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Ichthyotoxicity of gymnodinioid dinoflagellates: PUFA and superoxide effects in sheepshead minnow larvae and rainbow trout gill cells

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ABSTRACT: While 24 h exposure of sheepshead minnow fish larvae to purified monogalactosyl diglyceride (MGDG) lipids, containing octadecapentaenoic acid (OPA) exclusively or as a mixture of octadecatetraenoic acid, eicosapentaenoic acid, and OPA (OTA-EPA-OPA), caused sluggish swimming and gulping, it produced no mortalities even at concentrations up to 120 mg l^{-1}. In contrast, comparable concentrations and exposure times caused significant reductions in viability of rainbow trout gill cells. Pure EPA was the most harmful to gill cells (up to 98.5% viability loss in 60 h) followed by OPA-rich MGDG (45% loss), with OTA-rich MGDG (37% loss) the least toxic. OPA-pure MGDG was non-toxic to rainbow trout gill cells; however, surprisingly, pure palmitic acid was harmful (40% viability loss), and we conclude that gill cell line toxicity of the OPA-rich MGDG fraction was caused by admixture with palmitic acid. Screening of 15 Kareniaceae dinoflagellate species demonstrated that these species are low (on average 10 times less) producers of superoxide compared to the ichthyotoxic raphidophyte *Chattonella marina*. No mortality of sheepshead minnow fish larvae occurred when exposed to superoxide alone or superoxide combined with either OPA-rich MGDG or OTA-rich MGDG. Superoxide showed a slight impact on viability of rainbow trout gill cells. In conclusion, synergistic interactions between free fatty acids and reactive oxygen species as previously claimed for raphidophytes could not be confirmed. Gill damaging effects from EPA were conclusively demonstrated, however; when these co-occurred with OTA, a higher loss of viability was observed (up to 37%), suggesting a magnified toxic effect. Contradictory literature claims as to the ichthyotoxicity of OPA (nontoxic in our work) may relate to the presence of chemical impurities.

KEY WORDS: Kareniaceae · Ichthyotoxicity · Octadecapentaenoic acid · OPA · Polyunsaturated fatty acid · PUFA· Superoxide

INTRODUCTION

Fragile, athecate, Kareniaceae species have been associated with mortalities of finfish worldwide, with the principal site of impact consistently observed to be the sensitive gills, thus causing hypoxia and impacts on blood osmolality. A full understanding of the fish-killing mechanism has been elusive however. In the absence of a specific toxin, such as brevetoxin in *Karenia brevis* and karlotoxin in *Karlodinium veneficum* (Mooney et al. 2009), research to find a toxic mechanism in other dinoflagellates such as *Karenia mikimotoi* has focused on lipids such as polyunsaturated fatty acids (PUFAs) (Arzul et al. 1998, Fossat et al. 1999, Sola et al. 1999, Gentien et al. 2007). Cold-adapted algae often have elevated levels of PUFAs to maintain cellular

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function, as these molecules remain fluid at lower temperatures (Valentine & Valentine 2004). Using bioassay-guided fractionation, the most common PUFAs investigated for toxic activity are octadecatetraenoic acid (OTA), octadecapentaenoic acid (OPA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These molecules are highly reactive, contain 4 to 6 double bonds, and are short-lived in the water column (Jüttner 2001). PUFAs are synthesized almost entirely in the plastid, with the majority of OPA and OTA present in plastid-related glycolipids, as mono- or digalactosyl diglycerides (MGDG and DGDG) (Bell et al. 1997, Leblond & Lasiter 2009), and with EPA and DHA dominant in the cell-membrane phospholipid fraction (Leblond & Chapman 2000, Adolf et al. 2007). Upon lysis of senescent cells and/or dinoflagellate cell implosion upon contact with fish gills, the algal cells may rupture and release a cocktail of reactive and potentially toxic lipids. Many Kareniaceae species contain appreciable relative levels of the PUFAs OPA, EPA, and DHA (Mooney et al. 2007). However, with the ichthyotoxic haptophyte *Chrysochromulina polylepis*, differences between toxic and non-toxic strains could not be simply attributed to OPA lipids (John et al. 2002).

In addition to PUFAs, reactive oxygen species (ROS), measured as superoxide, have been implicated as contributing to mass mortalities of fish (Kim et al. 1999, Yamasaki et al. 2004). ROS are produced by both animal and plant cells in biochemical processes. Living cells have protective systems designed to prevent oxidative damage. ROS are short-lived oxygen free radicals that include superoxide (O_2^-), the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). ROS are toxic in their own right, causing cellular damage by degrading organic molecules such as lipids, proteins, and nucleic acids, and consequently have been implicated in fish kills via damage to gills through lifting of the epithelial layer and affecting gas exchange (Shimada et al. 1991). Production of ROS is closely linked to photosynthesis, with higher levels produced during exponential growth and during the day (Marshall et al. 2002). Algal species producing significant levels of ROS investigated in conjunction with ichthyotoxic blooms include the dinoflagellates *Cochlodinium polykrikoides* (Kim et al. 1999, 2002) and *Karenia mikimotoi* (Yamasaki et al. 2004), and raphidophytes such as *Chattonella marina* (Oda et al. 1992a,b, Marshall et al. 2005a,b). Ichthyotoxicity to damselfish by EPA, as the free acid, increased 3-fold in the presence of superoxide and was claimed as the toxic principle in *Chattonella marina* (Marshall et al. 2003).

Here, we investigate superoxide production by Kareniaceae and explore synergistic ichthyotoxicity of purified MGDG, with OPA or OTA, using both whole fish bioassays as well as a novel gill cell line assay.

MATERIALS AND METHODS

Algal strains and growth conditions. For ROS experiments, non-axenic cultures of 23 species of toxic and non-toxic marine algae (Table 1) were maintained in ESAW (enrichment solution artificial seawater) (Berges et al. 2001), f/2 (f medium of Guillard and Ryther at half strength) (Andersen et al. 1997), or GSe (medium G with selenium added) (Blackburn et al. 1989) at 15 to 32 salinity and at 14, 17, or 20°C. A 12 h light:12 h dark cycle of 80 µmol photons photosynthetically active radiation (PAR) m^{-2} s^{-1} of cool-white fluorescent light was constant throughout the experiment. Cell numbers were determined using a Zeiss Axiovert microscope at ×100 magnification from well mixed cultures using a Sedgwick Rafter cell counter with a minimum of 200 cells counted per culture.

For lipid experiments, *Karlodinium veneficum* (Swan River strain) and *Amphidinium carterae* (CCMP 1314) were grown as per Mooney et al. (2010) on ESAW medium as semi-continuous batch cultures in glass vessels (40 l) with aeration and CO_2 bubbling and with pH regulation ensuring a range of 8.0 to 8.4. At late exponential growth, after 2 to 4 wk, 30 l were harvested and the culture was replenished to 40 l with medium. A 12 h light:12 h dark cycle of 80 µmol photons PAR m^{-2} s^{-1} of cool-white fluorescent light was used throughout the experiments.

Reactive oxygen species. Levels of superoxide free radical (O_2^-) in 22 species of marine algae were measured using the luciferin analogue 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazol [1,2-*a*]-pyrazin-3-one (MCLA) method (Oda et al. 1995) using a Berthold Autolumat LB 953 luminometer. MCLA (5×10^{-6} M) was standardized against superoxide dismutase (SOD) (5×10^{-6} M) (Sigma). Solutions of 100 µl of 5×10^{-6} M MCLA added to triplicate 2 ml cultures were analysed in triplicate for chemiluminescence at 530 nm and standardized with the addition of 100 µl of superoxide dismutase (5×10^{-6} M). Superoxide was generated by periodic addition of 5×10^{-6} M xanthine to 10 to 30 units l^{-1} of xanthine oxidase in filtered seawater (Oda et al. 1992a,b, 1995, Halliwell & Gutteridge 1999, Marshall et al. 2003). Superoxide free radicals for fish exposures were generated as described above and measured using a Polarstar Optima plate reader (BMG Labtech) at 530 nm.

Lipid extraction and purification. Filters containing 2.6×10^{10} and 3×10^{10} cells of *Karlodinium veneficum* (Swan River strain) and *Amphidinium carterae* (CCMP 1314), respectively, were extracted following a modified Bligh & Dyer (1959) method using an initial chloroform: methanol:water (1:2:0.8, v/v/v) single-phase solution. The addition of chloroform and Milli-Q water with a final chloroform:methanol:water ratio of 1:1:0.9

Table 1. Algal species screened in the present study. See 'Materials and methods' for media: GSe, f/2 and ESAW

Species	Authority	Culture code	Temp (°C)	Medium and salinity	Source locality	Isolator and date
Dinophyta						
Alexandrium catenella[a]	(Wheldon & Kofoid) Balech	AC.TRA02	17	GSe28	Spring Bay, Tasmania	C. Bolch, 17.10.1997
Amphidinium carterae[b]	Hulburt	CCMP 1314	20	f/2 15	Falmouth, MA, USA	R. Guillard, 1954
Karenia brevis[a]	(Davis) Hansen & Moestrup	KBCCMP718	20	GSe28	Florida, USA	W. Wilson, 1958
Karenia brevisulcata[a]	(Chang) Hansen & Moestrup	KDS.CWD82	17	GSe28	Wellington, New Zealand	L. MacEnzie
Karenia mikimotoi[a]	(Miyake & Kominami ex Oda) Hansen & Moestrup	KMNZ63	17	f/2 32	New Zealand	A. Haywood, 1994
Karenia papilionacea[a]	Haywood & Steidinger	PL01	20	GSe/2 35	Port Lincoln, South Australia	M. de Salas, 2003
		AB01	20	GSe/2 35	Ansons Bay, Tasmania	M. de Salas, 2003
Karenia umbella[a]	de Salas, Bolch & Hallegraeff	KU.PL01	20	GSe/2 35	Port Lincoln, South Australia	M. de Salas, 2002
Karlodinium antarcticum[a]	de Salas	KDAN.SO10.1	12	GSe/2 35	Southern Ocean 50°S, 145°E	M. de Salas, 16.03.2006
Karlodinium australe[a]	de Salas, Bolch & Hallegraeff	KDA.DE12	12	GSe/2 35	Derwent River, Tasmania	M. de Salas, 27.09.2006
Karlodinium ballantinum[a]	de Salas	KDBM	17	f/2 32	Mercury Passage, Tasmania	M. de Salas, 01.02.2006
Karlodinium conicum[a]	de Salas	KDCSO15	17	f/2 32	Southern Ocean 44°41′S, 147°07′E	M. de Salas, 01.02.2006
Karlodinium corrugatum[a]	de Salas	KDGEAC07.2	17	f/2 32	Southern Ocean 44°41′S, 147°07′E	M. de Salas, 01.02.2006
Karlodinium decipiens[a]	de Salas & Laza	KDDSB01	17	f/2 32	Spring Bay, Tasmania	M. de Salas, 19.05.2005
Karlodinium veneficum[a,b]	(Ballantine) Larsen	KVSR01	20	ESAW 15	Swan River, Western Australia	M. de Salas, 9.03.2001
		KVHU01	20	ESAW 15	Huon River, Tasmania	M. de Salas, 10.01.05
		KVDE01	20	ESAW 15	Derwent River, Tasmania	
Takayama helix[a]	de Salas, Bolch, Botes & Hallegraeff	THNWB01	16	GSe/2 35	North West Bay, Tasmania	M. de Salas, 14.05.2001
Takayama tasmanica[a]	de Salas, Bolch & Hallegraeff	TTPL01	19	GSe/2 35	Port Lincoln, South Australia	M. de Salas, 16.06.2003
Takayama tuberculata[a]	de Salas	TTBSO11.1	12	GSe/2 35	Southern Ocean	M. de Salas
Raphidophyceae						
Chattonella marina[a]	(Subrahmanyan) Hara & Chihara	CM.DE01	17	f/2 35	Derwent River, Tasmania	M. de Salas
		CM.PL02	17	f/2 35	Port Lincoln, South Australia	J. M. LeRoi, 01.04.1996
Heterosigma akashiwo[a]	(Hada) Hada	HA.HB01	18	f/2 35	Hideaway Bay, Tasmania	M. de Salas
		HA.PL01	18	f/2 35	Port Lincoln, South Australia	M. de Salas
Cryptophyta						
Rhodomonas maculata[a]	(Butcher) Hill & Wetherbee	RMCS85	20	f/2 35	Dee Why, Australia	L. Borowitzka, 01.01.1978
Haptophyta						
Pavlova lutheri[a]	(Droop) Green	PVCS23	20	f/2 35	Halifax, Nova Scotia, Canada	
Prasinophyta						
Tetraselmis suecica[a]	(Kylin) Butcher	TSCS187	20	f/2 35	Brest, France	A. Dodson
Chlorophyta						
Dunaliella tertiolecta[a]	Butcher	DTCS175	20	f/2		

[a]Strains used for assessment of reactive oxygen species (as superoxide) production; [b]Strains used for lipid extraction

(v/v/v) yielded phase separation. The lower, lipid-containing, chloroform layer was concentrated *in vacuo* by rotary evaporation and the lipid extract transferred to vials, made to volume with 1 ml chloroform and 10 drops of methanol, and stored at –20°C in the dark. Lipid extracts (592 mg of *K. veneficum* and approx. 1.6 g of *A.carterae*) were applied to a silicic acid column (50 g of silicic acid in chloroform) and fractions eluted as follows: neutral lipids (chloroform), combined MGDG and DGDG (7:13 ratio of chloroform:acetone), SQDG (sulfoquinovosyldiacylglycerol) (7:1 chloroform: methanol), and phospholipids (1:1 chloroform: methanol) (Yongmanitchai & Ward 1992). The MGDG and DGDG fraction was further separated on another silicic acid column with 500 ml each of the following ratios of chemicals: 11:9 chloroform:acetone (for MGDG) and 7:13 chloroform: acetone (for DGDG). Purity of fractions was confirmed by TLC-FID (thin-layer chromatography and flame ionization detection) on Iatroscan rods (Iatron Laboratories) using acetone: acetic acid:water in the ratio 100:2:1 (Christie 1982).

Liquid chromatography–mass spectrometry (LC–MS) analysis of galactolipids. Galactolipid fractions were injected onto a C8 column (LiChrosphere 125 × 4 mm, 5 µm bead-size RP-8; Agilent) and subjected to a 1 ml min^{-1} 10 to 95% methanol:water gradient over 45 min using an Agilent 1100 HPLC. Galactolipid peaks were detected using an Agilent Diode Array Detector (DAD) (Model#G1315B) with a micro high-pressure flow cell (G1315B#020, 6 mm path length, 1.7 µl volume) at a wavelength of 210 nm (Fig. 1). The eluate from the DAD was split (10% to mass spectrometer: 90% to the fraction collector) using a graduated microsplitter valve (model P-470; Upchurch Scientific). The major portion of the eluate was fed into an Agilent 1100 fraction collector (Model G1364C), while the remaining portion was passed into the electro-spray nozzle of the mass spectrometer (Agilent G1956A SL) for ionization with the following spray chamber conditions using N_2 as the drying gas: 10 l min^{-1} flow rate, 60 psi pressure, 350°C temperature, 350 V fragmentor voltage, and 4000 V capillary voltage. The fatty acid composition of the collected fractions was determined after hydrolysis and methylation by gas chromatography (GC) as described in the next subsection. A solution of 1% formic acid in water was added at 0.1 ml min^{-1} to provide appropriate pH conditions for positive mode ionization. MGDG (18:2/18:2) was used as a calibration standard, verified by LC–MS (Guella et al. 2003). We observed no MS signal characteristic of amphidinol or karlotoxin in the purified MGDG or DGDG components.

Fatty acid identification. Confirmation of OPA and OTA components of MGDG was performed by sampling 10 µl of extract fractions, which were transmethylated using MeOH:HCl:$CHCl_3$ at a ratio of 10:1:1

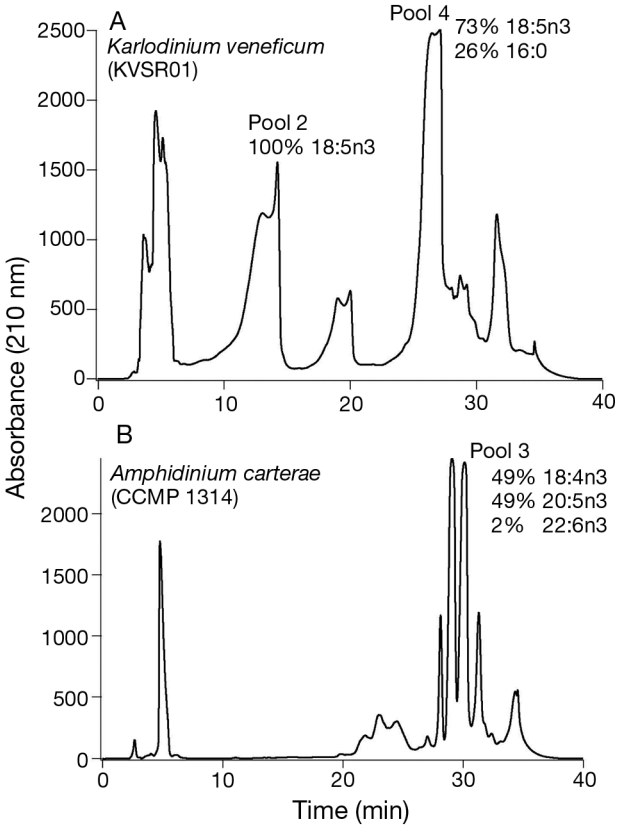

Fig. 1. HPLC chromatograms of galactolipid fractions measured at 210 nm for (A) octadecapentaenoic acid (OPA)-pure monogalactosyl diglyceride (MGDG) (Pool 2) and OPA-rich MGDG (Pool 4) isolated from *Karlodinium veneficum* (KVSR01) and (B) octadecatetraenoic acid (OTA)-rich MGDG (Pool 3) isolated from *Amphidinium carterae* (CCMP 1314). Included are the fatty acid composition, by GC, of each pool

(v/v/v) at 100°C for 60 min and, after the addition of water, extracted 3 times with a 4:1 ratio of hexane:chloroform to yield fatty acid methyl esters (FAME) (Mooney et al. 2007). Samples were made to volume in hexane containing C19:0 and C23:0 internal injection standards and analysed by GC. Identification of FAME was accomplished by comparing gas chromatography retention data with authentic quantitative standards (3B, GLC-68D, GLC-17AA¢) from NU-CHECK (Elysian) and qualitative standards from Matreya (Pleasant Gap). Peaks in some samples, including OPA, were also confirmed by GC–MS. The Hewlett-Packard 6890 GC (Agilent) used was equipped with a 30 m × 0.25 mm internal diameter capillary column with 0.25 mm film thickness (DB Wax; J and W Scientific), and a FID at 300°C. The GC was run in 'constant flow rate' mode at 1.5 ml min^{-1} with H_2 as the carrier gas. The column-temperature profile was as follows: 50°C for 0.5 min, hold at 195°C for 15 min after ramping at 40°C min^{-1}, and hold at 220°C for 7 min after ramping

at 2°C min^{-1}. Total run time was 38.13 min. The mass of FAME was determined by comparison of the response factor from the FID for each FAME in the quantitative standard (NU-CHECK) and of the internal C19:C21 standards run with each sample. The relative distribution (% FAME) was calculated based on the peak area of a given peak divided by the total peak area of identified FAME in a sample. OPA-rich and OTA-rich MGDG compositions were identified by comparison with retention time of laboratory standards and published literature (Harvey et al. 1988, Mansour et al. 1999b). The fatty acid composition of the suite of MGDGs and the commercial fatty acids hexadecanoic or palmitic acid (PA) (Sigma) and eicosapentaenoic acid (EPA) (1167, Matreya, LLC) is shown in Table 2; these lipids were tested for ichthyotoxicity and epithelial gill cell line viability.

Fish larvae bioassays. Larvae of the sheepshead minnow *Cyprinodon variegatus* (3 to 5 d old) maintained at 18°C, 25 salinity, and pH 8.1 were obtained from Aquatic Biosystems Larvae were acclimated to 20°C and either 15 or 32 salinity for 24 h prior to exposure to lipids and ROS. Larvae were transferred to 12-well plates (Falcon, Becton Dickinson) with 3 larvae per well in a total volume of 1 ml water in duplicate wells (6 fish per treatment), and acclimated for 3 h. Larvae were not fed during the experiment. Control experimental larvae were exposed to 1 ml medium (ESAW or f/2) or 10 µl methanol (treatments contained lipids dissolved in 10 µl methanol). Challenged experimental larvae were exposed to 0.02–120 mg l^{-1} lipid and/or xanthine–xanthine oxidase. The upper concentration of 120 mg l^{-1} lipid is equivalent to approximately 5×10^{11} cells l^{-1} based on a total lipid estimation of 0.25 ng per cell. Larvae were observed every 30 min for the first 6 h and at 24 h prior to termination of the experiment, when all fish were preserved in 4F/1G preservative (4% formalin, 1% glutaraldehyde in phosphate buffered saline) (McDowell & Trump 1976) and stored for histopathological analysis. Observation of superoxide production from xanthine and xanthine oxidase revealed that 10 µl xanthine (5×10^{-6} M) and 10 µl xanthine oxidase (5×10^{-6} M) produced similar levels of superoxide to Kareniaceae cultures in well-plates. Addition of 50 µl xanthine (5×10^{-6} M) and 50 µl xanthine oxidase (5×10^{-6} M) generated similar levels of superoxide as *Chattonella marina* cultures. Larvae were exposed to superoxide (low and high levels) and OPA-rich and OTA-rich MGDG (2 to 120 mg l^{-1}) as standalone treatments and in combination.

Table 2. Summary of fatty acid composition (%) of lipid treatments on larval sheepshead minnow and rainbow trout gill cells. OPA: octadecapentaenoic acid; OTA: octadecatetraenoic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; PA: palmitic acid; MGDG: monogalactosyl diglyceride

Treatment	Fatty acid				
	OPA	OTA	EPA	DHA	PA
OPA-rich MGDG[a,b]	73				26
OTA-rich MGDG[a,b]		49	49	2	
OPA-pure MGDG[a]	100				
OTA/EPA MGDG[a]	23	51	26		
Commercial PA[b]					99
Commercial EPA[b]			99		

[a]Larval sheepshead minnow treatment
[b]Gill cell RTgill-W1 treatment

Gill cell line assay. The rainbow trout (*Oncorhynchus mykiss*) epithelial gill cell line RTgill-W1 was obtained from the American Type Culture Collection. The cells were grown in 25 cm^2 culture-treated flasks 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}), and routinely maintained at 19°C (±1°C) in the dark. Confluent flasks were treated with 0.25% trypsin with 0.02% EDTA in Hank's balanced salt solution (59428C, Sigma) for detachment and then counted using a haemocytometer and seeded at a concentration of 2×10^5 cells ml^{-1} in a final volume of 100 µl in quadruplicate in 96-well flat bottomed microplates (3860-096, Iwaki). The gill cells were exposed to OPA-rich and OTA-rich MGDG (0.02 to 120 mg l^{-1}) for 60 h in the dark. These fatty acids were dissolved in MeOH and mixed with L-15ex medium (Schirmer et al. 1997) for the exposure assays. The final concentration of MeOH was 1%. The commercial fatty acids PA and EPA were also tested at the same concentrations. Gill cell viability was determined using the indicator dye alamarBlue (DAL1025, Invitrogen) (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). The results are expressed as a percentage of the readings compared to the controls. A more detailed account of the gill cell line bioassays can be found in Dorantes-Aranda et al. (2011).

RESULTS

Lipid ichthyotoxicity to sheepshead minnow larvae

Purified lipids, OPA-rich MGDG, and OTA-rich MGDG (Table 2) were added to fish larvae in wells at concentrations ranging from 0.02 to 120 mg l^{-1}. No fish

mortalities were observed in all treatments and at all concentrations. Above 1 mg l^{-1} of OPA-rich MGDG and OTA-rich MGDG, fish were visibly affected: sluggish swimming and, as the concentration increased, gulping at the surface, inability to maintain an upright position in the water column, and delayed movements occurred. No mortalities, however, were observed in either treatment after 24 h. Similarly, no fish mortalities were observed in control treatments.

Lipid cytotoxicity on RTgill-W1

Most of the fatty acids had a negative effect on the gill cells, except for OPA-pure MGDG, which contrary to expectation had a positive effect on the gill cells. Viability increased by 10 and 17% during the first 24 h when exposed to 20 and 120 mg l^{-1}, respectively (Fig. 2). However, the combination of OPA (73%) and PA (26%) in OPA-rich MGDG exhibited a significant toxic effect, but only at a high concentration of 120 mg l^{-1} ($\alpha = 0.05$) (Fig. 3A). This toxic effect was time-dependent with maximum toxicity after 60 h and cell viability reduced to 55.4%. A commercial preparation of palmitic free fatty acid (Table 2) had a similar effect at 120 mg l^{-1}, except for the effect being more gradual and with cell damage increasing with concentration and time of exposure (Fig. 3B). OTA-rich MGDG showed both a time- and concentration-dependent effect (Fig. 3C). The highest and fastest toxicity was registered in combinations where EPA was present, especially at high concentrations of EPA. In the mixture containing 26% of EPA, the gill cells were 99.8% viable during the first 12 h of exposure to 120 mg l^{-1}, but viability decreased to 19.9% in combination with 47% EPA (Fig. 3B). Combination with 5% of EPA did not show high toxicity, with the gill cells only losing 10% viability during 60 h exposure at the highest concentration of 120 mg l^{-1}. Toxicity of EPA was confirmed when using a commercial preparation, which produced the highest loss (from ~40 down to 1.5%) of gill cell viability observed in this study when exposed to 120 mg l^{-1} of 100% EPA for 12 and 60 h ($\alpha = 0.05$), respectively (Fig. 3D).

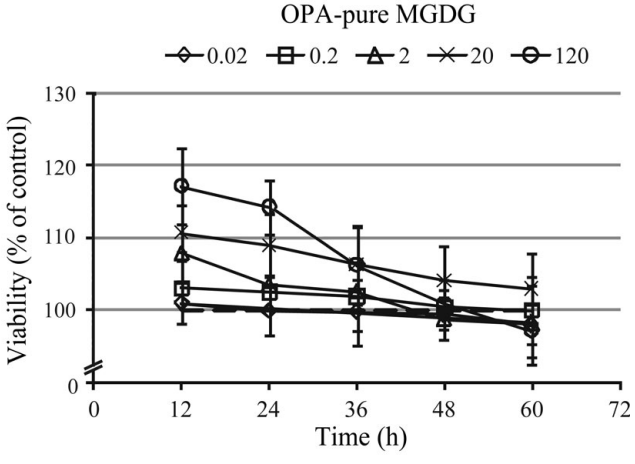

Fig. 2. Effect of octadecapentaenoic acid (OPA)-pure monogalactosyl diglyceride (MGDG) on rainbow trout gill cells (RTgill-W1) at 0.02 to 120 mg l^{-1} (presented as percent viability compared to controls, mean ± SD, n = 4)

ROS production and ichthyotoxicity and cytotoxicity

Superoxide production per cell by 15 species of Kareniaceae ranged from 1 to 22 cellular chemiluminescence units (CCU) compared to 100 to 110 CCU by 2 strains of *Chattonella marina* (Fig. 4). The highest cellular production of superoxide in Kareniaceae was recorded in *Takayama* species (17 to 22 CCU), and the lowest was observed in the 3 strains of *Karlodinium veneficum* (from Swan River, Derwent River, and Huon River; see Table 1), *Karlodinium decipiens*, and *Karlodinium ballantinum* (1 CCU). Non-toxic aquaculture feed algae, *Dunaliella tertiolecta*, *Pavlova lutheri*, and *Tetraselmis suecica* all recorded low cellular superoxide production (1 CCU). Isolates of the same species, *Karenia papilionacea* (2 strains), *Karlodinium veneficum* (3 strains) and *C.marina* (2 strains) produced comparable cellular superoxide levels. Exposure of larval finfish to the equivalent of low and high levels of superoxide (20 and 100 CCU, respectively), from periodic addition of xanthine and xanthine oxidase, did not result in mortalities. Slower swimming was observed, but no symptoms corresponding to gill insult (mucus production or epithelial gill lifting) occurred. Similarly, no fish mortalities were observed in control treatments. Synthetically produced ROS, using 5 to 25 µM xanthine and 30 U l^{-1} of xanthine oxidase on gill cells from rainbow trout, resulted in only minor (<14%) loss of viability of rainbow trout gill cells (Fig. 5).

Synergistic ichthyotoxicity

No fish mortalities were observed upon exposure to low and high superoxide and treatments with lipids, OPA-rich, and OTA-rich MGDG at 0.02 to 120 mg l^{-1}, after 24 h exposure. Above 20 mg l^{-1} of OPA-rich MGDG and OTA-rich MGDG combined with low or high superoxide treatments, fish were visibly affected, with gulping at the surface and difficulty swimming upright; however, no mortalities were observed in either treatment after 24 h exposure.

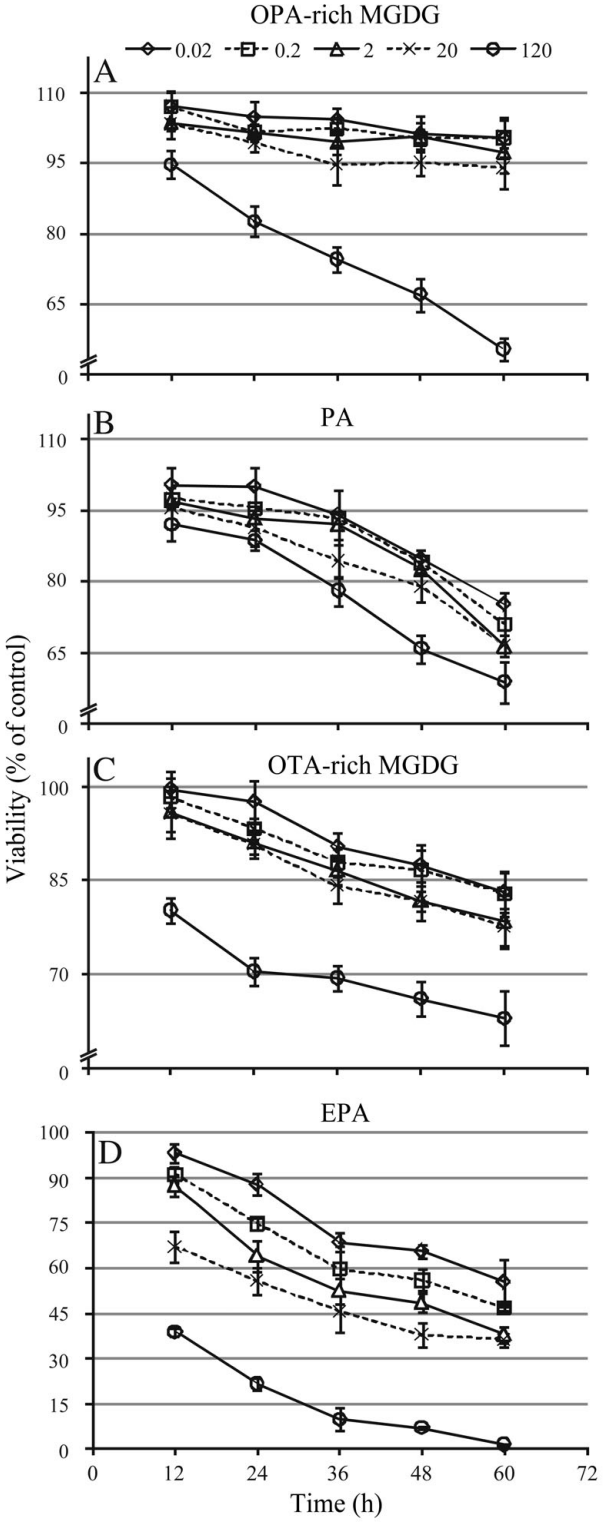

Fig. 3. Exposure of rainbow trout gill cells (RTgill-W1) to (A) octadecapentaenoic acid (OPA)-rich monogalactosyl diglyceride (MGDG), (B) commercial palmitic acid (PA), (C) octadecatetraenoic acid (OTA)-rich MGDG and (D) commercial eicosapentaenoic acid (EPA) at 0.02 to 120 mg l^{-1} (presented as percent viability compared to controls, mean ± SD, n = 4)

DISCUSSION

PUFAs

The hemolytic, antibacterial, and anti-algal activity of PUFAs (Ikawa 2004) are well-documented; however, the fish-killing ability of these molecules remains controversial.

Hemolytic activity by the raphidophyte *Fibrocapsa japonica*, in the presence of PUFAs, increased with light intensity (de Boer et al. 2009), and analyses of hemolytic fractions from *Karenia mikimotoi* have previously confirmed that OPA was present (Yasumoto et al. 1990, Arzul et al. 2000).

Many toxic and non-toxic algae contain OPA, a PUFA found primarily as a mono- or digalactosyl glycolipid in the chloroplast (Leblond & Lasiter 2009). Experiments with toxic (LC_{50} at 4×10^3 cell ml^{-1}) and non-toxic (no mortality up to 4×10^5 cell ml^{-1}) strains of the haptophyte *Chrysochromulina polylepis*, both of which contained OPA (16 and 19%, respectively), suggested another causative agent was responsible for brine shrimp toxicity (John et al. 2002). PUFA ichthyotoxicity against fry of the fathead minnow *Pimephales promelas* from another haptophyte, *Prymnesium parvum*, suggests increasing potency with increasing fatty acid chain length and degree of unsaturation; however, this does not fully account for naturally occuring fish killing blooms of this species (Henrikson 2010).

Our exposure of sheepshead minnow larvae for 24 h to high concentrations of OPA-rich MGDG caused sluggish swimming and gulping but no mortality even at concentrations up to 120 mg l^{-1}. Inactivity of OPA-rich MGDG was found at 50 µg ml^{-1} *in vitro* against leukemia cells L-1210 and P-338 (Oshima et al. 1994) (Table 3). Conversely, cytolytic activity of OPA-rich MGDG and OPA-OTA-rich MGDG against heart cells of oysters was found at concentrations > 0.5 µg l^{-1} using bioassay-guided fractionation (Hiraga et al. 2002).

Experiments using synthetic OPA have confirmed toxicity to sea bass (Sola et al. 1999) and trout hepatocytes (Fossat et al. 1999) (Table 3). The toxicity of synthetic OPA to the dinoflagellate *Karenia mikimotoi* itself was also demonstrated, but to a lesser extent than against other algae, and has been proposed as a factor in causing dominance of *K. mikimotoi* biomass in the pycnocline layer (Gentien et al. 2007). Levels of intracellular free fatty acid in 8 species of Kareniaceae cultures were between 2 and 11% of total lipid, with *K. mikimotoi* at 7.5% (Mooney et al. 2007) and 8% (Parrish et al. 1993). The latter workers found only 1 and 2% of toxic OPA and EPA, respectively, in extracellular media, which suggests that PUFAs are short-lived in culture media and the water column (Jüttner 2001).

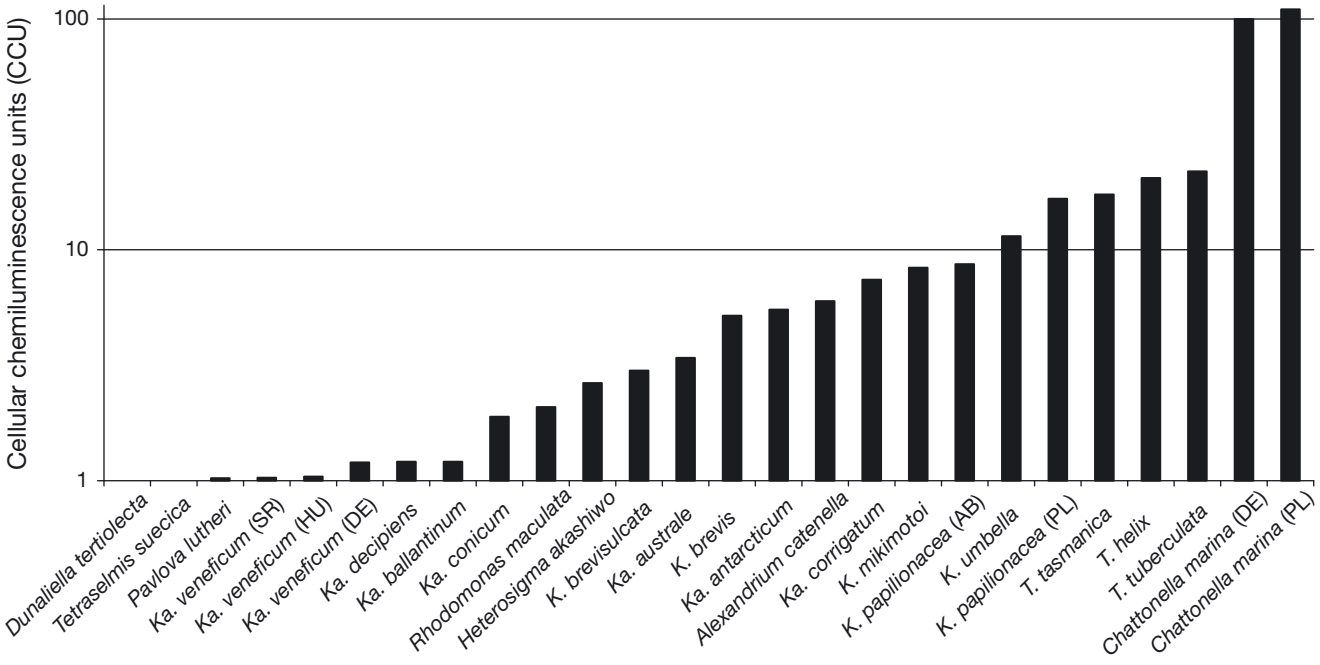

Fig. 4. Superoxide production in 22 species of marine algae expressed as cellular chemiluminescence units (CCU). *K.* = *Karenia*, *Ka.* = *Karlodinium*, *T.* = *Takayama*. Strains are indicated in brackets after species (see Table 1): SR = Swan River, HU = Huon River, DE = Derwent River, AB = Ansons Bay, PL = Port Lincoln

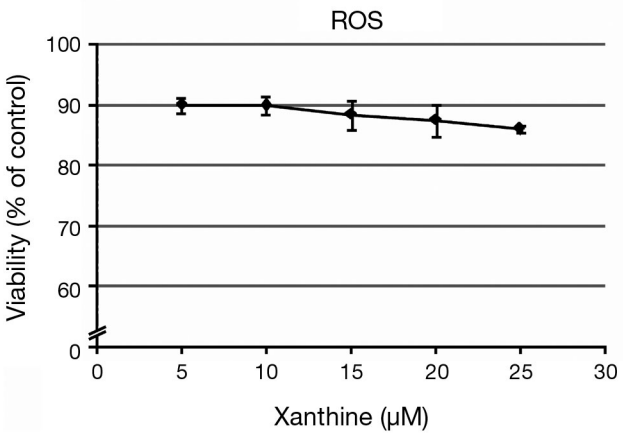

Fig. 5. Influence of reactive oxygen species (ROS), as synthetically produced superoxide using 5 to 25 µM xanthine and 30 U l^{-1} of xanthine oxidase, on rainbow trout gill cells (RTgill-W1) (presented as percent viability compared to controls, mean ± SD, n = 4)

The rainbow trout gill cell line RTgill-W1 was much more sensitive to PUFAs harvested from the dinoflagellates *Karlodinium veneficum* and *Amphidinium carterae* than intact sheepshead minnow fish larvae (Table 3). In our work, the OPA-pure MGDG fraction was nontoxic to sensitive rainbow trout gill cell lines and even improved cell viability. This may be due to the ability of the gill cells to metabolize certain fatty acids, as demonstrated previously with cultured fish cells (Ghioni et al. 2001). Surprisingly, a commercial preparation of the saturated free fatty acid PA caused significant fish gill cell damage (40% viability loss in 60 h). PA has been previously demonstrated to cause cell death of human melanoma cell lines (de Sousa Andrade et al. 2005), and the loss of viability from exposure to the OPA-rich MGDG (73% OPA and 26% PA) preparation in our work is therefore almost certainly caused by PA. The apparent toxic mechanism of PA exposure is loss of membrane integrity and/or DNA fragmentation, as observed in hamster lung cells exposed to PA; a large amount of triacylglycerol accumulated in these lung cells which caused cytoplasmic clefts and perturbed cell functions and caused cell injury (Urade & Kito 1982). Contradictory literature claims as to the ichthyotoxicity of OPA (in our work nontoxic to sheepshead minnow larvae and RTgill-W1 cells) may be due to the presence of byproducts in previous studies. As previously acknowledged by Yasumoto et al. (1990), oxidation artifacts can be generated in water samples by being frozen and thawed for lipid extraction, in purified hemolytic fractions of PUFA-rich MGDG, DGDG, and also in the preparation of the free fatty acid OPA.

OTA and EPA have been previously claimed to cause hemolysis (Fu et al. 2004, de Boer et al. 2009) and lipid peroxidation. EPA has been claimed to exhibit a synergistic effect with reactive oxygen species, most likely producing toxic lipid peroxidation byproducts and hence faster mortality of fish (Marshall et al. 2003). In the present work, loss of gill cell

Table 3. Comparative biological activity of octadecapentaenoic acid (OPA), octadecatetraenoic acid (OTA), eicosapentaenoic acid (EPA) and reactive oxygen species (ROS). For fatty acid concentration of monogalactosyl diglyceride (MGDG) treatments see Table 2. CCU: cellular chemiluminescence units, TCU : total chemiluminescence units; IC_{50}: half maximal (50%) inhibitory concentration of an antagonistic substance; comparable to EC_{50} for agonistic substances; LD_{50}: lethal dose which kills 50% of exposed test animals (or 25% for LD_{25}); EC_{50}: median effective concentration

Chemical	Bioassay	Concentration (mg l^{-1})	Toxicity	Source
Galactolipids				
OPA-pure MGDG	Rainbow trout gill cells RTgill-W1	0.2–120	None	Present study
OPA-rich MGDG	Sheepshead minnow larvae	0.2–120	None	Present study
	Rainbow trout gill cells RTgill-W1	0.02–20	None	Present study
	Rainbow trout gill cells RTgill-W1	120	45% viability loss in 60 h	Present study
	Leukemia cells L-1210, P-338	50	None	Oshima et al. (1994)
	Heart cells of oysters	>0.5	Cytolytic	Hiraga et al. (2002)
OPA-OTA rich MGDG	Heart cells of oysters	>0.5	Cytolytic	Hiraga et al. (2002)
	Rainbow trout gill cells RTgill-W1	120	23% viability loss after 60 h	Present study
OTA-rich MGDG	Sheepshead minnow larvae	0.21–120	None	Present study
	Rainbow trout gill cells RTgill-W1	0.02–20	17–22% viability loss	Present study
	Rainbow trout gill cells RTgill-W1	120	37% viability loss in 60 h	Present study
Free fatty acids				
OPA	Sea bass gill (ATPase activity)	0.16	IC_{50}	Sola et al. (1999)
	Trout hepatocytes (ATPase activity)	0.866	IC_{50}	Fossat et al. (1999)
	Karenia mikimotoi	0.15	LD_{50}	Gentien et al. (2007)
	Diatom growth	1	Inhibit	Gentien et al. (1998)
OTA	Damselfish	25	LD_{25}	Marshall et al. (2003)
	Hemolytic	25	EC_{50}	de Boer et al. (2009)
PA	Rainbow trout gill cells RTgill-W1	0.02	25% viability loss	Present study
	Rainbow trout gill cells RTgill-W1	120	40% viability loss	Present study
EPA	Zooplankton	10	LC_{50}	Jüttner (2001)
	Damselfish	2.7	LD_{50}	Marshall et al. (2003)
	Artemia	50 µg ml^{-1}	80% mortality	Pezzolesi et al. (2010)
	Diatom	1.5		Arzul et al. (1998)
	Hemolytic	4	EC_{50}	de Boer et al. (2009)
	Rainbow trout gill cells RTgill-W1	0.02	44% viability loss	Present study
	Rainbow trout gill cells RTgill-W1	20	64% viability loss	Present study
	Rainbow trout gill cells RTgill-W1	120	98.5% viability loss in 60 h	Present study
Reactive oxygen species				
Synthetic ROS	Damselfish	100 CCU	None	Marshall et al. (2003)
	Sheepshead minnow larvae	100 CCU	None	Present study
	Rainbow trout gill cells RTgill-W1	57×10^4 TCU[a]	10–14% viability loss in 2 h	Present study

[a]Calculated from Marshall et al. (2003) as the enzymatic system tested in this study was similar, using periodic addition of 5×10^{-6} M xanthine to 10–30 units l^{-1} of xanthine oxidase dissolved in L-15ex medium

viability was observed in all 4 combinations of fatty acids containing OTA, EPA, or both. When co-occurring, a higher loss of viability was observed (up to 37%), suggesting a magnified toxic effect. The present work confirms the harmful effects of the fatty acids OTA, EPA, and PA. Commercial preparations of EPA and PA also supported claimed toxicity to fish gill cells.

ROS

Levels of production of superoxide by Kareniaceae are 10-fold less than known high superoxide producers such as *Chattonella marina*. The absence of sheepshead minnow larval finfish mortality and absence of impact on rainbow trout gill cell lines exposed to xanthine or xanthine oxidase agrees with observations by Marshall et al. (2003) on damselfish, *Acanthochromis polycanthus*. The measurement of superoxide by the MCLA method is potentially influenced by other ROS, such as hydrogen peroxide and the hydroxyl radical, as reaction of these ROS with MCLA results in generation of additional superoxide radicals (Kambayashi & Ogino 2003, Wardman 2007). In our work, we observed no mortality of sheepshead minnow larvae exposed to superoxide and OPA-rich MGDG or OTA-rich MGDG, at high lipid and superoxide concentrations. Our results differ from those of Marshall et al. (2003), where EPA toxicity increased 3-fold in conjunction with superoxide. These differences may be attributable to the form of fatty acid used (FFA versus MGDG), the type of PUFA (EPA versus OPA or OTA), the fish species (damselfish versus sheepshead minnow), or fish maturity (adult versus larvae).

Damselfish LD_{50} toxicity of the free acid EPA was 2.7 mg l^{-1}, whereas only 25% of fish died with free OTA (referred to as stearidonic acid) at 25 mg l^{-1} (Marshall et al. 2003), indicating vast differences in PUFA activity (Table 3).

CONCLUSIONS

The observed difference in activity of PUFAs as membrane lipids (as glycolipids) compared to synthetic or purified forms (as FFA or FAME) suggests that additional components or by-products are responsible, which potentially could be introduced during preparation or storage of fatty acids. PUFAs are stable against peroxidation in aqueous systems (Miyashita et al. 1993), whereas those preserved in organic solvents such as chloroform and hexane are more easily oxidized (Halliwell & Gutteridge 1999). PUFAs, EPA, and DHA, are claimed to have anti-oxidant properties, making synergistic ichthyotoxic activity with ROS unlikely (Okuyama et al. 2008).

Rupturing of algal cells and the release of reactive lipids combined with superoxide and contact with sensitive fish gills has been proposed, in the absence of a known toxin, as a possible mechanism of killing fish (Okaichi 1983), including lipids present in the free fatty acid form (Marshall et al. 2003). Many Kareniaceae contain high relative levels of OPA (14 to 35%) and DHA (8 to 23%) (Mooney et al. 2007); however, not all these dinoflagellate species are implicated in fish kills. Similarly, toxic and non-toxic strains of the haptophyte *Chrysochromulina polylepis* containing 16 to 19% OPA indicate that these fatty acids are not responsible for brine shrimp toxicity (John et al. 2002).

The gill cell line RTgill-W1 has proved to be a promising new tool for ichthyotoxic assessment of fish-killing microalgae. Scanning and transmission electron microscopy confirmed membrane disruption of gill cells after exposure to *Chattonella marina* and PA (Dorantes-Aranda et al. 2011). This supports claims that gills are a potential target organ for ichthyotoxic compounds from harmful microalgae. In the present study, sheepshead minnow larvae were not affected by fatty acids, while gill cells were. This suggests that for fish to be impacted by fatty acids their gills need to be well developed. Deeds et al. (2006) also found that juvenile sheepshead minnow were more sensitive to karlotoxin than were larvae. The application of RTgill-W1 has much more ecological relevance than using mammalian cells or brine shrimp.

Synergistic interactions between free fatty acids and reactive oxygen species as claimed for raphidophytes could not be confirmed. Gill cell damaging effects from EPA and OTA (but not OPA) were conclusively demonstrated, however, and remain candidates of ichthyotoxicity when well-defined toxins such as brevetoxin or karlotoxin can be ruled out as causative factors. The rainbow trout gill cell viability bioassay results are in agreement with observed symptoms and mortalities of sheepshead minnow larvae. Further work using the gill cell line will ascertain closer relationships between loss of viability and lethal dose and concentration to fish.

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LITERATURE CITED

Adolf JE, Place AR, Stoecker DK, Harding LW Jr (2007) Modulation of polyunsaturated fatty acids in mixotrophic *Karlodinium veneficum* (Dinophyceae) and its prey, *Storeatula major* (Cryptophyceae). J Phycol 43:1259–1270

Andersen RA, Morton SL, Sexton JP (1997) Provasoli-Guillard National Center for Culture of Marine Phytoplankton 1997 list of strains. J Phycol 33(Suppl.):1–75

Arzul G, Gentien P, Bodennec F (1998). Potential toxicity of microalgal polyunsaturated fatty acids (PUFAs). In: Baudimant G, Guézennec JH, Roy P, Samain JF (eds) Marine lipids. IFREMER, Plouzané, p 53–62

Bell MV, Dick JR, Pond DW (1997) Octadecapentaenoic acid in a raphidophyte alga, *Heterosigma akashiwo*. Phytochemistry 45:303–306

Berges JA, Franklin DJ, Harrison PJ (2001) Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the last two decades. J Phycol 37:1138–1145

Blackburn SI, Hallegraeff GM, Bolch CJS (1989) Vegetative production and sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum* from Tasmania, Australia. J Phycol 25:577–590

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:912–917

Christie WW (1982). Lipid analysis.Pergamon Press, Oxford

de Boer MK, Tyl MR, Fu M, Kulk G and others (2009) Haemolytic activity within the species *Fibrocapsa japonica* (Raphidophyceae). Harmful Algae 8:699–705

de Sousa Andrade LN, Martins de Lima T, Curi R, de Lauro Castrucci AM (2005) Toxicity of fatty acids on murine and human melanoma cell lines. Toxicol In Vitro 19:553–560

Deeds JR, Reimschuessel R, Place AR (2006) Histopathological effects in fish exposed to the toxins from *Karlodinium micrum* (Dinophyceae). J Aquat Anim Health 18:136–148

Dorantes-Aranda JJ, Waite TD, Godrant A, Rose AL, Tovar CD, Woods GM, Hallegraeff GM (2011) Novel application of a fish gill cell line assay to assess ichthyotoxicity of harmful marine microalgae. Harmful Algae

Fossat B, Porthé-Nibelle J, Sola F, Masoni A, Gentien P, Bodennec G (1999) Toxicity of fatty acid 18:5n3 from *Gymnodinium* cf. *mikimotoi*: II. intracellular pH and K^+ uptake in isolated trout hepatocytes. J Appl Toxicol 19:275–278

Fu M, Koulman A, van Rijssel M, Lützen A, de Boer MK, Tyl MR, Liebezeit G (2004) Chemical characterisation of three haemolytic compounds from the microalgal species *Fibrocapsa japonica* (Raphidophyceae). Toxicon 43:355–363

Gentien P (1998) Bloom dynamics and ecophysiology of the *Gymnodinium mikimotoi* species complex. In: Andersen DM, Cembella AD, Hallegraeff GM (eds) Physiological ecology of harmful algal blooms. Springer-Verlag, Berlin, NATO ASI Series, G 41, p 155–173

Gentien P, Lunven M, Lazure P, Youenou A, Crassous MP (2007) Motility and autotoxicity in *Karenia mikimotoi* (Dinophyceae). Philos Trans R Soc Lond B 362:1937–1946

Ghioni C, Porter AEA, Sadler IH, Tocher DR, Sargent JR (2001) Cultured fish cells metabolize octadecapentaenoic acid (all-cis δ3,6,9,12,15-18:5) to octadecatetraenoic acid (all-cis δ6,9,12,15-18:4) via its 2-trans intermediate (trans δ2, all-cis δ6,9,12,15-18:5). Lipids 36:145–153

Guella G, Frassanito R, Mancini I (2003) A new solution for an old problem: the regiochemical distribution of the acyl chains in galactolipids can be established by electrospray ionisation tandem mass spectrometry. Rapid Commun Mass Spectrom 17:1982–1994

Halliwell B, Gutteridge JMC (1999). Free radicals in biology and medicine. Oxford University Press, Oxford

Harvey HR, Bradshaw SA, O'Hara SCM, Eglington G, Corner EDS (1988) Lipid composition of the marine dinoflagellate *Scrippsiella trochoidea.* Phytochemistry 27:1723–1729

Henrikson JC, Gharfeh MS, Easton AC, Easton JD and others (2010) Reassessing the ichthyotoxin profile of cultured *Prymnesium parvum* (golden algae) and comparing it to samples collected from recent freshwater bloom and fish kill events in North America. Toxicon 55:1396–1404

Hiraga Y, Kaku K, Omoda D, Sugihara K, Hosoya H, Hino M (2002) A new digalactosyl diacylglycerol from a cultured marine dinoflagellate *Heterocapsa circularisquama.* J Nat Prod 65:1494–1496

Ikawa M (2004) Algal polyunsaturated fatty acids and effects on plankton ecology and other organisms. UNH Cent Freshw Biol Res 6:17–44

John U, Tillmann U, Medlin LK (2002) A comparative approach to study inhibition of grazing and lipid composition of a toxic and non-toxic clone of *Chrysochromulina polylepis* (Prymnesiophyceae). Harmful Algae 1:45–57

Jüttner F (2001) Liberation of 5,8,11,14,17-eicosapentaenoic acid and other polyunsaturated fatty acids from lipids as a grazer defense reaction in epilithic diatom biofilms. J Phycol 37:744–755

Kambayashi Y, Ogino K (2003) Reestimation of *Cyprina* luciferin analogs (MCLA) as a chemiluminescence probe to detect active oxygen species — cautionary note for use of MCLA. J Toxicol Sci 28:139–148

Kim CS, Lee SG, Lee CK, Kim HG, Jung J (1999) Reactive oxygen species as causative agents in the ichthyotoxicity of the red tide dinoflagellate *Cochlodinium polykrikoides.* J Plankton Res 21:2105–2115

Kim D, Oda T, Muramatsu T, Kim D, Matsuyama Y, Honjo T (2002) Possible factors responsible for the toxicity of *Cochlodinium polykrikoides*, a red tide phytoplankton. Comp Biochem Physiol C 132:415–423

Leblond JD, Chapman PJ (2000) Lipid class distribution of highly unsaturated long chain fatty acids in marine dinoflagellates. J Phycol 36:1103–1108

Leblond JD, Lasiter AD (2009) Mono- and digalactosyldiacylglycerol composition of dinoflagellates. II. *Lepidodinium chlorophorum, Karenia brevis* and *Kryptoperidinium foliaceum*, three dinoflagellates with aberrant plastids. Eur J Phycol 44:199–205

Mansour MP, Volkman JK, Jackson AE, Blackburn SI (1999b) The fatty acid and sterol composition of five marine dinoflagellates. J Phycol 35:710–720

Marshall JA, Hovenden M, Oda T, Hallegraeff GM (2002) Photosynthesis does influence reactive oxygen species production in the red tide alga *Chattonella marina* (Raphidophyceae). J Plankton Res 24:1231–1236

Marshall JA, Nichols PD, Hamilton B, Lewis RJ, Hallegraeff GM (2003) Ichthyotoxicity of *Chattonella marina* (Raphidophyceae) to damselfish (*Acanthochromis polycanthus*): the synergistic role of reactive oxygen species and free fatty acids. Harmful Algae 2:273–281

Marshall JA, Ross T, Pyecroft S, Hallegraeff G (2005a) Superoxide production by marine microalgae. II. Towards understanding ecological consequences and possible functions. Mar Biol 147:541–549

Marshall JA, Salas M, Oda T, Hallegraeff G (2005b) Superoxide production by marine microalgae. I. Survey of 37 species from 6 classes. Mar Biol 147:533–540

McDowell EM, Trump BF (1976) Histologic fixatives suitable for diagnostic light and electron microscopy. Arch Pathol Lab Med 100:405–414

Miyashita K, Nara E, Ota T (1993) Oxidative stability of polyunsaturated fatty acids in an aqueous solution. Biosci Biotechnol Biochem 57:1638–1640

Mooney BD, Nichols PD, de Salas MF, Hallegraeff GM (2007) Lipid, fatty acid and sterol composition of eight species of Kareniaceae (Dinophyta): chemotaxonomy and putative lipid phycotoxins. J Phycol 43:101–111

Mooney BD, De Salas MF, Hallegraeff GM, Place AR (2009) Survey for karlotoxin production in 15 species of gymnodinioid dinoflagellates (Kareniaceae, Dinophyta). J Phycol 45:164–175

Mooney BD, Hallegraeff GM, Place AR (2010) Ichthyotoxicity of four species of gymnodinioid dinoflagellates (Kareniaceae, Dinophyta) and related karlotoxins. Harmful Algae 9:557–562

Nakayama GR, Maureen CC, Nova MP, Parandoosh Z (1997) Assessment of the Alamar Blue assay for cellular growth and viability in vitro. J Immunol Methods 204:205–208

Oda T, Akaike T, Sato K, Ishimatsu A, Takeshita S, Muramatsu T, Maeda H (1992a) Hydroxyl radical generation by red tide algae. Arch Biochem Biophys 294:38–43

Oda T, Ishimatsu A, Shimada S, Takeshita S, Muramatsu T (1992b) Oxygen-radical-mediated toxic effects of the red tide flagellate *Chattonella marina* on *Vibrio alginolyticus.* Mar Biol 112:505–509

Odat T, Moritomi J, Kawano I, Hamaguichi S, Ishimatsu A, Muramatsu T (1995) Catalase- and superoxide dismutase-induced morphological changes and growth inhibition in red tide phytoplankton *Chattonella marina*. Biosci Biotech Biochem 59:2044–2048

Okaichi T (1983) Marine environmental studies on outbreaks of red tides in neritic waters. J Oceanogr Soc Jpn 39: 267–278

Okuyama H, Orikasa Y, Nishida T (2008) Significance of antioxidative functions of eicosapentaenoic and docosahexaenoic acids in marine microorganisms. Appl Environ Microbiol 74:570–574

Oshima Y, Yamada Sh, Matsunaga K, Moriya T, Ohizumi Y (1994) A monogalactosyl diacylglycerol from a cultured marine dinoflagellate, *Scrippsiella trochoidea.* J Nat Prod 57:534–536

Pagé B, Pagé M, Noël C (1993) A new fluorometric assay for cytotoxicity measurements *in vitro.* Int J Oncol 3:473–476

Parrish CC, Bodennec G, Sebedio JL, Gentien P (1993) Intra- and extracellular lipids in cultures of the toxic dinoflagellate, *Gyrodinium aureolum.* Phytochemistry 32:291–295

Pezzolesi L, Cucchiari E, Guerrini F, Pasteris A and others (2010) Toxicity evaluation of *Fibrocapsa japonica* from the Northern Adriatic Sea through a chemical and toxicological approach. Harmful Algae 9:504–514

Schirmer K, Chan AGJ, Greenberg BM, Dixon DG, Bols NC (1997) Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol In Vitro 11:107–119

Shimada M, Akagi N, Nakai Y, Goto H and others (1991) Free radical production by the red tide alga, *Chattonella antiqua.* Histochem J 23:361–365

Sola F, Masoni A, Fossat B, Porthé-Nibelle J, Gentien P, Bodennec G (1999) Toxicity of fatty acid 18:5n3 from *Gymnodinium* cf. *mikimotoi*: I. morphological and biochemical aspects on *Dicentrarchus labrax* gills and intestine. J Appl Toxicol 19:279–284

Urade R, Kito M (1982) Perturbation of lipid metabolism by palmitic acid in Chinese hamster V79-R cells. J Biochem 91:1639–1649

Valentine RC, Valentine DL (2004) Omega-3 fatty acids in cellular membranes: a unified concept. Prog Lipid Res 43: 383–402

Wardman P (2007) Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. Free Radic Biol Med 43:995–1022

Yamasaki Y, Kim DI, Matsuyama Y, Oda T, Honjo T (2004) Production of superoxide anion and hydrogen peroxide by the red tide dinoflagellate *Karenia mikimotoi.* J Biosci Bioeng 97:212–215

Yasumoto T, Underdahl B, Aune T, Hormazabal V, Skulberg OM (1990). Screening for hemolytic activity and ichthyotoxic components of *Chrysochromulina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters. In: Graneli E, Sundstrom B, Edler L, Anderson DM (eds) Toxic marine phytoplankton.. Elsevier, New York, NY, p 436–440

Yongmanitchai W, Ward OP (1992) Separation of lipid classes from *Phaeodactylum trichornutum* using silica cartridges. Phytochemistry 31:3405–3408

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