell disruption, possibly associated with a higher production of superoxide anion and EPA, which may be quickly oxidized to produce more toxic derivates, such as aldehydes.

1. Introduction

Marine microalgae produce polyunsaturated fatty acids (PUFA), some of which have been associated with ichthyotoxicity (Marshall et al., 2003; Henrikson et al., 2010; Mooney et al., 2011). Among these fatty acids, eicosapentaenoic acid (EPA, 20:5ω3) is produced in high amounts by fish-killing raphidophytes (Marshall et al., 2002b). Palmitic acid on the other hand, which is a saturated fatty acid (16:0), is common to most marine microalgae, including toxic and non-toxic dinoflagellates and raphidophytes, such as *Heterosigma akashiwo*, *Chattonella antiqua*, *C. marina*, *C. subsalsa*, *Karenia mikimotoi*, *Karlodinium veneficum*, and *Cochlodinium polykrikoides* (Dorantes-Aranda et al., 2009; Giner et al., 2008; Marshall et al., 2002b; Mooney et al., 2007; Nichols et al., 1987). Both EPA and palmitic acid have been conclusively documented to be toxic towards fish gill cells at ecologically relevant concentrations (Mooney et al., 2011; chapter 6).

The precise mechanism of how fatty acids cause ichthyotoxicity is still unclear. Some of the effects caused by fatty acids, when assessed *in vitro*, include haemolysis, loss of cell viability, cell growth inhibition, loss of membrane integrity and DNA fragmentation (Urade and Kito, 1982; Fu et al., 2004; de Boer et al., 2009; Dorantes-Aranda et al., 2011).

Fu et al. (2004) reported the PUFA octadecatetraenoic (OTA, 18:4ω3), eicosapentaenoic (EPA, 20:5ω3) and arachidonic (AA, 20:4ω6) acids from the ichthyotoxic raphidophyte *Fibrocapsa japonica* as the three main haemolytic compounds against human erythrocytes. De Boer et al. (2009) also associated haemolytic activity of *F. japonica* against rat erythrocytes as caused by these three fatty acids; however, the higher the haemolytic activity observed, the less it was associated with PUFA, suggesting that PUFA play a role, albeit only in part, in the

ichthyotoxicity of *F. japonica*. Other research with *Chattonella marina* suggested a synergistic effect between EPA (as free acid) and the reactive oxygen species, superoxide anion O_2-, causing faster fish mortalities in combination than when challenged against damselfish separately (Marshall et al., 2003).

Generation of aldehydes from fatty acids represents a chemical defense for some diatoms against grazers (Pohnert et al., 2002). Aldehydes that have shown toxicity to zooplankton predators include (in decreasing order of toxicity) 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal, decanal, undecanal and 2*E*,4*E*-heptadienal showing lethal effects (Taylor et al., 2005). Miralto et al. (1999) successfully isolated and identified the three aldehydes 2*E*,4*Z*,7*Z*-decatrienal, 2*E*,4*E*,7*Z*-decatrienal and 2*E*,4*E*-decadienal from the diatoms *Thalassiosira rotula*, *Skeletonema costatum* and *Pseudo-nitzschia delicatissima*, which caused low hatching success of eggs from copepods that fed on these diatoms.

Lipid production in microalgae can depend on nitrate concentration (Rodolfi et al., 2009), while phosphate also seems to influence lipid composition in phytoplankton with triacylglycerol (storage lipids) synthesis being triggered by phosphorus and nitrogen stress while polar lipids are reduced (Lombardi and Wangersky, 1991). Growth phase, which is related to nutrient stress, also affects lipid production of microalgae (Mansour et al., 2005), with a decrease in EPA production reported for diatoms during stationary phase (Brown et al., 1996).

The aim of this chapter was to characterize lipid classes, with special emphasis on fatty acids, of three strains of *Chattonella marina*, grown under different conditions of light and phosphate concentrations, and harvested at different growth phases. Free fatty acids derived from these algae and commercial aldehydes were tested for toxicity on gill cells and any correlation between lipid composition and variation in toxicity examined.

2. Methods

2.1. Culture of *Chattonella marina*

The three strains of *Chattonella marina* CMPL01 from Port Lincoln, South Australia, N-118 from the Seto Inland Sea, Japan, and CMCV-1 from Bahía Concepción, B.C.S., Mexico were used for this section (see Table 3.1 in chapter 3 for full details).

Chattonella marina was grown in duplicate in 500 mL Erlenmeyer flasks containing 200 mL of GSe medium (Blackburn et al., 1989) at 35 of salinity, enriched with either 20 or 200 μM K_2HPO_4, and maintained at 19°C with light intensities of 100, 300 or 500 μmol photons m^{-2} s^{-1} (cool white fluorescent lamps) under a 12/12-hr light/dark cycle. Cultures were harvested at three growth phases (different cultures were used for each phase). Mid-exponential phase was reached at day 8, early stationary at day 14 and late stationary at day 18. Another batch of cultures were grown in GSe with 200 μM K_2HPO_4 at 100 and 500 μmol photons m^{-2} s^{-1}, and harvested in the mid-exponential and early stationary growth phases. These cultures were subjected to sonication before harvesting in order to rupture the cells. Cultures were filtered through precombusted glass-fiber filters (Whatman GF/C). *Chattonella marina* was also grown in 4-L flasks containing 2 L of GSe medium with 200 μM K_2HPO_4 and exposed to 100 μmol photons m^{-2} s^{-1}. These cultures were harvested in the mid-exponential growth phase by centrifugation, and used for toxicity assays using fish gill cells. Cell counts were performed prior to harvesting in all cases using a Sedgwick-Rafter cell and an inverted light microscope. All samples were stored at -70°C in the dark until analysis.

2.2. Lipid extraction and analysis

Filters and pellets containing algae were extracted using a modified Bligh and Dyer (1959) method with a chloroform/methanol/water (1:2:0.7, v/v/v) single-phase solution. The samples were extracted overnight and the phases separated by adding chloroform and water (final chloroform/methanol/water ratio, 1:1:0.9, v/v/v). The

lower chloroform layer was drained and concentrated *in vacuo* using rotatory evaporation. Lipids were transferred to glass vials and stored at -20°C in the dark until analysis. This fraction represented the total lipid extract.

Lipid class abundance was determined using an Iatroscan MK V thin-layer chromatography-flame ionization detector (TLC-FID) analyzer (Iatron laboratories, Tokyo, Japan). Samples were spotted, using 1-µL disposable micropipettes, onto silica gel SIII chromarods (5 µm particle size). Chromarods were developed in a glass tank lined with pre-extracted filter paper for 25 min, containing a solvent mix of hexane/diethyl ether/acetic acid (60:17:0.1, v/v/v; Volkman and Nichols, 1991). Once the lipid classes were separated, the chromarods were oven dried (100°C, 10 min) and analyzed immediately to minimize adsorption of atmospheric contaminants. DAPA software (Kalamunda, Western Australia) was used to quantify the peaks for each lipid class. The FID was calibrated for each compound class with phosphatidylcholine, cholesterol, oleic acid, hydrocarbon (squalene), wax ester (derived from fish oil) and triacylglycerol (TAG, derived from fish oil).

For all samples an aliquot of the total lipid was trans-methylated in methanol/chloroform/hydrochloric acid (10:1:1, v/v/v) for 2 hrs at 85°C. After addition of water, the mixture was extracted three times with hexane/chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME) which were concentrated under a stream of nitrogen gas. An aliquot of the total lipid extract from the three *C. marina* strains corresponding to mid-exponential growth phase cultures grown in GSe with 200 µM K_2HPO_4 under 100 µmol photons m^{-2} s^{-1}, was saponified using 5% KOH in MeOH/mQH_2O, and heated for 3 hrs at 85°C. Free sterols were extracted three times with hexane/chloroform (4:1, v/v) and converted to their trimethylsilyl ethers adding N,O-bis(trimethylsilyl)trifluoroacetamide (BST-FA). All total lipid extracts were trans-methylated using MeOH/HCl/$CHCl_3$ (10:1:1, v/v/v; 85°C, 2 hrs). After cooling, milli-Q water was added and then the free fatty acids were extracted three times with hexane/chloroform (4:1, v/v).

Samples were made up with a known volume of the internal injection standard (19:0 FAME), and analyzed by gas chromatography using an Agilent Technologies 7890N GC (Palo Alto, Ca, USA) equipped with an Equity-1 cross-

linked methyl silicone fused silica capillary column (15 m × 0.1 mm i.d.), and an FID. Helium was used as the carrier gas. Samples were injected by a split/splitless injector and an Agilent Technologies 7683B Series autosampler in splitless mode at an oven temperature of 120°C. After 1 min, the oven temperature was increased to 270°C at 10°C min^{-1}, then to 310°C at 5°C min^{-1}. FAME and sterols were identified by comparison with retention time of authentic and laboratory standards. Gas chromatography-mass spectrometry (GC-MS) analyses were performed for selected samples for confirmation of sterol and FAME identification using a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, TX, USA). The GC was fitted with a capillary column of similar polarity to that described previously.

2.3. *In vitro* assays

2.3.1. Exposure of gill cells to free fatty acids

Free fatty acids (see Fig. 7.1 for chemical structure) extracted from *Chattonella marina* after base saponification of the total lipids, were tested on fish gill cells to assess their impact on cell viability. Gill cells were treated and seeded as specified in chapter 2, section 2.3.2. The free fatty acid fractions (Table 7.1) were dissolved in MeOH and mixed with L-15/ex medium (Schirmer et al., 1997) for the exposure assays. The final concentration of MeOH was 1 % (v/v). The gill cells were washed with phosphate buffer saline (PBS) and exposed to the free fatty acid fractions for 1, 2, 3 and 4 hrs at 19°C in light (100 μmol photons m^{-2} s^{-1}) and dark conditions simultaneously. Viability was measured in all conditions as per chapter 2, section 2.2.

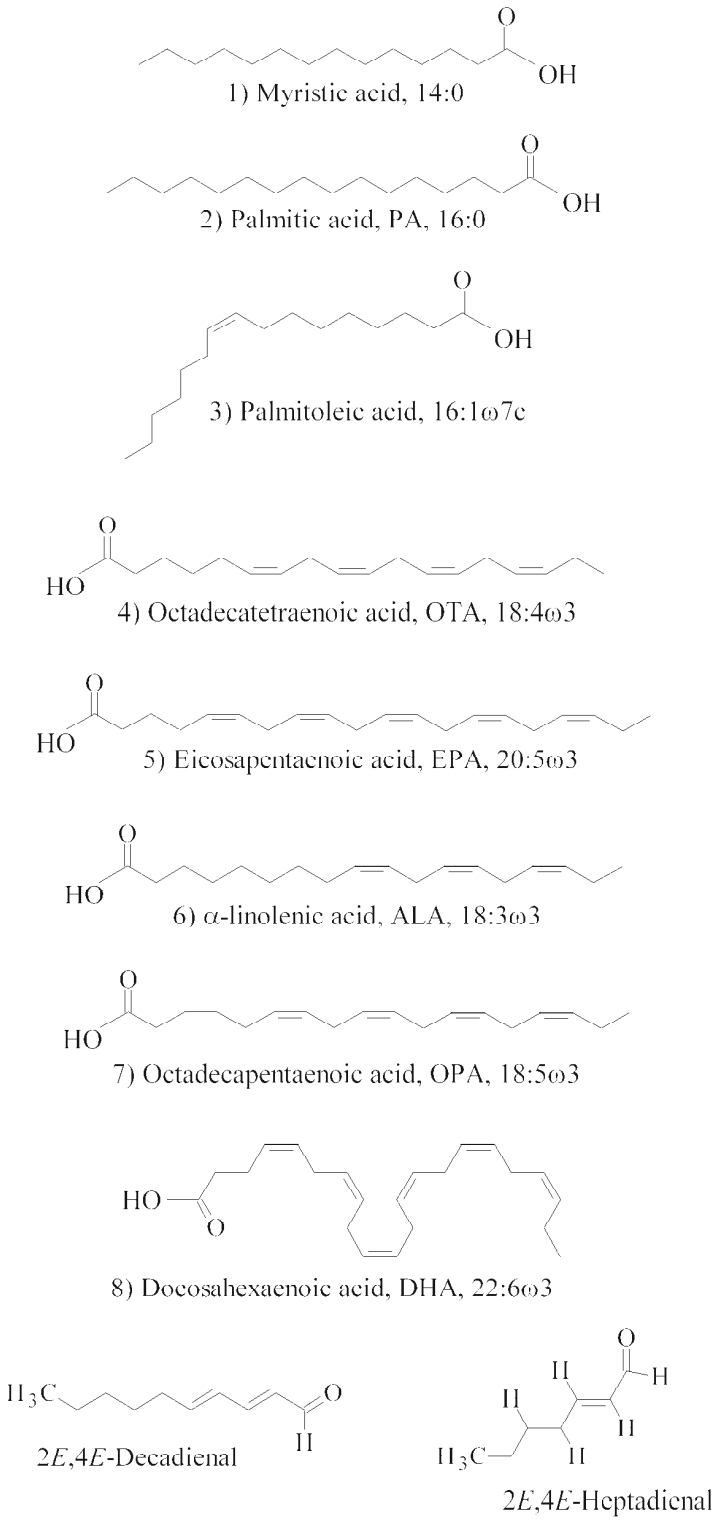

Figure 7.1. Chemical structures of major (1 – 5) and some minor (6 – 8) fatty acids from *Chattonella marina*, and two commercial aldehydes (2*E*,4*E*-Decadienal and 2*E*,4*E*-Heptadienal) that were tested on gill cells for toxicity. Toxicity of the three minor fatty acids (6 – 8) was documented in chapter 6.

Table 7.1. Concentrations (μg mL^{-1}) of major fatty acids from extracts of three *C. marina* strains tested on gill cells.

Common name	Lipid numbers	CMCV-1	CMPL01	N-118
Myristic	14:0	0.7	0.7	0.4
Palmitic	16:0 PA	1.4	1.6	1.4
Palmitoleic	16:1ω7c	0.4	0.7	0.5
Ocatadecatetraenoic	18:4ω3 OTA	1.1	1.0	1.2
Eicosapentaenoic	20:5ω3 EPA	1.0	1.0	1.0

2.3.2. Exposure of gill cells to aldehydes

It has been reported that some polyunsaturated fatty acids can be oxidized to produce by-products, denominated oxylipins (compounds derived from oxidative transformation of fatty acids), including aldehydes (Kieber et al., 1997; Pohnert et al., 2002). Some of the aldehydes isolated from diatoms and haptophytes include decadienals, decatrienals, heptadienals and octadienals, which have shown adverse effects on invertebrates (Miralto et al., 1999; Pohnert et al., 2002; Hansen et al., 2004; Jüttner, 2005). For this reason, two commercial preparations of aldehydes were tested for toxicity.

The aldehydes 2*E*,4*E*-decadienal (180513, Sigma) and 2*E*,4*E* heptadienal (180548, Sigma) were tested on the gill cells at concentrations ranging between 0.011 and 15.2 μg mL^{-1} at 1, 2, 3 and 4 hrs in dark and light conditions at 19°C. See Fig. 7.1 for chemical structure of these aldehydes. Gill cells were seeded as described previously. Aldehydes were dissolved in ethanol and mixed with L-15/ex medium (final EtOH concentration of 1%, v/v) to achieve each experimental concentration. Cell viability was measured in all conditions.

2.3.3. Data analysis

Data were subjected to analysis of variance (ANOVA). Normality of the data was tested with the Kolmogorov-Smirnov method and homogeneity of variances was assessed with Hartley's F_{max} tests. When multiple comparisons were required, the *a posteriori* Tukey test was performed to determine any difference among treatments. Principal component analysis (PCA) was performed for fatty acids content results. The significance level considered in all statistical tests was 95% (α=0.05). The software Statistica 8.0 was used for all statistical analyses.

3. Results

3.1. Lipid profiles for three *Chattonella marina* strains

3.1.1. Lipid classes

Five lipid classes were found in *Chattonella marina*, including triacylglycerols (TAG), free fatty acids (FFA), sterols (ST), diacylglycerols (DAG) and polar lipids (PL). The most abundant class in all strains was polar lipids (includes glyco- and phospholipids), ranging between 51 and 94% of the total lipid content (Table 7.2). Polar lipids were generally higher in the mid-exponential growth phase (88.3 – 94%) for all strains compared to early (51 – 89.1%) and late stationary phases (75 – 93.4%). In contrast, diacylglycerol levels in *C. marina* were low, representing no more than 2.6% of total lipids.

3.1.2. Triacylglycerols

Relative levels of triacylglycerol in *Chattonella marina* were generally low with some variation observed among the three strains. Mexican CMCV-1 showed similar TAG levels in the mid-exponential (0.3 – 1.2%) and early stationary growth phases (0.2 – 1.9%), with higher levels in ruptured cells. Australian CMPL01 did not show any changes between growth phases either, but Japanese N-118 contained, in

most cases, lower levels of TAG in the mid-exponential phase (0.1 – 1.7%) than in the stationary phase (0.3 – 10.4%). When comparing between nutrient concentrations, CMCV-1 showed higher TAG at 200 μM K_2HPO_4 (0.6 – 1.2%) than at 20 μM (0.3 – 0.4%) in the mid-exponential phase. TAG levels were similar in the early and late stationary phases. Australian CMPL01 contained lower TAG at 20 μM, especially in the mid-exponential growth phase. Japanese N-118 had higher levels of TAG at 20 μM in the mid-exponential phase, with a shift observed at 200 μM in the early stationary phase, especially at the highest light intensity of 500 μmol photons m^{-2} s^{-1}, where TAG increased up to 10.4% for ruptured cells (Table 7.2).

Table 7.2. Lipid class profile (as % of total lipids) of three strains of intact and ruptured ([a]) cells of *Chattonella marina* harvested in the mid-exponential and early stationary growth phases as a function of light and phosphate (n=2). Highest values of free fatty acids for each growth phase are in bold and shaded numbers. TAG, triacylglycerols; FFA, free fatty acids; ST, sterols; PL, polar lipids.

Mid-exponential phase													
µmol photons m^{-2} s^{-1}	µM K_2HPO_4	Mexican CMCV-1				Australian CMPL01				Japanese N-118			
		TAG	FFA	ST	PL	TAG	FFA	ST	PL	TAG	FFA	ST	PL
100	20	0.4	5.5	1.2	92.4	0.3	3.4	1.6	93.8	0.3	2.7	1.8	93.2
	200	0.6	6.3	1.8	91.1	0.6	4.1	1.8	91.1	0.1	4.1	2.4	91.6
	200[a]	0.6	3.8	2.9	91.9	0.6	4.6	3.5	90.6	0.6	3.7	0.7	94.3
300	20	0.3	3.8	2.4	93.1	0.2	3.8	3.1	91.4	1.3	4.1	2.0	91.0
	200	1.2	6.1	1.5	91.1	0.4	4.8	2.4	91.3	0.4	4.5	2.7	90.6
500	20	0.3	4.4	3.2	91.6	0.2	4.9	4.7	88.3	1.7	4.1	2.3	90.9
	200	0.6	**6.5**	3.5	89.1	0.5	5.9	3.8	88.3	1.2	7.7	3.0	86.5
	200[a]	0.7	2.0	5.1	90.9	1.7	**11.4**	2.3	83.4	1.2	**10.2**	4.7	82.5
Early stationary													
100	20	0.3	5.9	3.6	88.9	0.5	11.7	3.2	84.4	0.6	7.1	1.7	86.7
	200	0.2	9.0	3.9	81.9	0.7	10.1	2.6	82.0	0.8	9.5	3.3	86.1
	200[a]	1.6	12.0	1.1	84.8	0.9	12.3	3.4	83.1	1.0	10.8	1.2	85.9
300	20	0.2	9.0	5.6	83.0	0.4	7.1	5.3	85.5	0.3	7.0	3.3	89.1
	200	0.2	**13.7**	4.6	80.3	0.5	14.4	3.7	78.0	0.5	12.2	2.5	83.4
500	20	0.3	7.4	4.9	86.0	0.4	15.4	4.0	79.0	1.1	17.0	5.9	74.8
	200	0.3	9.8	5.4	83.2	0.4	10.2	6.2	81.3	1.0	11.1	4.3	83.4
	200[a]	1.9	12.0	4.0	81.8	0.8	**16.3**	6.6	76.1	10.4	**33.2**	2.5	50.8

3.1.3. Sterols

Three duplicate samples of each strain corresponding to cultures grown in GSe medium at 200 μM K_2HPO_4 under 100 μmol photons m^{-2} s^{-1} and harvested in the mid-exponential phase were analysed for sterol composition. 24-Ethylcholesterol was the major sterol in *C. marina*, ranging between 74.1 and 84%. CMCV-1 was the strain with the highest content of this sterol, with 47.7 pg $cell^{-1}$. In contrast, CMPL01 and N-118 showed a lower content (~36 pg $cell^{-1}$). Isofucosterol ranged between 9.9% for CMCV-1 and 18.2% for N-118 (Table 7.3). Cholesterol was the sterol in lowest relative levels, with similar levels in all strains (1.2 – 1.6%).

Table 7.3. Sterol composition (% of total sterols with standard deviations) in *Chattonella marina* grown in GSe medium with 200 μM K_2HPO_4 under 100 μmol photons m^{-2} s^{-1}, and harvested in the mid-exponential phase. Strain samples with [a] are from Marshall et al. (2002b). Numbers in parenthesis indicate sterol content in pg $cell^{-1}$.

Sterol	**CMCV-1**	**CMPL01**	**N-118**	**CMPL01[a]**	**N-118[a]**
Cholesterol	1.2±0.3 (0.7)	1.6± 0.0 (0.7)	1.6±0.1 (0.8)	17.1	22.9
24-Dihydrozymosterol	5.0±1.0 (2.9)	4.0±0.5 (1.7)	6.1±1.2 (3.0)	4.5	2.9
24-Ethylcholesterol	84.0±1.7 (47.7)	84.3±0.7 (36.7)	74.1±1.2 (36.0)	68.4	64.0
Isofucosterol	9.9±0.3 (5.6)	10.1±0.1 (4.4)	18.2±0.3 (8.9)	2.3	3.6
Total pg $cell^{-1}$	10.8	6.4	8.4	9.8	8.3

3.1.4. Free fatty acids

The relative abundance of FFA in the total lipid extract of samples was variable, generally ranging between 2.7 to 18.3%. However, a culture of ruptured cells of the Japanese N-118 strain grown under high light intensity of 500 μmol photons m^{-2} s^{-1} showed significantly higher FFA levels (33.2%) (Table 7.2). FFA

levels (%) and content (pg cell^{-1}) were usually lower in the mid-exponential growth phase for the three strains, and higher in the early and late stationary phases. Mexican CMCV-1 showed a higher content of FFA in the mid-exponential growth phase compared to CMPL01 and N-118. Phosphate concentration in culture media did not influence FFA levels in the mid-exponential phase, except for ruptured cells of Australian CMPL01 and Japanese N-118 strains grown at 200 μM and high light intensity. Stationary phases showed higher FFA levels and content. High light intensities affected Japanese N-118 in the early and late stationary phases, where higher FFA levels and content were observed (Table 7.2).

3.1.5. Fatty acid composition

The main fatty acids produced by the three *C. marina* strains were myristic (14:0), palmitic (16:0, PA), palmitoleic (16:1ω7), octadecatetraenoic (18:4ω3, OTA), and eicosapentaenoic (20:5ω3, EPA) acids. The saturated palmitic acid was the most abundant of all fatty acids (21.4 – 35.8%), followed by the polyunsaturated fatty acids octadecatetraenoic and eicosapentaenoic (8.5 – 25.3%) for samples from intact cells. The three strains showed similar levels of PA grown at low phosphate concentrations (20 μM), and at low light intensities for the Mexican and Australian strains (100 μmol photons m^{-2} s^{-1}), and at high light intensity for the Japanese strain (500 μmol photons m^{-2} s^{-1}). EPA abundance was also similar for the three strains, but with the highest relative levels (22.3 – 25.3%) in cultures grown at high phosphate concentrations (200 μM) and high light intensities (300 to 500 μmol photons m^{-2} s^{-1}) (Table 7.4). Ruptured algal cells showed higher levels of EPA (up to 29.4%) (Table 7.5).

Table 7.4. Fatty acid composition of three *Chattonella marina* strains from different geographical locations (CMCV-1 Mexico, CMPL01 Australia, and N-118 Japan). These data include those culture conditions (intact cells) where the strains had the highest levels of palmitic (PA, 16:0) and eicosapentaenoic (EPA, 20:5ω3), as shown in bold numbers.

Strain	CMCV-1		CMPL01		N-118	
μmol photons m^{-2} s^{-1}	100	500	100	500	500	300
μM K_2HPO_4	20	200	20	200	20	200
Growth phase	E-Stat	E-Stat	L-Stat	L-Stat	L-Stat	E-Stat
Saturates						
14:0	12.6	8.9	12.7	9.3	7.1	6.5
16:0 PA	**34.3**	24.5	**35.8**	25.5	**31.1**	21.4
18:0	2.4	2.7	3.3	1.7	4.1	1.9
Monounsaturates						
16:1ω7c	8.6	5.2	12.3	7.0	6.0	6.3
16:1ω13trans	5.4	3.9	6.0	3.3	3.5	4.6
18:1ω9c	4.0	2.8	3.5	3.1	3.1	2.7
18:1ω7c	1.0	1.1	0.8	0.4	0.6	0.7
Polyunsaturates						
18:4ω3 OTA	8.1	11.8	5.9	10.9	12.0	15.0
18:2ω6 LA	3.7	2.7	2.5	2.6	2.6	3.3
18:3ω3 ALA	3.2	4.0	2.2	3.0	2.6	3.0
20:4ω6 ARA	2.8	3.5	1.3	1.9	2.3	3.3
20:5ω3 EPA	8.5	**22.3**	8.8	**23.5**	20.1	**25.3**
22:5ω6 DPA	1.1	2.2	1.1	1.7	1.0	1.5
22:6ω3 DHA	0.6	1.5	1.0	3.0	1.0	1.2
Sum SFA	49.8	36.5	52.9	37.5	42.9	30.1
Sum MUFA	20.5	13.8	23.5	14.9	14.2	15.4
Sum PUFA	29.7	49.7	23.6	47.6	42.9	54.5

Mean of duplicate cultures and samples for each treatment. The fatty acids 20:0, 22:0, 24:0, 14:1ω5, 16:1ω9, 17:1ω8, 18:1ω5, 18:1, 20:1ω11, 18:3ω6, 20:3ω6, 20:4ω3, 20:2ω6, 22:4ω6, 22:5ω3 were ≤1.0% and were not included. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids (all double bonds of *cis* geometry unless otherwise stated); PUFA, polyunsaturated fatty acids. E-Stat and L-Stat, early and late stationary phase, respectively.

Table 7.5. Comparison of fatty acid composition (as % of total fatty acids) in *Chattonella marina* grown in GSe containing 200 µM K_2HPO_4 under 100 µmol photons m^{-2} s^{-1}, and harvested in the mid-exponential and early stationary growth phases. Strain codes with [a] are from mid-exponential and intact cell cultures, [b] are from mid-exponential and ruptured cell cultures, [c] are from early stationary and ruptured cells cultures, and [d] from Marshall et al. (2002b). Values represent means of duplicate cultures and samples for each treatment. See Table 7.4 footnotes.

	CMCV-1[a]	CMCV-1[b]	CMCV-1[c]	CMPL01[a]	CMPL01[b]	CMPL01[c]	CMPL01[d]	N-118[a]	N-118[b]	N-118[c]	N-118[d]
Saturates											
14:0	10.8	8.5	7.5	10.5	8.1	8.9	11.9	6.0	6.1	5.6	5.4
16:0, PA	21.8	20.9	22.7	24.2	21.3	20.9	22.3	23.3	20.7	20.5	21.2
18:0	3.6	1.3	1.1	1.7	0.7	0.7	0.8	1.9	0.6	0.5	1.6
Monounsaturates											
16:1ω7c	6.3	5.7	5.7	10.3	7.1	6.2	9.4	8.1	7.1	6.3	10.1
16:1ω13trans	3.3	3.1	2.5	3.7	2.9	2.3	0.2	4.7	5.1	3.2	2.6
18:1ω9c	2.9	2.5	2.8	2.8	2.5	3.1	4.5	2.7	2.3	2.1	9.9
18:1ω7c	0.7	0.9	0.6	0.6	0.6	0.4	1.7	0.4	0.5	0.3	1.7
Polyunsaturates											
18:4ω3 OTA	17.6	18.6	14.3	15.2	18.4	13.7	13.7	19.2	20.5	17.8	12.7
18:2ω6 LA	4.0	3.2	3.1	2.6	2.5	1.9	1.9	3.8	3.1	2.8	1.5
18:3ω3 ALA	3.7	3.7	4.0	2.9	3.5	4.6		3.2	3.3	3.1	
20:4ω6 ARA	3.8	3.3	1.4	2.2	1.9	1.4	1.7	3.4	3.7	2.3	5.5
20:5ω3 EPA	**15.6**	**21.4**	**27.1**	**15.9**	**23.0**	**27.4**	**22.9**	**16.9**	**21.5**	**29.4**	**18.5**
22:5ω6 DPA	1.6	2.1	2.0	2.0	2.4	2.6	2.4	1.9	1.5	1.6	1.4
22:6ω3 DHA	1.0	1.6	1.9	1.9	2.7	3.5	3.4	1.2	1.2	1.6	3.3
Sum SFA	36.2	30.7	31.3	36.4	30.1	30.5	35.0	31.2	27.4	26.6	28.2
Sum MUFA	13.2	12.2	11.6	17.4	13.1	12.0	15.8	15.9	15.0	11.9	24.3
Sum PUFA	47.3	53.9	53.8	42.7	54.4	55.1	46.0	49.6	54.8	58.6	42.9

A principal component analysis of fatty acid composition of the three geographic strains is presented in Fig. 7.2. Australian CMPL01 mostly grouped in the first quadrant (top left), Japanese N-118 in the third quadrant (bottom left), and Mexican CMCV-1 in the second and fourth quadrants (top right and bottom right, respectively). Some cultures of N-118 corresponding to stationary phase fell in quadrant 1, and were therefore more related to CMPL01, especially the culture grown under high light intensity and high phosphate concentration, which produced significantly higher amounts of FFA (Table 7.2). Other cultures from the mid-exponential phase of N-118 fell in quadrant 3, more related to CMCV-1. One culture of CMPL01 from low phosphate and low light fell in quadrant 2, separated from the rest of the samples of this strain, corresponding to a culture with the lowest FFA levels and very low EPA abundance. In general, CMPL01 exhibited the least variation under all environmental conditions (Fig. 7.2).

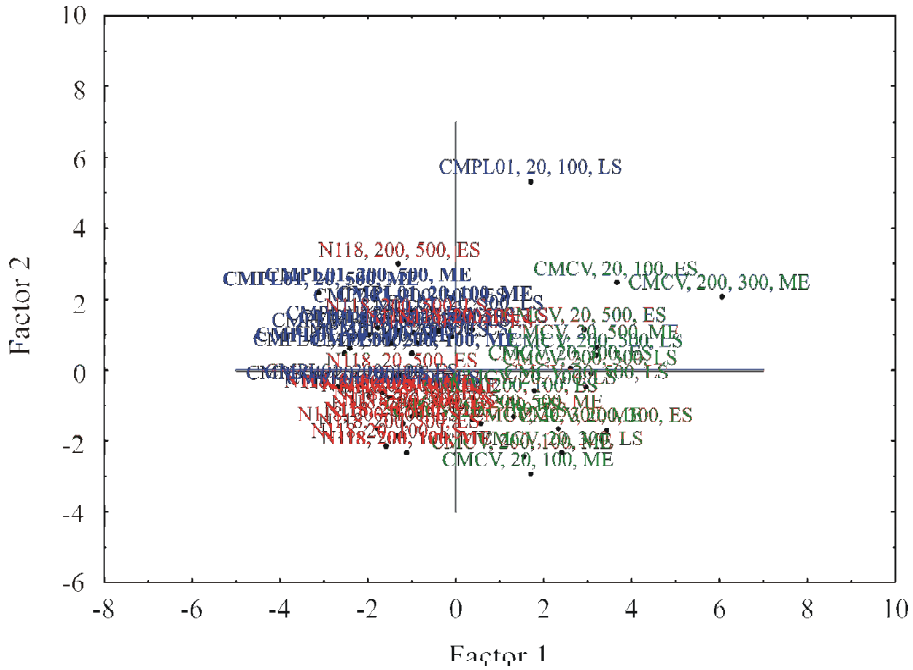

Figure 7.2. Principal component analysis of fatty acid composition (as % of total fatty acids) of three strains of *Chattonella marina* harvested at three different growth phases. CMCV-1, Mexican (green); CMPL01, Australian (blue); N-118, Japanese (red). The first number after the strain code represents the phosphate concentration in µM, followed by the light intensity in µmol photons m^{-2} s^{-1} in which cultures were

grown, and finally the growth phase in which cultures were harvested: ME mid-exponential, ES early stationary, and LS late stationary. Ruptured cells of CMPL01 and N-118 in the mid-exponential growth phase were the most toxic to RTgill-W1 cells as shown in chapter 3.

3.2. Toxicity of free fatty acid fractions and aldehydes towards RTgill-W1 cells

Three free fatty acid fractions extracted from *Chattonella marina* were obtained after base saponification of the total lipids from 2-L cultures (one from each strain), and tested on the gill cells. Palmitic acid showed the highest abundance (1.4 – 1.6 μg mL^{-1}), followed by octadecatetraenoic (OTA, 1.0 – 1.2 μg mL^{-1}) and eicosapentaenoic acid (EPA, 1.0 μg mL^{-1}) (Table 7.1).

The origin of the fatty acid fractions had no significant influence on gill cell viability. The lowest concentration tested, 0.06 μg mL^{-1}, did not affect the gill cells during the 4-hr exposure. However, cell viability decreased ~50% and 85% after 1-hr exposure to 0.79 μg mL^{-1} in dark and light conditions, respectively. Exposure in light conditions also had a higher impact at 1.58 μg mL^{-1}; gill cells were almost 0% viable at concentrations higher than 3.17 μg mL^{-1} in both dark and light conditions at 1 hr. Exposures for more than 2 hrs resulted in complete loss of viability of gill cells at concentrations higher than 0.79 μg mL^{-1} in either dark or light conditions (Fig. 7.3).

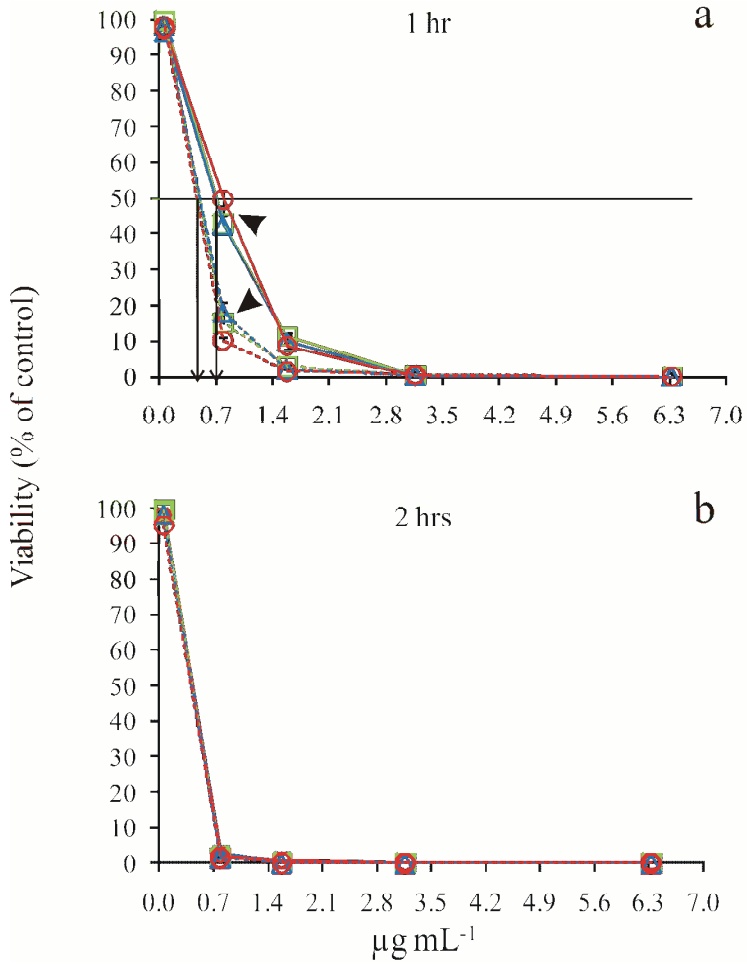

Figure 7.3. Exposure of gill cells to free fatty acid fractions from *Chattonella marina* strains: CMPL01 (Δ blue), CMCV-1 (□ green) and N-118 (o red) for 1 hr (a) and 2 hrs (b). The exposure was carried out simultaneously in light (dashed line) and dark (solid line) conditions. Arrows show differences between light and dark conditions at 0.79 µg mL^{-1}. LC_{50} values at 1-hr exposure were 0.44 and 0.68 µg mL^{-1} for light and dark conditions, respectively. Symbols represent the mean and bars the standard deviation of cell viability from quadruplicate wells containing the free fatty acid fractions.

The commercial preparations of the aldehydes 2*E*,4*E*-decadienal and 2*E*,4*E*-heptadienal (putative FFA oxidation products) had no effect on gill cells at concentrations of 0.015 and 0.011 µg mL^{-1}, respectively, during the first 2 hrs of exposure. However, cell viability decreased by 87% and 73% after 4 hrs of exposure

to these concentrations of decadienal and heptadienal, respectively. Heptadienal was less toxic than decadienal at ≤0.015 μg mL^{-1} during the first hour, but both aldehydes decreased cell viability by 99% at concentrations >1.1 μg mL^{-1}. These aldehydes showed similar toxicity at 4 hrs at all concentrations, with loss of viability greater than 96% at concentrations of ≥0.11 μg mL^{-1}. LC_{50} calculations for decadienal and heptadienal at 1 hr were 0.34 and 0.36 μg mL^{-1}, respectively (Fig. 7.4). As shown in Figure 7.5, both decadienal and heptadienal had a similar effect on gill cells at 0.015 and 0.011 μg mL^{-1}, respectively, during all exposure times. In contrast, decadienal was more toxic than heptadienal at 0.15 and 0.11 μg mL^{-1} between 1 and 3 hrs of exposure. LT_{50} values for decadienal and heptadienal when exposed to a concentration of 0.015 and 0.011 μg mL^{-1}, respectively, were 3.5 hrs and 3.6 hrs, respectively. However, gill cells lost viability faster at 0.15 and 0.11 μg mL^{-1} for decadienal and heptadienal, with LT_{50} of 1.9 hrs and 2.5 hrs, respectively (Fig. 7.5).

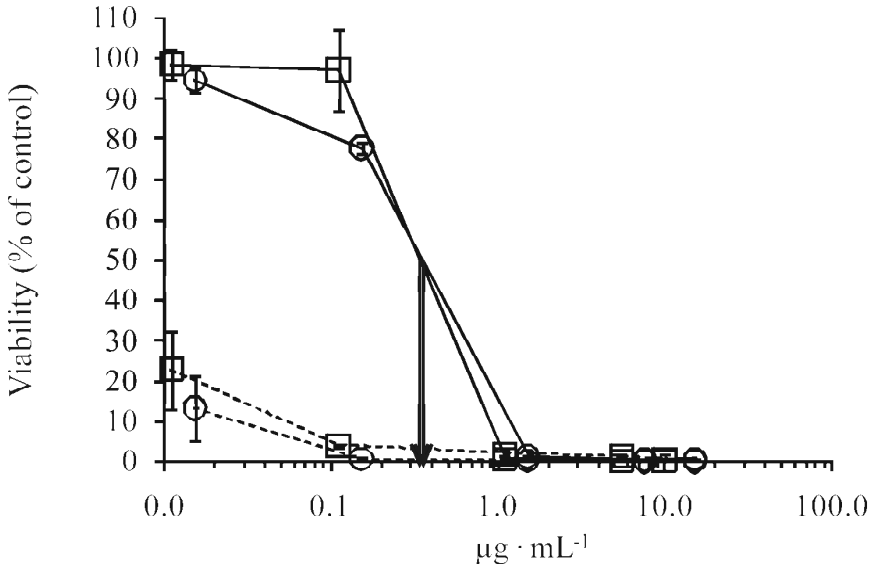

Figure 7.4. Exposure of gill cells to 2*E*,4*E*-decadienal (o) and 2*E*,4*E*-heptadienal (□) for 1 hr (solid line) and 4 hrs (dashed line). LC_{50} at 1 hr for decadienal and heptadienal was 0.34 and 0.36 μg mL^{-1}, respectively (as indicated by arrows in "x" axis). Symbols represent the mean and bars the standard deviation of cell viability from quadruplicate wells containing the aldehydes.

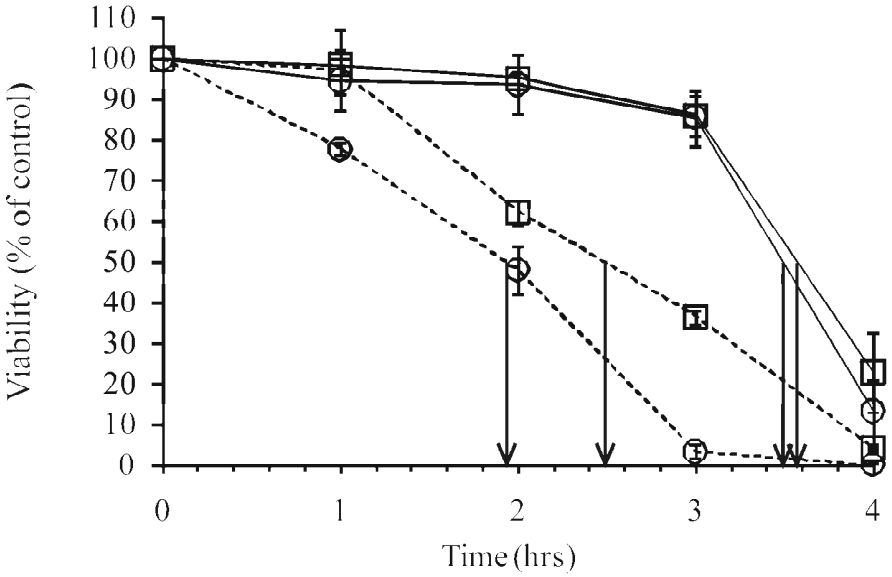

Figure 7.5. Exposure of gill cells to 2*E*,4*E*-decadienal (o) and 2*E*,4*E*-heptadienal (□) at concentrations of 0.015 and 0.011 µg mL^{-1}, respectively (solid line for both), and 0.15 and 0.11 µg mL^{-1}, respectively (dashed line for both). LT_{50} for decadienal and heptadienal was 3.5 hrs and 3.6 hrs at 0.015 and 0.011 µg mL^{-1}, respectively, and 1.9 hrs and 2.5 hrs at 0.15 and 0.11 µg mL^{-1}, respectively (as indicated by arrows on "x" axis). Symbols represent the mean and bars the standard deviation of cell viability from measurements on quadruplicate wells containing the aldehydes.

4. Discussion

4.1. Chemotaxonomy

Fatty acid and sterol profiles have been previously used for chemotaxonomic discrimination of genera of raphidophytes (Marshall et al., 2002b). In the present study, lipid classes were characterized as a function of growth conditions including phosphate concentration in the growth medium, light intensity and growth phase. Triacylglycerol levels were < 2% in all conditions, sterols were < 7% with cholesterol levels (1.2 – 1.6%) much lower than those reported by Marshall et al. (2002b), who found 17.1 and 22.9% for CMPL01 and N-118, respectively. However, isofucosterol levels (9.9 – 18.2%) were higher than those observed by Marshall et al. (2002b) (2.3 – 3.6%). Polar lipids were the most abundant lipid class (≥51%) in all conditions.

A similar relation of low cholesterol levels with high fucosterol levels, and viceversa, has been observed in brown algae (Fleury et al., 1994). The shift from low (2.3 – 3.6%, Marshall et al., 2002b) to higher (9.9 – 18.2%, present work) fucosterol levels appears to be an adaptation of *Chattonella marina* growing under summer-like conditions (19°C) continuously (both strains have been kept in these conditions for 9 years after Marshall et al. did the same analysis) since the same pattern has been observed in brown algae with higher fat and fucosterol levels in algae collected in summer time compared to those from winter, and in algae from shallow waters (more exposed to sunlight and high water temperatures) compared to algae from deeper habitats (Black and Cornhill, 1951; Idler and Wiseman, 1970), which also coincides with the results found here that when *C. marina* was grown at high light intensities (500 μmol photons m^{-2} s^{-1}) it showed higher levels of fatty acids (2 – 33.2%) and sterols (2.3 – 6.6%) than when it was grown at lower light intensities (100 μmol photons m^{-2} s^{-1}) (2.7 – 12.3% fatty acids, 0.7 – 3.9% sterols) (Table 7.2).

Since toxicity of *Chattonella marina* seems to be caused, at least in part, by free fatty acids (FFA), ruptured algal cells were also analyzed in order to determine whether higher FFA were found after sonication. Higher levels of FFA were observed for Australian and Japanese *C. marina* but only when grown under high

light intensities. Increases from 5.9 to 11.4 and 7.7 to 10.2% of FFA (as total lipid class) were found between intact and ruptured cells for Australian and Japanese strains in the mid-exponential growth phase, respectively.

Individual fatty acids were also characterized from intact and ruptured cells. The major fatty acids found in *C. marina* under all growth conditions were myristic (14:0), palmitic (16:0), palmitoleic (16:1ω7c), octadecatetraenoic (OTA, 18:4ω3) and eicosapentaenoic (EPA, 20:5ω3) acids, which is consistent with the major fatty acids reported for the raphidophytes *Heterosigma akashiwo*, *Chattonella antiqua* (Nichols et al., 1987), and *C. marina* (Marshall et al., 2002b; Giner et al., 2008). Polyunsaturated fatty acids accounted for 43 – 59% of the total fatty acids in most cases.

When intact cells were analyzed, EPA was more abundant (22.3 – 25.3%) in the stationary phase under high phosphate concentrations and light intensities, compared to cultures from the exponential phase. These values were higher than those reported by Marshall et al. (2002b) for Australian CMPL01 (22.9%) and Japanese N-118 (18.5%) *C. marina*. However, when algal cells were ruptured by sonication, EPA levels were higher (21.4 – 29.4%) than levels measured in intact cells (8.5 – 25.3%). In general, Japanese N-118 *C. marina* was the highest producer of EPA (14.3 – 29.4%), and Mexican CMCV-1 the lowest (7.9 – 27.1%). Australian and Japanese strains had been previously compared, but here lipid and fatty acids profile of the tropical Mexican strain CMCV-1 were incorporated for the first time.

4.2. Toxicity

In contradiction to earlier claims of brevetoxin production by *C. marina* (Haque and Onoue, 2002), using more sophisticated LC MS/MS methods no evidence was found for this ichthyotoxin in any of the *C. marina* strains (Marshall et al., 2003). Similarly, earlier studies (Marshall et al., 2003; Mooney et al., 2011, chapter 4) conclusively demonstrated that ROS on their own are only weakly ichthyotoxic (summarised in Table 7.6). Accordingly, other ichthyotoxic mechanisms by this raphidophyte had to be pursued. Studies of the mechanisms of

chemical defense of diatoms have previously been suggested to involve the liberation of free fatty acids, such as EPA, upon stress, especially when cells were disintegrated mechanically (Jüttner, 2001). Although EPA in FFA form is not always present in live cells, it can be produced by hydrolysis of lipids in very fast reactions upon disruption of algal cells (Jüttner, 2005). The liberated free fatty acids are subject to oxidative degradation, which can be enhanced by the presence of reactive oxygen species (ROS) (Halliwell and Gutteridge, 1984). Since *Chattonella marina* is notable for its ability to produce both superoxide (Marshall et al., 2005; Mooney et al., 2011) and PUFA, these combined factors may increase the production of more toxic compounds thereby causing adverse effects on fish gills (Fig. 7.6). For instance, Marshall et al. (2003) found that free EPA on its own was able to cause fish mortality, but EPA combined with superoxide anion accelerated fish mortality. The exact mechanism by which superoxide induces oxidation of PUFA is unclear but could be associated with the fact that superoxide facilitates reduction and uptake of iron by *Chattonella marina* (Garg et al., 2007) with the reduced form of iron possibly reacting with hydrogen peroxide, the disproportionation product of superoxide, to produce strongly oxidizing hydroxyl radicals via the Fenton reaction (Halliwell and Gutteridge, 1984).

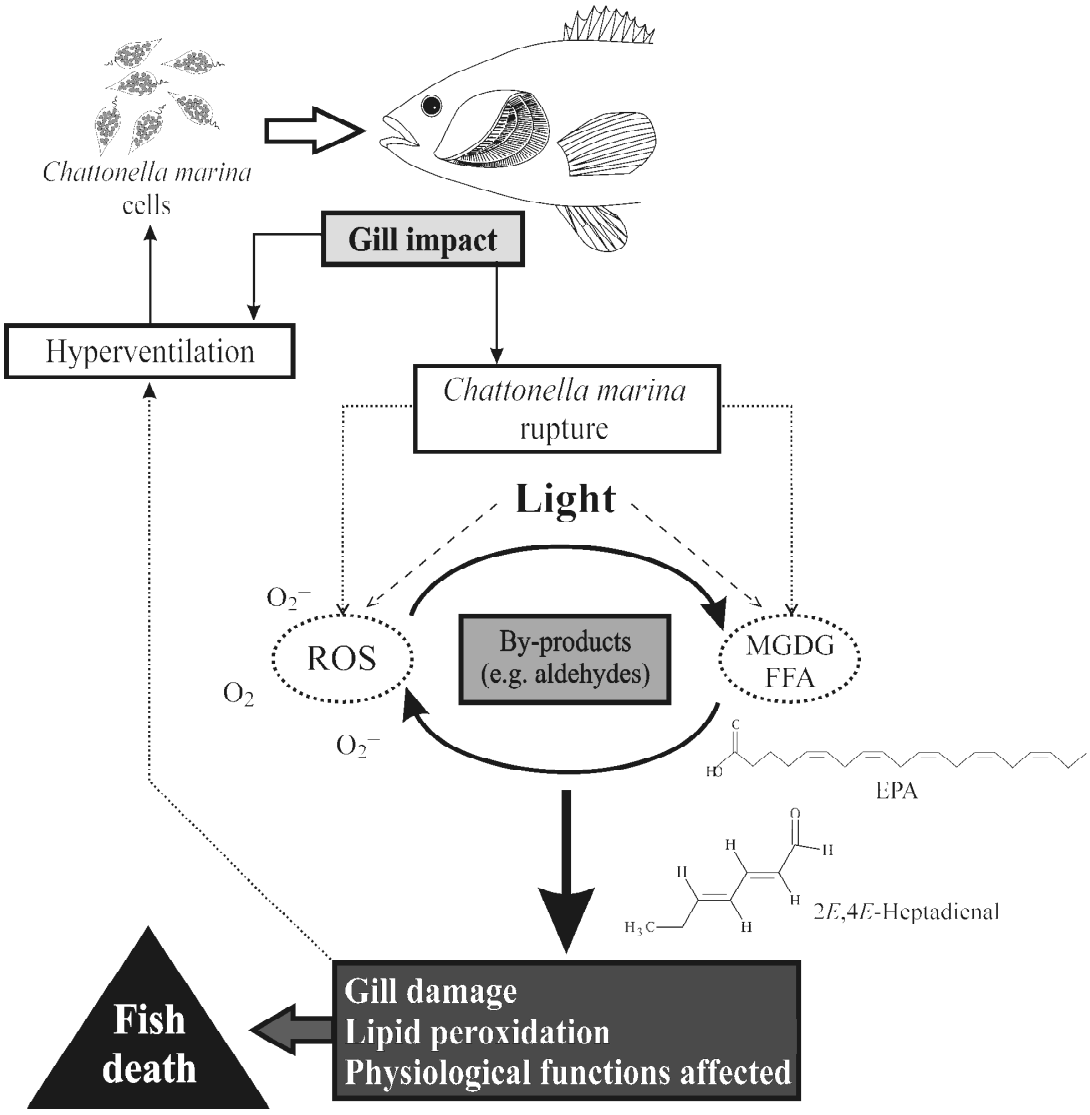

Figure 7.6. Diagram modified from Marshall et al. (2003) showing the proposed pathway by which *Chattonella marina* causes mortality of fish. Algal cells are ruptured after impacting on the fish gills, releasing high amounts of free fatty acids (e.g. EPA) (possibly derived from monogalactosyl diglycerides) and reactive oxygen species, such as superoxide anion. Light enhances production of superoxide anion and stimulates oxidization of free fatty acids to produce more toxic by-products (e.g. aldehydes) that cause gill damage and alter physiological functions leading to death.

By-products derived from degradation of fatty acids include aldehydes, carboxylic acids, alkanes and alcohols, which have shown toxic effects on marine invertebrates (Kieber et al., 1997; Roméas et al., 1999; Pohnert et al., 2002; Jüttner,

2005). Additionally, a fast release of aldehydes immediately after cell disruption has also been found in diatoms (Pohnert, 2000). Although this kind of defense had been reported exclusively for diatoms, it has been found that the haptophyte *Phaeocystis pouchetii* is also able to produce the polyunsaturated aldehyde 2*E*,4*E*-decadienal (Hansen et al., 2004). d'Ippolito et al. (2006) reported generation of the aldehyde 2*E*,4*Z*-octadienal from hexadecatrienoic acid (16:3ω4) through the lipoxygenase-dependent intermediate (9S)-9-hydroperoxyhexadeca-6,10,12-trienoic acid, and that synthesis of 2*E*,4*Z*,7*Z*-decatrienal involves mainly EPA by a 11R-lipoxygenase in *Thalassiosira rotula*. Thus, production of aldehydes by diatoms is suggested to occur through enzymatic reactions, involving oxidation of polyunsaturated fatty acids using lipoxygenase and lyase enzymes that facilitate the transport and removal of intermediate hydroperoxides by transformation into aldehydes (Pohnert, 2000; d'Ippolito et al., 2006).

Previous results showed toxicity towards RTgill-W1 cells by glycolipid containing fatty acid fractions extracted from red tide dinoflagellates containing OTA and EPA (Mooney et al., 2011, chapter 6). The toxic effects did not occur as rapidly as found here (12 – 60 hrs vs ≤4 hrs), and higher concentrations of the fatty acids were required to induce loss of gill cell viability (≤56 μg EPA mL^{-1} in that study vs 1 μg EPA mL^{-1} here). These observations support the proposition that the form of fatty acids plays an important role in ichthyotoxicity. In that study monogalactosyl diglycerides (MGDG) were used compared to the free fatty acid form, which appears to be more highly reactive. Production of aldehydes from MGDG of the marine diatom *Skeletonema costatum* has also been reported (d'Ippolito et al., 2004). As summarised in Table 7.6, free fatty acids had highest toxicity towards gill cells. An average content of PUFA of 156.5 pg $cell^{-1}$ was found in *C. marina*, and considering the algal density in blooms, it is possible for ecologically relevant concentrations of up to 0.56 μg mL^{-1} to occur, which may undergo further degradation to FFA or aldehydes (Kieber et al., 1997).

Marshall et al. (2003) found an LC_{50} of 2.7 μg mL^{-1} upon exposure of damselfish to pure EPA (as free fatty acid) at 2.6 hrs. In contrast, the LC_{50} value (considering only EPA relative content in the fatty acid fractions) for gill cells in the

present study was 23 to 34 times lower at 1 hr in light and dark conditions, at 0.08 and 0.12 μg EPA mL^{-1} respectively. This difference most likely is due to the *in vitro* assay using gill cells from rainbow trout being significantly more sensitive than *in vivo* assays working with whole fish.

Light was also an important factor in a 1-hr exposure at concentrations of 0.79 and 1.58 μg mL^{-1}. Apart from cell viability, PUFA (including EPA) from the raphidophyte *Fibrocapsa japonica* have been associated with hemolytic activity with a strong dependency on light intensity (de Boer et al., 2009). Kuroda et al. (2005) reported potent hemolytic activity of an extract from *C. marina*, but only in light conditions, with no hemolytic activity observed in the dark. As shown previously (Dorantes-Aranda et al., 2011; chapters 2, 3 and 5) membrane damage on gill cells occurred after exposure to free fatty acids and ruptured cells of *C. marina*, which confirms current results that ruptured cells were more toxic than intact cells (i.e. for the CMPL01 strain), coinciding with higher EPA and superoxide production.

LC_{50} values for gill cells after a 1-hr exposure to 2*E*,4*E*-decadienal and 2*E*,4*E*-heptadienal were 0.34 and 0.36 μg mL^{-1}, respectively. Decadienal was more toxic than heptadienal as has also been found for crustaceans and phytoplankton upon exposure to these aldehydes. However, for these *in vivo* assays, effects were observed in a wide range of concentrations, 0.14 – 6.39 μg mL^{-1} for 2*E*,4*E*-decadienal, and 0.65 – 38.55 μg mL^{-1} for 2*E*,4*E*-heptadienal. The effects reported include reduction of swimming, inhibition of egg production and hatching, and embryonic development in crustacean grazers, reduction of growth in phytoplankton, and mortality (Pohnert et al., 2002; Jüttner et al., 2005; Taylor et al., 2005; Ribalet et al., 2007).

These results showed that cell rupture and light are key factors in ichthyotoxicity by *C. marina*. It has been previously recognised that a glycocalyx, a polysaccharide-containing structure on the cell surface of *Chattonella* (Yokote and Honjo, 1985) that is easily released upon physical stimulation of cells, is involved in toxicity (Kim and Oda, 2010). The glycocalyx possesses oxidoreductase enzymes, such as NADPH oxidase, which generate superoxide by utilizing NAD(P)H as an electron source to reduce O_2 to O_2^- (Kawano et al., 1996). Separation of the

glycocalyx from *C. marina* may have occurred during sonication and associated oxidoreductase enzymes may have stimulated production of O_2^- since a higher generation in ruptured than in intact cells was observed. Findings from chapter 5 also demonstrated that a sudden change of *C. marina* from darkness to light, simulating vertical migration of *C. marina* in the water column from turbid subsurface to well-lit surface waters, or resuspension of benthic populations by storms, resulted in increased O_2^- production. Thus it appears that photolysis, particularly in the presence of the glycocalyx, stimulates production of superoxide as has been suggested by Marshall et al. (2002a).

Table 7.6. Toxicity towards RTgill-W1 cells of fatty acids, superoxide anion and *Chattonella marina* (CMPL01, Australian; N-118, Japanese; CMCV-1, Mexican) reported in the present and our previous studies (Mooney et al., 2011).

Toxic material	**Experimental concentration**	**Toxicity to RTgill-W1 cells (% loss of viability)**	**Ecologically relevant concentration**
Intact cells *C. marina*	7×10^6 cells L^{-1}	CMPL01: 55% in 2 hrs N-118: 60% CMCV-1: 35% (chapter 3)	Natural fish-killing blooms: CMPL01: up to 6.6×10^4 cells L^{-1} (Munday and Hallegraeff, 1998) N-118: 0.2 – 3×10^4 cells L^{-1} (Watanabe et al., 1991) Mexico: up to 3.6×10^6 cells L^{-1} (Band-Schmidt et al., 2005)
Ruptured cells *C. marina*	7×10^6 cellsL^{-1}	CMPL01: 71% N-118: 65% CMCV-1: 35%	
Superoxide anion	~ 60×10^4 CU[a]	10 – 14% in 2 hrs (Mooney et al., 2011; chapter 4)	
Palmitic acid (commercial preparation)	0.02 – 120 µg mL^{-1}	25 – 40% in 60 hrs (Mooney et al., 2011; chapter 6)	21.4 – 35.8% of total fatty acids (intact cells); 20.5 – 22.7% (ruptured cells)
Eicosapentaenoic acid (commercial preparation)	0.02 – 120 µg mL^{-1}	44 – 98.5% in 60 hrs (Mooney et al., 2011; chapter 6)	8.5 – 25.3 % of total fatty acids (intact cells) 21.4 – 29.4% (ruptured cells)
FFA fractions from *C. marina*	0.06 – 6.3 µg TFFA[b] mL^{-1} containing: 0.006 – 0.6 µg mL^{-1} 14:0	LC_{50} at 1 hr: 0.44 µg mL^{-1} (light) 0.68 µg mL^{-1} (dark)	*C. marina* had, on average, 156 pg PUFA $cell^{-1}$, and considering that it occurs at 6.6×10^4 to 3.6×10^6 cells L^{-1}, this is equivalent to 0.01 to

	0.02 – 1.5 μg mL^{-1} 16:0 0.005 – 0.5 μg mL^{-1} 16:1ω7c 0.01 – 1.1 μg mL^{-1} 18:4ω3 0.01 – 1.0 μg mL^{-1} 20:5ω3	100% at >3.17 μg mL^{-1} (at 1 hr)	0.56 μg PUFA mL^{-1}, which may undergo further oxidative degradation
2*E*,4*E*-decadienal	0.015 – 15.2 μg mL^{-1}	LC_{50} at 1 hr: 0.34 μg mL^{-1}	
2*E*,4*E*-heptadienal	0.011 – 9.9 μg mL^{-1}	LC_{50} at 1 hr: 0.36 μg mL^{-1}	

[a] Synthetically produced mixing 5 to 25 μM xanthine + 30 U L^{-1} xanthine oxidase, being equivalent to production by *C. marina* at 6×10^6 cells L^{-1} under normal culture conditions (intact cells from exponential growth phase) (Marshall et al., 2003).

[b] Total free fatty acids.

4.3. Strain variations

In the eutrophic Japanese Seto Inland Sea (from where N-118 was isolated), water turbidity limits penetration of sun light (Imai et al., 1998; 2006). Tidal currents also generate turbidity in the Seto Inland Sea (Senjyu et al., 2001) and N-118 does not tolerate high irradiance compared to Australian CMPL01 (Marshall and Hallegraeff, 1999). Diurnal tidal regimes affect the dynamics of *C. marina* blooms causing an increase in superoxide anion production and, when algal cells lyse, production of EPA including the FFA form. A similar scenario was observed in a South Australian bloom where *C. marina* (strain CMPL01, up to 6.6×10^4 cells L^{-1}) was associated with a major mortality of farmed tuna in Boston Bay (Munday and Hallegraeff, 1998). This region receives significantly higher irradiance than the Seto Inland Sea (Marshall and Hallegraeff, 1999); raphidophyte cells had been building up in bottom waters and water had been turbid for 2 to 3 months prior to the mortality event, when ocean surges and strong winds resuspended bottom cells into the well-lit surface waters leading to a major mortality event (Munday and Hallegraeff, 1998). These conditions may also have ruptured algal cells and increased production of both superoxide (as shown in chapter 4) and EPA in FFA form.

Mexican strain CMCV-1 is larger (30 – 40 µm wide × 80 – 120 µm long) than CMPL01 and N-118 (30 – 40 µm wide × 70 – 80 µm long), which renders it less vulnerable to cell rupture thus leading to less production of both EPA and superoxide anion, and consequently this is the least toxic of the three strains. The Mexican *C. marina* strain CMCV-1 was not isolated from a bloom, and no mortalities were associated with its occurrence (Band-Schdmidt et al., 2004). Since then, three blooms of *C. marina* (up to 3.6×10^6 cells L^{-1}) have been reported in the Mexican Pacific, but only two events were associated with wild fish mortalities. There is no available water turbidity data prior to these events; however, a shift from diatom to dinoflagellate and raphidophyte dominance has been linked to eutrophication in the region (Barraza-Guardado et al., 2004; Band-Schmidt et al., 2005; Cortés-Altamirano et al., 2006).

In conclusion, *Chattonella marina* was more toxic to fish gill cells upon rupture (notably CMPL01) with resultant high production of EPA and superoxide anion. Free fatty acid fractions decreased gill cell viability up to 100% in only 1 hr at concentrations >3.17 μg mL^{-1}. Light was a critical factor since it enhanced toxicity of fatty acids compared to dark conditions at 1 hr at concentrations of 0.79 and 1.58 μg mL^{-1}, suggesting a fast oxidation by ROS and light. Toxicity of the fatty acid-derived aldehydes 2*E*,4*E*-decadienal and 2*E*,4*E*-heptadienal (LC_{50} at 1 hr of 0.34 μg mL^{-1} and 0.36 μg mL^{-1}, respectively) confirmed this suggested mode of action.

Conclusions

- Two simple and sensitive assays using the cell line RTgill-W1 from rainbow trout gills were developed for the assessment of toxicity of algal extracts and living red tide phytoplankton. These assays were used to test and confirm toxicity of fatty acid fractions from the raphidophyte *Chattonella marina*, and the dinoflagellates *Amphidinium carterae* and *Karlodinium veneficum*, as well as karlotoxins extracted from the latter. Cytotoxicity of several microalgal species, including raphidophytes, dinoflagellates and haptophytes, was demonstrated and compared using these *in vitro* bioassays. Due to the reproducibility and sensitivity of these assays, strain differences in toxicity were detected.

- Toxicity of superoxide was associated only with *C. marina* but not with other red tide species including dinoflagellates, haptophytes and other raphidophytes. Production of superoxide by *C. marina* was influenced by growth phase, with highest production in the exponential phase, and by iron content in growth medium, with highest production at 1 μM and lowest at 0.01 μM. Dark adaptation had a major impact on production of superoxide by *C. marina* since it produced highest superoxide levels after a sudden change from the dark to light. This discovery has an important relevance to vertical migration by *C. marina*, suggesting that adverse effects during blooms of this raphidophyte may occur during peak sunlight hours.

- *Chattonella marina* was more toxic to fish gill cells upon cell rupture with resultant high production of eicosapentaenoic acid and superoxide anion. This could be due to cell fragility that influences toxicity since the Mexican strain was the least toxic among the six strains tested and it was also larger than the others. Light was a critical factor since it increased the extent of production of superoxide anion of dark-adapted cultures, and also enhanced toxicity of fatty acids compared to dark conditions, suggesting a fast oxidation by reactive oxygen species and light. Eicosapentaenoic acid is demonstrated to be the major contributor to toxicity by *C. marina*.

- Mitigation of *Chattonella marina* blooms could be potentially achieved by creating shading in the area where fish pens are located and adding strong ligands to bind iron and thus diminish production of superoxide by this raphidophyte and reduce any possible photo-oxidation of fatty acids from *C. marina.*

- Future work is recommended, including:

    - ✓ Full characterisation of lipid peroxidation products under different environmental conditions, such as dark/light, with and without humic substances to determine the reaction products between fatty acids and reactive oxygen species.

    - ✓ Possible production of aldehydes by *C. marina*. This study may be related to the previous point since fatty acids produced by this microalga may be subject to degradation, and reactive oxygen species may produce aldehydes as a result of lipid peroxidation.

    - ✓ Assessment of biomarkers for oxidative stress (e.g. superoxide dismutase, catalase, glutathione peroxidase, lipid peroxidation) in gill cells after exposure to *C. marina* and its free fatty acids.

    - ✓ Pursue mitigation of gill cell damage by adding antioxidant agents (superoxide dismutase, catalase, cysteine, glutathione) while being exposed to *C. marina* and its free fatty acids to determine whether the effect of reactive oxygen species or by-products can be suppressed.

    - ✓ Further characterisation of gill cell damage at the ultrastructural level (TEM micrographs) upon exposure to *C. marina* and its extracts.

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