Algicidal activity of polyunsaturated fatty acids derived from *Ulva fasciata* and *U. pertusa* (Ulvaceae, Chlorophyta) on phytoplankton

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Abstract

Isolation of algicidal compounds from *Ulva fasciata* revealed that the algicidal substances were the polyunsaturated fatty acids (PUFAs) as hexadeca-4,7,10,13-tetraenoic acid (HDTA) C16:4 n-3, octadeca-6,9,12,15-tetraenoic acid (ODTA) C18:4 n-3, α-linolenic acid (ALA) C18:3 n-3 and linoleic acid (LA) C18:2 n-6. Fatty acid compositions of four species of Ulvaceae (*U. fasciata*, *U. pertusa*, *U. arasakii* and *U. conglobota*) were analyzed by capillary gas chromatography to investigate the relationship with the algicidal activity. The results indicate that highly algicidal species, *U. fasciata* and *U. pertusa*, showed higher contents of C16:4 n-3, C18:3 n-3, and C18:4 n-3. Concentrations of these PUFAs released from the seaweed in the culture medium were also analyzed. These PUFAs were found to be significantly active against *Chattonella antiqua*, *C. marina*, *Fibrocapsa japonica*, *Heterosigma akashiwo*, *Karenia mikimotoi*, moderately effective against *Heterocapsa circularisquama*, *Prorocentrum minimum*, *P. sigmoides*, *Scrippsiella trochoidea*, whereas low effective against *Alexandrium catenella* and *Cochlodinium polykrikoides*. It is suggested that the PUFAs are useful mitigation agents to remove several harmful effects without causing detrimental effects on surrounding marine living organisms.

Key words: algicidal compound, red tide phytoplankton, *Ulva fasciata*, *Ulva pertusa*

Introduction

The red tide phytoplankton known as harmful algal blooms (HAB) is reported to occur in world-wide, with warnings for public health and fisheries industries. Chemical agents such as copper sulfate (Steidinger, 1983) and hydrogen peroxide (Ryu et al., 1998) have

been performed as algicidal substances but are known to have a wide-spectrum in sterilizing effects. Viruses (Garry et al., 1998), bacteria (Imai et al., 1995), planktonic ciliates (Kamiyama, 2002) and heterotrophic dinoflagellates (Nakamura, 2002) as biological control also have been proposed. However, these methods also have the potential of disastrous environmental consequences (Jeong et al., 2000).

Several studies have been done aimed at development of a novel, low cost, environmentally benign method to control HAB by utilizing macroalgae. Studies on the allelochemicals of macroalga to microalgae were initially done by Kakisawa et al. (1988), in which the algicidal substance of octadeca-6,9,12,15-tetraenoic acid (ODTA) C18:4 n-3 from the brown alga *Cladosiphon okamuranus* was demonstrated to be toxic against 21 phytoplankton. Nagayama et al. (2003) have investigated toxic effect of the brown alga *Ecklonia kurome* on red tide phytoplankton and several phlorotannins were isolated as the active principles. Jeong et al. (2000) also screened extracts of macroalgae from the coast of Korea for growth inhibition of *Cochlodinium polykrikoides* and found *Corallina pilulifera, Ulva pertusa, Ishige foliacea* and *Endarachne binghamiae* were significantly active. Jin & Dong (2003) showed comparative studies on the algicidal effects of two different strains of *U. pertusa* on *Heterosigma akashiwo* and *Alexandrium tamarense*. Nan et al. (2004) reported that algicidal interaction between green alga *U. pertusa* and eight phytoplankton species. Recently, Wang et al. (2006) showed effect of green alga *U. pertusa* and red alga *Gracilaria lemaneiformis* on the growth of *H. akashiwo* in co-culture.

We screened the methanol extracts of thirty-seven species of macroalgae (10 Chlorophyta, 13 Phaeophyta and 14 Rhodophyta) collected from the coast of Nagasaki Prefecture, Japan, for algicidal activity against red tide phytoplankton, and found that

the green algae *U. fasciata* and *U. pertusa* (Ulvaceae, Chlorophyta) had the higher algicidal activity than the other macroalgae tested (Alamsjah et al., 2005). We also isolated and identified hexadeca-4,7,10,13-tetraenoic acid (HDTA) C16:4 n-3, C18:4 n-3 (ODTA), α-linolenic acid (ALA) C18:3 n-3 and linoleic acid (LA) C18:2 n-6 as the active principles. These polyunsaturated fatty acids (PUFAs) are found to be significantly active against several red tide phytoplankton at low concentrations and are promising for the chemical agents for HAB control. Herein, we describe the summary of our study on the algicidal effects of *Ulva* species involving isolation and structure determination of active principles, structure-activity relationship (SAR) study for fatty acids, quantitative analysis of the algicidal compounds released from the plant body, and comparison of fatty acid composition among green seaweed of the family of Ulvaceae.

Materials and methods

Plant material

Green algae of the Ulvaceae (*U. fasciata*, *U. pertusa*, *U. arasakii*, and *U. conglobota*) were collected in May 2005 – April 2006. Ecological damage during harvesting was prevented by not removing the algae stems. All samples were brought to the laboratory in plastic bags containing seawater to prevent evaporation, and then washed with distilled water to separate potential contaminants. For convenience, macroalgae tissues were dried completely for 1 d at room temperature and then ground to powder using a blender.

Phytoplankton species and culture

Axenic phytoplankton species of *Alexandrium catenella* NIES-677, *Chattonella antiqua*

NIES-1, *C. marina* NIES-3, *Eutreptiella gymnastica* NIES-381, *Fibrocapsa japonica*, NIES-462, *Heterocapsa triquetra* NIES-7, *Heterosigma akashiwo* NIES-4, *Karenia mikimotoi* NIES-680, *Nephroselmis astigmatica* NIES-252, *N. viridis* NIES-486, *Oltmannsiellopsis unicellularis* NIES-359, *Prorocentrum sigmoides* NIES-683, *Pterosperma cristatum* NIES-221, *Pyramimonas* aff. *amylifera* NIES-251, *P. parkeae* NIES-254, *Scrippsiella sweeneyae* NIES-684 and *S. trochoidea* NIES-369 were obtained from the National Institute for Environmental Studies, Japan. The other species, *Nannochloropsis oculata* ST-6, *Cochlodinium polykrikoides* ND-14, *Heterocapsa circularisquama* ND-12, *Prorocentrum minium* ND-34, *Pinnularia* sp. NB-1, *Skeletonema costatum* NB-2, *Stephanopyxis palmeriana* NB-3 and *Rhizosolnia hebetata f. semispina* NB-4 had been maintained at Marine Plant Science laboratory, Faculty of Fisheries, Nagasaki University of Japan. The phytoplankton species were cultured aseptically in Guillard`s f/2 medium (Sigma). These experiments were maintained at 20^{o}C, 40 μmol m^{-2} s^{-1} using 40 watts fluorescent lamps FL40SD (Toshiba) with 12L:12D cycle.

Extraction and isolation of algicidal compounds from U. fasciata

U. fasciata (4.36 kg, dry wt) was crushed into small pieces using a blender and extracted twice with MeOH (10.5 L, 4 d and 12.5 L, 2 d). After removing the MeOH under reduced pressure, the residue, containing about 300 mL of water, was partitioned between water (700 mL) and hexane (1,200 mL). The hexane extract was concentrated, taken up in hexane (350 mL), and extracted with 4M aqueous HCl (100 mL x 3) followed by 50% aqueous MeOH (150 mL x 2 and 300 mL). The hexane layer was dried (Na_2SO_4) and concentrated to leave an deep green oily residue (12.95 g), which

was chromatographed on silica gel (63-230 μm, 240 g) eluted with a gradient of hexane-EtOAc (9:1, 5:1, 1:1, then 0:1) to give four fractions (FL1-FL4). The active fraction, FL3 (2.35 g), eluted with hexane:EtOAc=1:1, was then chromatographed on Octadecyl-S (50 μm, 38 g) eluted with 90% MeOH to give four fractions (FL3-1 to FL3-4). FL3-2 (319.4 mg) and FL3-3 (548.8 mg) were active. HPLC purification of FL3-2 (319.4 mg) (Cosmosil 5C18-MS-II, 20 x 250 mm, 80% CH_3CN, flow rate 5 mL min^{-1}) yielded compound 1 (73.5 mg). Part of FL3-3 (82 mg) was also separated by HPLC in the same manner to yield compound **1** (13.1 mg), compound **2** (21.4 mg), and compound 3 (22.1 mg). FL3-1 (158 mg) was purified with TLC RP-18 (Merck, 20 x 20 cm, layer thickness 0.25 mm) using 85% CH_3CN as the solvent to give compound **4** (16.9 mg) along with compound **3**.

Spectral data (IR, EIMS, HRMS, ^1H and ^{13}C NMR) for compounds **1**-**3**: see reference Alamsjah et al., 2005.

Compound **4** (linoleic acid). EIMS 281 (M^+). ^1H NMR (400 MHz, $CDCl_3$) δ 0.89 (3H,t, J=6.6 Hz), 1.23-1.41 (14H, m), 1.63 (2H, m), 2.01-2.10 (4H, m), 2.34 (2H, t, J=7.6 Hz), 2.77 (2H, m), 5.28-5.43 (4H, m), 7.26 (1H, br.). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.7 (CH_2), 24.8 (CH_2), 25.7 (CH_2), 27.2 (CH_2 x 2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.7 (CH_2), 31.5 (CH_2), 33.9 (CH_2), 127.9 (CH=C), 128.2 (CH=C), 130.0 (CH=C), 130.2 (CH=C), 178.7(C=O).

Measurements of fatty acid composition

The dried sample of each green macroalgae (10 g) was extracted using 150 mL of methanol (Wako Pure Chemical) for 3 d. The extraction procedure was repeated twice and the extracts were combined. The 50 mg of the crude extract was esterified by

exposure to 2.5 mL of 3% HCl/MeOH for overnight. After concentrating under reduced pressure, the residue was taken up in CH_2Cl_2 and washed with 5% $NaHCO_3$ solution. The CH_2Cl_2 layer was separated and dried over Na_2SO_4. After concentration, the residue was passed through a short column on silica gel (63-230 μm, 500 mg) using CH_2Cl_2 as the eluent. Finally, the methyl esters fraction was diluted in 1 mL of hexane and analyzed using a GC-2014 gas chromatograph (Shimadzu) equipped with a capillary column (CP-Sil 88 for FAME fused silica WCOT, 50 m x 0.25 mm i.d., 0.2 μm film thickness, Chrompack, Hewlett-Packard Co.) and a flame ionization detector (FID). The detector and the injector temperature were maintained at 300^{o}C. The initial oven temperature was programmed at 170◦C for 15 min, and then, was allowed to rise to 230 ^{o}C at a rate of 5 ^{o}C min^{-1}, and was kept at the final temperature for 5 min. Nitrogen was used as the carrier gas at a flow rate of 24 mL min^{-1}. A standard fatty acid methyl ester mixture C8-C22 and C14-C22 (Supelco) was used for identification of the peaks.

Structure activity relationship study for fatty acids (C16, C18, C20 and C22)

The fatty acids, palmitic acid C16:0, palmitoleic acid *cis*-C16:1 n-7, stearic acid C18:0, oleic acid C18:1 n-9, α-linolenic acid C18:3 n-3, arachidic acid C20:0, arachidonic acid C20:4 n-6, eicosapentaenoic acid C20:5 n-3, behenic acid C22:0, and docosahexaenoic acid C22:6 n-3 were purchased from Tokyo Kasei Kogyo. The other fatty acids, palmitelaidic acid *trans*-C16:1 n-7, linolic acid C18:2 n-6, eicosenoic acid C20:1 n-9 and erucic acid C22:1 n-9 were from MP Biomedicals, Wako Pure Chemical, Sigma and Alfa Aesar, respectively. C16:4 n-3 (HDTA) and C18:4 n-3 (ODTA) used for the algicidal assay were the natural compounds isolated from *U. fasciata*.

Algicidal assay of C16-C22 fatty acids against H. akashiwo and C. marina

H. akashiwo and *C. marina* were cultured aseptically in sterilized Guillard`s f/2 medium (Sigma) at 20 ^{o}C under illumination of a fluorescent lamp (40 μmol photons m^{-2} s^{-1}) under a cycle of 12 h light and 12 h dark. Cells in the exponential growing phase were used throughout the experiments. To each of the phytoplankton cell suspension (cell density ca. 3 x 10^4 cells mL^{-1}) in a 24-well micro plate (Iwaki), varying amounts of the fatty acid solution in MeOH (< 2 μL) were added to make the final concentration of 100, 75, 50, 25, 15, 5, 2, 1 and 0.5 μg mL^{-1}. After 4 h cultivation, survivability and mortality of cells were calculated under microscope observation (x 20). Algicidal activity was calculated using a formula: Algicidal activity = (dead cells/living and dead cells) x 100%. All data in this study were tested by means of ANOVA-test ($p<0.05$) and continued by multiple range test for comparing means in an analysis of variance. Essentially the same procedure was used for the algicidal assay of C16:4 n-3, C18:3 n-3 and C18:4 n-3 on various phytoplankton (Table 4).

Algicidal compounds released from dry powder and fresh tissue of U. fasciata in seawater

The dry powder of *U. fasciata* (1.5 g L^{-1}, size 150 μm, autoclaved and non autoclaved) were mixed in autoclaved of seawater (ASW) using a stirrer for 6, 12, 24, 36 and 48 h on 20 ^{o}C in dark condition, and filtered through a No. 2 filter paper. The filtrate (8 mL) and 2 mL of *H. akashiwo* (cells density 3 x 10^5 cells mL^{-1}) were mixed in a Petri dish of 5-cm diameter to check algicidal activity at 4 h. Then the water solution was adjusted at pH 2 using 1 M HCl, extracted 3 times with CH_2Cl_2 (100 mL each). The extracts were combined, dried over Na_2SO_4, and concentrated. The extract was esterified by the

procedure described in “measurement of fatty acid composition”.

For analysis of algicidal compounds released from fresh tissue of *U. fasciata*, samples were performed as 50 g of whole material or cutting tissue (0.5 x 0.5 cm^2 size segments). Fresh tissue sample was rinsed with ASW and were further maintained in 1 L flat bottom aeration flasks with ASW containing 1% of antibiotic mixture (penicillin G 1 g and streptomycin sulfate 2 g in distilled water 100 mL) for 2 d. The water rinsed of cutting tissue was collected and then analyzed for fatty acid composition as described for the dry powder.

Statistics

All the experiments in this study were done separately in at least triplicate and tested by ANOVA test ($p<0.05$). Simple regression and correlation analysis was used to examine data for possible relationships and to obtain LC_{50} (median lethal concentration) values (Nakai et al., 1999). For sigmoid curves, the y values (percentage) were converted by probit transformation using Microsoft Excel®.

Results

Structural determination of algicidal compounds from U. fasciata

Four algicidal compounds (compounds **1**-**4**) were isolated from *U. fasciata* and their structures were elucidated as C16:4 n-3 (HDTA), C18:4 n-3 (ODTA), C18:3 n-3 (ALA) and C18:2 n-6 (LA) on the basis of spectroscopic information (Alamsjah et al., 2005). Structures of all compounds isolated were verified by comparison of authentic samples obtained from commercial sources or by a synthesis (unpublished data).

Fatty acid composition of macroalgae

Fatty acid compositions of methanol extracts of four macroalgae of the Ulvaceae were analyzed using GLC and the result is shown in Table 1. All the macroalgae showed similar fatty acid composition, palmitic acid C16:0, C16:4 n-3 (HDTA), C18:3 n-3 (ALA) and C18:4 n-3 (ODTA) being predominant, except for *U. arasakii,* which had higher concentration of C18:2 n-6 (LA) and lower ratio of C18:4 n-3.

Correlation between total active PUFAs amount and algicidal activity

Table 2 shows quantitative analysis of the three highly active compounds, C16:4 n-3 (HDTA), C18:3 n-3 (ODTA), and C18:4 n-3 (ALA) in dry tissue of four macroalgae and the algicidal activity of their methanol extracts. Total average of active PUFAs of C16:4 n-3, C18:3 n-3 and C18:4 n-3 from *U. fasciata* and *U. pertusa* were 1338.21 and 1094.44 mg 100 g^{-1}, respectively, which were 2 to 10-fold of those of the other two species. It also shows that the algicidal activities correlate well with the amount of total active PUFAs in dry tissues. Dry powder of *U. fasciata* and *U. pertusa* itself also showed potent activity on *H. akashiwo,* with 4 h LC_{50} values were 0.4 and 0.7 g L^{-1}, respectively. Furthermore, algicidal activity of *U. fasciata* and *U. pertusa* based on seasonal variation were checked (Figure 1). Graph A and C show the monthly change in the total amount of the active PUFAs and algicidal activity to *H. akashiwo* expressed in LC_{50} value of *U. fasciata* (A) and *U. pertusa* (C). There seems to be a trend that the amount of active PUFAs produced by the green algae decline in June and July, and consequently the algicidal activities decrease. However, the correlation analyses (Graph B and D) indicated that the algicidal activity of *U. fasciata* and *U. pertusa* were not different significantly ($p>0.05$) based on seasonal variation.

Structure activity relationship study for fatty acids

Sixteen fatty acids of the numbers of carbon from C16 to C22 were evaluated for algicidal activity against *H. akashiwo* and *C. marina*. Highly unsaturated fatty acids, C16:4 n-3 (HDTA), C18:3 n-3 (ALA), C18:4 n-3 (ODTA), arachidonic acid C20:4, eicosapentaenoic acid C20:5 and docosahexaenoic acid C22:6 showed strong activity with 4 h LC_{50} values less than 5 and 8 μg mL^{-1} on *H. akashiwo* and *C. marina*, respectively (Table 3). In this study, C18:2 n-6 (LA) also showed strong activity against *H. akashiwo*, however it showed lower activity against *C. marina*. Among the fatty acids of the same carbon number, the activity increased as the number of unsaturation increased, while the number of carbons was irrelevant. Interestingly, palmitoleic acid C16:1 n-7 had moderate activity while longer fatty acids having the same unsaturation (oleic acid C18:1, eicosenoic acid C20:1 and eruric acid C22:1) were almost or completely inactive.

Algicidal activity on phytoplankton

Algicidal activities of C16:4 n-3, C18:3 n-3, and C18:4 n-3 were evaluated against 25 species of phytoplankton including red tide phytoplankton species (Table 4). Algicidal profiles of these fatty acids were almost similar. C16:4 n-3, C18:3 n-3, and C18:4 n-3 were found to be significantly active against *C. antiqua*, *C. marina*, *Fibrocapsa japonica*, *H. akashiwo*, *Karenia mikimotoi*, moderately effective against *Heterocapsa circularisquama*, *Prorocentrum minimum*, *P. sigmoides*, *Scrippsiella trochoidea*, whereas low effective against *A. catenella* and *Coch. polykrikoides*.

Algicidal compounds (C16:4 n-3, C18:3 n-3 and C18:4 n-3) released from U. fasciata

C16:4 n-3, C18:3 n-3, and C18:4 n-3 released from autoclaved and non-autoclaved dry powder of *U. fasciata* in seawater were different significantly ($p<0.05$) (Table 5). Released amount of the total PUFAs reached maximum at 6 h, and afterward, gradually decomposed with time. At 6 h, 46.72% of the total active PUFAs contained in the dry powder were released into the seawater. After 48 h, remaining active PUFAs decreased to 2% of the maximum amount. No significant difference was observed in the released amount between whole and cut tissues (Table 6).

Discussion

The algicidal principles of *U. fasciata* and *U. pertusa* were found to be PUFAs such as C16:4 n-3, C18:3 n-3, and C18:4 n-3 (Alamsjah et al., 2005), one of which, C18:4 n-3, was the same compound that had been isolated as the allelopathic substance of *C. okamuranus* (Kakisawa et al., 1988). Other researchers have also reported that green algae such as *U. pertusa*, *Monostroma lattisium* and *Enteromorpha* sp. obtained in Japanese water contain large amount of PUFA composed mainly of C16:4 n-3, C18:3 n-3, and C18:4 n-3 (Takagi et al., 1985).

The higher toxicity of C16:4 n-3, C18:3 n-3, and C18:4 n-3 may be also reflected by the amphiphatic property of PUFAs, which probably disrupts the membrane integrity of phytoplankton cells. Chemical structural feature such as the number of unsaturated double bonds may also be involved in the biological activity of these unsaturated fatty acids. These results are supported by earlier experiments by Kakisawa et al. (1988), Suzuki et al. (1996) and Sellem et al. (2000). Chiang et al. (2004) showed that the highest toxicity from the green colonial alga *Botryococcus braunii* was

observed with C18:3 n-3 (ALA), followed by C18:2 n-6 (LA) and C18:1 n-9. In this study, PUFAs were also more toxic than saturated fatty acids against *H. akashiwo* and *C. marina*. Morohashi et al. (1991) determined that unsaturated fatty acids of the *cis*-configuration were more effective than saturated acids. Interestingly, C18:3 n-3 (ALA) and C16:4 n-3 (HDTA) also showed lethal effect on phytoplankton, to the same extent as C18:4 n-3 (ODTA) (Kakisawa et al. 1988), which is active against phytoplankton without cell coverings and inactive to ones with rigid cell walls. Kakisawa et al. (1988) also showed that C18:4 n-3, the allelochemical of the brown alga *C. okamuranus*, had different responses against phytoplankton.

The fatty acid composition profiles of each macroalga of the family Ulvaceae were almost similar, having a high concentration of C16 and C18 PUFAs. These data are in agreement with other researchers conclusions that a dominance of C16 and C18 PUFAs are characteristic of green macroalgae (Jamieson & Reid, 1972; Ackman & McLachlan, 1977). In this study, the ratio of total PUFAs to total fatty acids (PUFA : FA ratio) of the family Ulvaceae showed high growth rates, as Ahlgren et al. (1992) suggested that PUFA : FA ratio could be useful indicator of the physiological status of algae. This is because PUFAs are usually stable major components of cell membranes while the saturated fatty acids are environmentally-sensitive storage products (Sargent et al., 1987). Thus, the biosynthetic substances of *Ulva* species are really promising for practical harmful algal bloom control. Ikawa (2004) tried to explain the inhibitory effects of PUFAs on phytoplankton, which it may have a disrupting influence on the lipid bilayer of natural biological membranes of eukaryotic cells. Murata et al. (1989) and Oda et al. (1992) also reported that the toxic activity of PUFAs may be due to oxidation products derived through photo oxidation or metabolic processes, whereas

Kogteva and & Bezuglov (1998) determined that the potent activity of PUFAs may act as second messengers to modulate functionally active proteins.

Although fatty acids are hydrophobic substances with a very limited solubility in water, Kattner et al. (1983) studying the lipid concentration during a spring phytoplankton bloom in the northern North Sea in Europe estimated that fatty acid constituted about 3% of total dissolved organic matter. The total fatty acid concentration amounted to about 1.15 μmol L^{-1} before the bloom and increased to a maximum of 5 μmol L^{-1} during a bloom. In this study, the C16:4 n-3, C18:3 n-3, and C18:4 n-3 from autoclaved of dry powder of *U. fasciata* were higher to release in seawater than non-autoclaved of dry powder of *U. fasciata* in time-dependent manner. It means decomposition of algicidal activity of *U. fasciata* was caused by biological agent (e.g. bacteria) and chemical reaction such as photooxidation. Similarly, algicidal activity of autoclaved of dry powder of *U. fasciata* also showed higher than non-autoclaved of dry powder of *U. fasciata* in time-dependent manner under controlled conditions in the laboratory, while Ikawa (2004) have mentioned that effective levels of PUFA soluble in long term exposure to low levels under natural conditions can have effects which are only observed with higher level acute doses. Scutt (1964) also reported that fresh filtrates of *Chlorella vulgaris* did not contain auto inhibitors and that only after storage for several days did auto inhibitory activity appear. It was probably fatty acids from fresh tissues of macroalgae in seawater were released in gradually concentration.

The main factors generally considered when selecting harmful algal bloom control methods are effectiveness, toxicity, cost and practicability. Taking into account the characteristics described so far, algicidal compound of *Ulva* species (Ulvaceae, Chlorophyta) is promising for controlling HAB species because (1) the *U. fasciata* and

U. pertusa have high inhibition effect on several red tide phytoplankton without recovery at a relatively low concentration, (2) they have high biodegradability, ecological acceptability, and a relatively environmentally benign nature. In conclusion, the PUFAs may useful mitigation agents to remove harmful red tide phytoplankton such as *C. marina* and *H. akashiwo* without causing detrimental effects on surrounding marine living organisms.

The world-wide distribution of *Ulva* species will also give an opportunity to evaluate the genetic variability for their toxic effects on HAB. Further research on the precise mechanism and mode of action of the algicidal substances from *Ulva* species to cause death of HAB species may shed new light on the algicidal activity and application of *Ulva* species as effective control agents.

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References

Ackman RG., McLachlan J (1977) Fatty acids in some Nova Scotian marine seaweeds: a survey for octadecapentaenoic and other biochemically novel fatty acids. Proc. N. S. Inst. Sci. 28: 47-64.

Ahlgren G, Gustaffson IB, Boberg M (1992) Fatty acid content and chemical composition of freshwater microalgae. J. Phycol. 28: 37-50.

Alamsjah MA, Hirao S, Ishibashi F, Fujita Y (2005) Isolation and structure determination of algicidal compounds from *Ulva fasciata*. Biosci. Biotechnol. Biochem. 69: 2186-2192.

Alamsjah MA, Ishibe K, Kim D, Yamaguchi K, Ishibashi F, Fujita Y, Oda T (2007) Selective toxic effects of polyunsaturated fatty acids derived from *Ulva fasciata* on red tide phytoplankter species. Biosci. Biotechnol. Biochem. 71: 265-268.

Chiang IZ, Huang WY, Wu JT (2004) Allelochemicals of *Botryococcus braunii* (Chlorophyceae). J. Phycol. 40: 474-480.

Garry RT, Hearing P, Cosper EM (1998) Characterization of a lytic virus infectious to the bloom-forming microalga *Aureococcus anophagefferens* (Pelagophyceae). J. Phycol. 34: 616-621.

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

Imai I, Ishida Y, Sakaguchi K, Hata Y (1995) Algicidal marine bacteria isolated from northern Hiroshima Bay. Fish. Sci. 61: 624-632.

Jamieson GR, Reid EH (1972) The component fatty acids of some marine algal lipids. Phytochem. 11: 1423-1432.

Jeong JH, Jin HJ, Sohn CH, Suh KH, Hong YK (2000) Algicidal activity of the seaweed *Corallina pilulifera* against red tide microalgae. J. Appl. Phycol. 12: 37-43.

Jin Q, Dong S (2003) Comparative studies on the allelopathic effects of two different strains of *Ulva pertusa* on *Heterosigma akashiwo* and *Alexandrium tamarense*. J. Exp. Mar. Biol. Ecol. 293: 41-55.

Kakisawa H, Asari F, Kusumi T, Toma T, Sakurai T, Oohusa T, Hara Y, Chiharai M (1988) An allelopathic fatty acid from the brown alga *Cladosiphon okamuranus*. Phytochem. 27: 731-735.

Kamiyama T (2002) Regulation of harmful algal blooms by planktonic ciliates. In Hiroishi S, Imai I, Ishimaru T (eds), Prevention and Extermination Strategies of Harmful Algal Blooms. Nihon Suisan Gakkai Kansyu 134, Kouseisya Kouseikaku, Tokyo, Japan, 89-101 (in Japanese).

Kattner G, Gercken G, Hammer KD (1983) Development of lipids during a spring plankton bloom in the northern North Sea. II. Dissolved lipids and fatty acids. Mar. Chem. 14: 163-173.

Kogteva GS, Bezuglov VV (1998) Unsatutared fatty acids as endogenous bioregulators. Biochem. (Moscow) 63: 6-15.

Morohashi M, Tsuchiya K, Mita T, Kawamura M (1991) Identification of (Na, K) ATPase inhibitor in brine shrimp, *Artemia salina*, as long chain fatty acids. J. Comp. Physiol. B. 161: 69-72.

Murata H, Sakai T, Endo M, Kuroki A, Kimura M, Kumanda K (1989) Screening of removal agents of a red tide plankton *Chattonella marina* with special reference to the ability of the free radicals derived from the hydrogen peroxide and polyunsaturated fatty acids. Nippon Suisan Gakkaishi 55: 1075-1082.

Nagayama K, Shibata T, Fujimoto K, Honjo T, Nakamura T (2003) Algicidal effect of phlorotannins from the brown alga *Ecklonia kurome* on red tide microalgae. Aquacult. 218: 601-611.

Nakai S, Inoue Y, Hosomi M, Murakami A (1999) Growth inhibition of blue-green algae by allelopathic effects of macrophytes. Water Sci. Technol. 39: 47-53.

Nakamura Y (2002) Control of red tides by heterotrophic dinoflagellates. In Hiroishi S, Imai I, Ishimaru T (eds), Prevention and Extermination Strategies of Harmful Algal Blooms. Nihon Suisan Gakkai Kansyu 134, Kouseisya Kouseikaku, Tokyo, Japan, 81-88 (in Japanese).

Nan C, Zhang H, Zhao G (2004) Allelopathic interactions between the macroalga *Ulva pertusa* and eight microalgal species. J. Seares. 52: 259-268.

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

Ryu HY, Shim JM, Bang JD, Lee C (1998) Experimental chemical treatments for the control of dinoflagellate, *Cochlodinium polykrikoides* in the land-based culture of olive flounder *Paralichthys olivaceus*. Kor. J. Aquacult. 11: 285-294.

Sargent JR, Parkes RJ, Mueller-Harvey I, Henderson J (1987) Lipid biomarkers in marine ecology. In Sleigh MA (ed) Microbes in the sea. Ellis Horwood, Chichester, United Kingdom, 119-138.

Scutt JE (1964) Autoinhibitor production by *Chlorella vulgaris.* Am. J. Bot. 51: 581-584.

Sellem F, Pesando D, Bodennec G, Abed AE, Girard JP (2000) Toxic effects of *Gymnodinium cf. mikimotoi* unsaturated fatty acids to gametes and embryos of the sea urchin *Paracentrotus lividus*. Water Res. 34: 550-556.

Steidinger KA (1983) A re-evaluation of toxic dinoflagellate biology and ecology. Prog. Phycol. Res. 2: 147-188.

Suzuki M, Wakana I, Denboh T, Tatewaki M (1996) An allelopathic polyunsaturated fatty acid from red algae. Phytochem. 43: 63-65.

Takagi T, Asahi M, Itabashi Y (1985) Fatty acid composition of twelve algae from Japanese waters. Yukagaku 34, 1008-1012.

Wang Y, Yu ZM, Song XX, Zhang SD (2006) Effects of *Ulva pertusa* and *Gracilaria lemaneiformis* on growth of *Heterosigma akashiwo* (Raphidophyceae) in co-culture. Huan Jing Ke Xue 27: 246-252 (in Chinese).

Figure Legends

Figure 1. A and C**:** seasonal variation of total C16:4 n-3 (HDTA), C18:3 n-3 (ALA) and C18:4 n-3 (ODTA) concentration (mg 100 g^{-1}, white bars) in dry tissue of *Ulva fasciata* (A) and *Ulva pertusa* (C) and algicidal activity [4 h LC_{50} (mg L^{-1}), ●] on lyses of *Heterosigma akashiwo*. B and D: correlation analysis between total of C16:4 n-3 (HDTA), C18:3 n-3 (ALA) and C18:4 n-3 (ODTA) (mg 100 g^{-1}) and algicidal activity [4 h LC_{50} (mg L^{-1})] for *Ulva fasciata* (B) and *Ulva pertusa* (D).

Figure 1

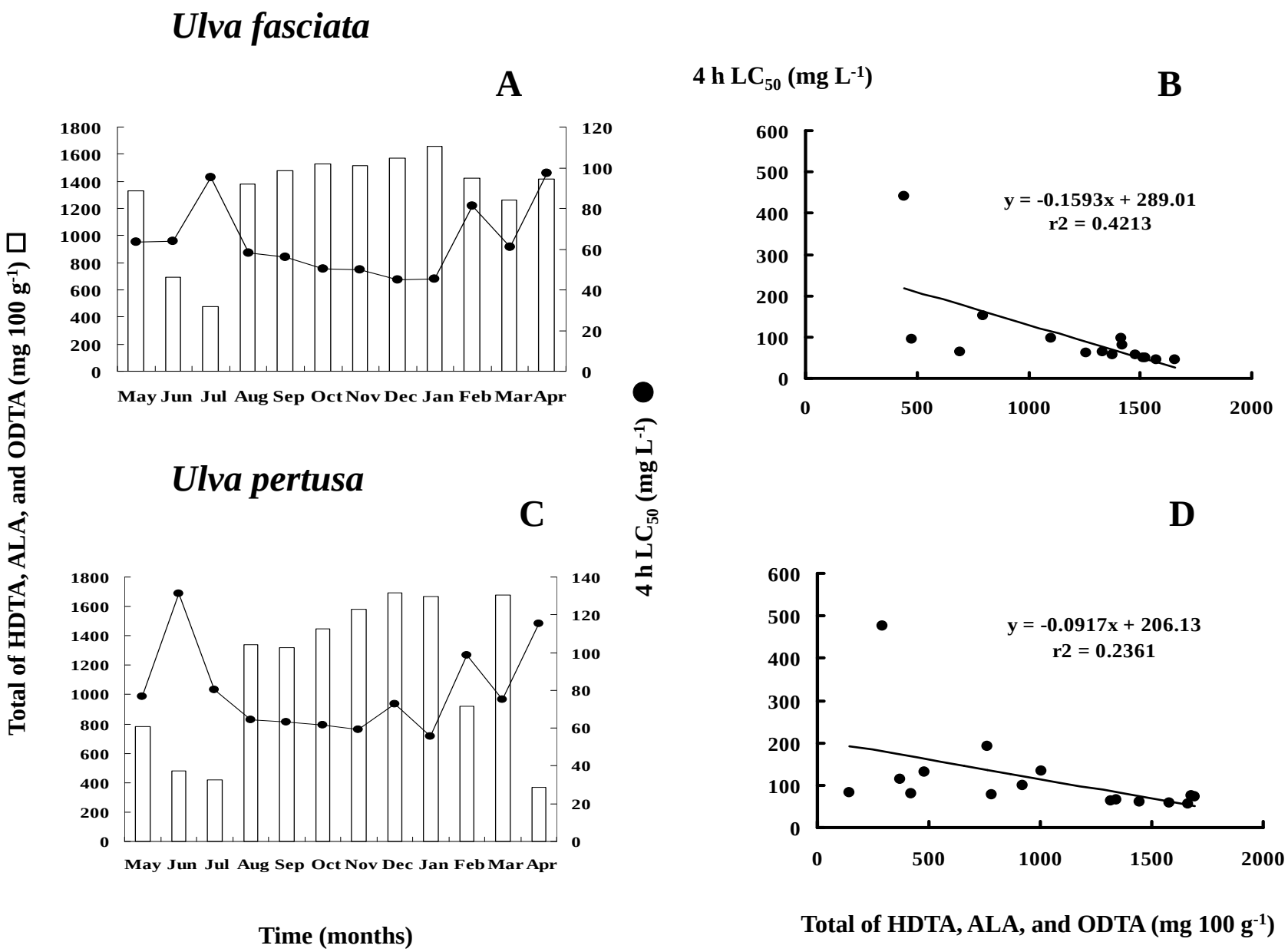
Ulva fasciata
A
B
Ulva pertusa
C
D
Total of HDTA, ALA, and ODTA (mg 100 g-1) □
4 h LC50 (mg L-1) ●
4 h LC50 (mg L-1)
y = -0.1593x + 289.01
r2 = 0.4213
y = -0.0917x + 206.13
r2 = 0.2361
May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr
Time (months)
Total of HDTA, ALA, and ODTA (mg 100 g-1)

Table 1. Fatty acid composition (mean ± SD, % of the total composition) of *Ulva fasciata, Ulva pertusa, Ulva arasakii and Ulva conglobota* from Nagasaki beach (collected May 2005 – April 2006).

Fatty acids		*Ulva fasciata* n = 13	*Ulva pertusa* n = 14	*Ulva arasakii* n = 1	*Ulva conglobota* n = 2
decanoic	C10:0	0.78±0.68	0.96±0.94	0.29	0.55±0.39
myristic	C14:0	0.70±0.36	0.68±0.46	0.44	1.03±1.02
myristoleic	*cis*-C14:1 n-9	1.81±1.44	2.07±1.19	0.87	3.35±0.53
palmitic	C16:0	29.32±5.41	27.36±4.10	25.43	34.16±0.33
hexadecatetraenoic	C16:4 n-3	10.57±2.53	12.73±2.66	11.53	9.28±1.87
stearic	C18:0	0.91±0.42	1.03±0.59	nd	2.39±0.43
elaidic	*trans*-C18:1 n-9	1.66±1.30	1.64±1.58	nd	3.35±0.53
oleic	*cis*-C18:1 n-9	5.12±3.52	5.15±3.53	7.40	6.31±2.52
linolic	C18:2 n-6	7.87±1.72	8.09±2.46	21.00	6.81±2.09
α-linolenic	C18:3 n-3	17.25±1.76	17.96±2.79	22.98	14.37±0.09
octadecatetraenoic	C18:4 n-3	20.49±7.39	20.56±6.81	5.36	11.84±7.14
eicosenoic	C20:1 n-9	1.98±1.93	1.35±0.89	0.42	1.77±0.21
behenic	C22:0	0.80±0.80	0.65±0.55	2.87	2.50±0.62
erucic	C22:1 n-9	2.46±1.49	2.88±1.59	1.42	2.57±0.37
PUFA:FA		0.56±0.09	0.59±0.10	0.61	0.42±0.07

nd: not detected; PUFA: FA: ratio of total polyunsaturated fatty acids to total fatty acids.

Table 2. The active compounds of HDTA, ALA, and ODTA/dry tissue (mean ± SD, mg 100 g^{-1}), 4 h LC_{50} of algicidal activity of methanol extracts (mean ± SD, mg L^{-1}*)* and dry powder (mean ± SD, g L^{-1}) from *Ulva fasciata, Ulva pertusa, Ulva arasakii and Ulva conglobota* on lyses of *Heterosigma akashiwo*.

	Ulva fasciata	*Ulva pertusa*	*Ulva arasakii*	*Ulva conglobota*
HDTA, ALA, ODTA/dry tissue	1338.21±358.02	1094.44±525.98	267.15	119.00±84.90
4 h LC_{50} of algicidal activity of methanol extracts	64.20±17.32	81.91±24.49	161.07	526.28±89.78
4 h LC_{50} of algicidal activity of dry powder	0.40±0.01	0.70±0.01	1.50	2.30±0.13

Table 3. Algicidal activity (4 h LC_{50} in μg mL^{-1}) of C16-C22 fatty acids against *Heterosigma akashiwo* and *Chattonella marina*.

Fatty acids		*Heterosigma akashiwo*	*Chattonella marina*
palmitic	C16:0	79.28	29.50
palmitelaidic	*trans*-C16:1 n-7	>100	43.75
palmitoleic	*cis*-C16:1 n-7	7.28	20.31
octadecatetraenoic	C16:4 n-3	1.93	5.34
stearic	C18:0	77.63	29.24
oleic	C18:1 n-9	34.01	24.85
linoleic	C18:2 n-6	3.46	23.90
α-linolenic	C18:3 n-3	2.59	7.11
hexadecatetraenoic	C18:4 n-3	0.83	4.02
arachidic	C20:0	94.38	34.07
eicosenoic	C20:1 n-9	>100	43.59
arachidonic	C20:4 n-6	2.34	6.85
eicosapentaenoic	C20:5 n-3	2.11	6.79
behenic	C22:0	>100	52.97
eruric	C22:1 n-9	>100	45.56
docosahexaenoic	C22:6 n-3	4.37	7.19

Table 4. Algicidal activity of HDTA, ALA, and ODTA (μg mL^{-1}) against phytoplankton. Each concentration of fatty acid was added to f/2 medium inoculated with approximately 3 x 10^4 cells mL^{-1} of phytoplankton for 4 h. A "+" symbol indicates more than 70% of mortality cells; "±" symbol indicates moderate of mortality cells, i.e. between 30 and 69%; "-" symbol indicates less than 30% of mortality cells. "NIES" strains were obtained from the National Institute for Environmental Studies, Japan. "ND", "NB", and "ST" strains were maintained at Marine Plant Science Laboratory, Faculty of Fisheries, Nagasaki University, Japan.

Plant family	Species	HDTA		ALA		ODTA	
		25	5	25	5	25	5
Raphidophyceae	*Chattonella antiqua* NIES-1	+	±	+	±	+	±
	Chattonella marina NIES-3	+	+	+	+	+	+
	Fibrocapsa japonica NIES-462	+	+	+	+	+	+
	Heterosigma akashiwo NIES-4	+	+	+	+	+	+
Dinophyceae	*Alexandrium catenella* NIES-677	-	-	-	-	±	-
	Cochlodinium polykrikoides ND-14	-	-	-	-	-	-
	Karenia mikimotoi NIES-680	+	+	+	+	+	+
	Heterocapsa circularisquama ND-12	±	±	±	±	±	±
	Heterocapsa triquetra NIES-7	±	-	±	-	+	±
	Prorocentrum minimum ND-34	±	-	±	-	±	-
	Prorocentrum sigmoides NIES-683	±	-	±	-	±	-
	Scrippsiella sweeneyae NIES-684	±	-	±	-	+	±
	Scrippsiella trochoidea NIES-369	+	±	+	±	+	±
Prasinophyceae	*Nephroselmis astigmatica* NIES-252	+	±	+	±	+	±
	Nephroselmis viridis NIES-486	+	±	+	±	+	±
	Pterosperma cristatum NIES-221	±	±	±	±	+	±
	Pyramimonas aff. *amylifera* NIES-251	+	+	+	+	+	+
	Pyramimonas parkeae NIES-254	+	±	+	±	+	±
Ulvophyceae	*Oltmannsiellopsis unicellularis* NIES-359	+	±	+	±	+	±
Euglenophycea	*Eutreptiella gymnastica* NIES-381	±	-	±	-	±	-
Bacillariophyceae	*Pinnularia* sp. NB-1	-	-	-	-	-	-
	Skeletonema costatum NB-2	-	-	-	-	-	-
	Stephanopyxis palmeriana NB-3	-	-	-	-	-	-
	Rhizosolenia hebetata f. semispina NB-4	-	-	-	-	-	-
Eustigmatophyceae	*Nannochloropsis oculata* ST-6	-	-	-	-	-	-

Table 5. Algicidal compounds [C16:4 n-3 (HDTA), C18:3 n-3 (ALA), and C18:4 n-3 (ODTA)] released from dry powder of *Ulva fasciata* in seawater (mg L^{-1}) and algicidal activity (percentage of mean ± SD) on *Heterosigma akashiwo*.

	Autoclaved		Non-autoclaved	
Time	HDTA, ALA, ODTA	Activity	HDTA, ALA, ODTA	Activity
1 h	-	-	4.82 (20.43)	100.00±0.00
2 h	-	-	5.18 (21.96)	100.00±0.00
3 h	-	-	5.68 (24.08)	100.00±0.00
4 h	-	-	7.38 (31.28)	100.00±0.00
5 h	-	-	9.20 (39.00)	100.00±0.00
6 h	11.90 (50.45)	100.00±0.00	11.02 (46.72)	100.00±0.00
12 h	7.84 (33.24)	100.00±0.00	1.32 (5.60)	63.51±4.75
24 h	5.26 (22.30)	100.00±0.00	0.64 (2.71)	10.93±1.33
36 h	3.62 (15.35)	100.00±0.00	0.56 (2.37)	7.08±3.35
48 h	2.84 (12.04)	100.00±0.00	0.26 (1.10)	2.77±0.87

- : not tested; ( ): percentage of HDTA, ALA, and ODTA released in sea water; 1.5 g L^{-1} of dry powder of *Ulva fasciata* in sea water contained 23.59 mg L^{-1} of HDTA, ALA, and ODTA.

Table 6. Algicidal compounds [C16:4 n-3 (HDTA), C18:3 n-3 (ALA), and C18:4 n-3 (ODTA)] released from fresh tissue of *Ulva fasciata* in seawater (mg L^{-1}) and algicidal activity (percentage of mean ± SD) on *Heterosigma akashiwo*.

	Whole tissue		Cutting tissue	
Antibiotic	HDTA, ALA, ODTA	Activity	HDTA, ALA, ODTA	Activity
Untreated	0.22 (0.28)	1.63±0.74	0.32 (0.41)	5.00±2.65
Treated	0.26 (0.34)	4.67±2.89	0.48 (0.62)	6.63±2.10

( ): percentage of HDTA, ALA, and ODTA released in sea water; 50 g L^{-1} of fresh tissue of *Ulva fasciata* in sea water contained 77.45 mg L^{-1} of HDTA, ALA, and ODTA.