Influence of non-nutrient environmental factors on *Ulva pertusa*'s inhibitory effects on *Heterosigma akashiwo* growth

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We studied the effects of four non-nutrient environmental factors (temperature, salinity, irradiance and pH) on the growth inhibition of the macroalgae Ulva pertusa *(Chlorophyta) upon the microalgae* Heterosigma akashiwo *(Rhaphidophyta). Experiments were conducted in single-factor incubation and various two-factor combination experiments in which temperature (10, 15, 25 and 30°C), salinity (10, 20, 30 and 40 g kg^{-1} water), irradiance (20, 100, 200 and 400 µmol m^{-2} s^{-1}), and pH (5.5, 7, 8.5 and 10) were varied systematically. The growth rates of* U. pertusa *and* H. akashiwo *and the rate of microalgal growth inhibition were altered significantly by changing some of the non-nutrient factors in both the single-factor and the two-factor experiments. The optimal growth conditions for* U. pertusa *were 20–25°C, salinity of 30 g kg^{-1}, irradiance level of 200–400 µmol m^{-2} s^{-1}, and pH 8.5–10; optimal conditions for* H. akashiwo *growth were 25°C, 30 g kg^{-1}, 100 µmol m^{-2} s^{-1} and pH 8.5, respectively. The growth inhibitory influence of* U. pertusa *on* H. akashiwo *was strongest at 25°C with low salinity (10 g kg^{-1}), high irradiance (400 µmol m^{-2} s^{-1}) and high alkalinity (pH = 10). The results of this study may be helpful in the development of methods for using green macroalgae to control the proliferation of microalgae in harmful algal blooms (HABs). In particular, these findings provide guidance regarding optimum levels of non-nutrient environmental factors in confined areas, such as aquaculture factories.*

INTRODUCTION

Since the discovery of allelopathy (Molisch, 1937), researchers have found numerous examples of allelopathic effects between primary producers as well as between primary producers and microorganisms. There is a growing interest in allelopathy among aquatic organisms. Indeed, allelopathy has been shown to have a remarkable influence on the structure and dynamic equilibrium of aquatic ecosystems (van Donk & van de Bund, 2002; Gross, 2003; Kim *et al.*, 2004; Gross *et al.*, 2007; Hu & Hong, 2008; Tang & Gobler, 2011; Slattery & Lesser, 2014).

Environmental factors such as temperature, salinity, irradiance, pH and nutrient availability change frequently in marine and freshwater ecosystems and these changes have a considerable impact on the growth and physiology of aquatic organisms (Taylor *et al.*, 2001; Cohen & Fong, 2004; Kakinuma *et al.*, 2006; Cade-Menun & Paytan, 2010; Kim *et al.*, 2013; Wallace & Gobler, 2015). Moreover, environmental conditions can abate (Keating, 1977; Hu & Hong, 2008) or enhance (Gross, 2003; Gross *et al.*, 2007) allelopathic interactions. Despite recognition of these influences, data on the dynamics of allelopathy in aquatic ecosystems are limited (Gross, 2003; Gross *et al.*, 2007; Hu & Hong, 2008; Wallace & Gobler, 2015).

Harmful algae blooms (HABs) generated by excessive growth of deleterious micro- and macroalgae in response to coastal eutrophication are becoming increasingly frequent along shorelines worldwide. Because of the severe economic and public health problems caused by HABs, researchers have begun to concentrate on how undesired algal growth can be controlled. Some promising control methods have been developed, including the use of yellow loess (Na *et al.*, 1996), clay (Sun *et al.*, 1999), copper sulphate (Steidinger, 1983), and some biological agents, including bacteria (Imai *et al.*, 1993; Kim *et al.*, 1998) and viruses (Garry *et al.*, 1998) that infect algae. Although short-term experiments have yielded promising results regarding the potential efficacy of these methods, there is apprehension concerning the potential for dangerous environmental consequences (Jeong *et al.*, 2000; Hu & Hong, 2008; Tang & Gobler, 2011).

With the aim of developing efficient HAB control methods that do not risk harming the environment, researchers have been examining how the growth of troublesome HAB species could be inhibited by allelopathic substances released by other aquatic organisms (Jin & Dong, 2003; Jin *et al.*, 2005; Hu & Hong, 2008; Nan *et al.*, 2008; Tang & Gobler, 2011). Many aquatic organisms have been shown to secrete allelopathic substances against HAB species (Nakai *et al.*, 1999, 2000; Jeong *et al.*, 2000; Lee *et al.*, 2000; Jin & Dong, 2003, 2005; Nelson *et al.*, 2003; Alamsjah *et al.*, 2005, 2008; Hu & Hong, 2008; Tang & Gobler, 2011; Ye *et al.*, 2014). Aquatic organisms that are endemic to aquatic ecosystems, such as macrophytes and macroalgae, are attractive potential mediators of targeted allelopathic HAB control because they

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should not cause the severe environmental problems associated with chemical treatments, which have broad-spectrum toxicity affecting diverse aquatic organisms (Gross, 2003; Gross et al., 2007; Hu & Hong, 2008). Unlike many synthetic toxins, allelochemicals secreted by aquatic organisms are both highly efficacious at very low concentrations and naturally degradable (Nakai et al., 1999, 2000; Jin & Dong, 2003; Hu & Hong, 2008). Furthermore, many macrophyte and macroalgae species have a high nutrient assimilation capacity, thus reducing eutrophication of aquatic ecosystems and mitigating harmful microalgal blooms (Jin et al., 2005; Tang & Gobler, 2011; Ye et al., 2014).

Moreover, some investigators are exploring how environmental factors may affect the occurrence of HABs (Taylor et al., 2001; Yan et al., 2002; Kakinuma et al., 2006; Shikata et al., 2007; Valenti et al., 2010; Butron et al., 2012; Kim et al., 2013; Wallace & Gobler, 2015). Researchers have obtained evidence suggesting that HAB algae can gain competitive advantages through their allelopathic effects on other marine organisms (Smayda, 1997; Nelson et al., 2003; Hu & Hong, 2008; Slattery & Lesser, 2014). If so, it may be possible to suppress HABs by adjusting environmental factors in confined areas, including small water areas in aquaculture factories, in a manner that enhances algae growth inhibition by other aquatic organisms. However, few researchers have focused on the relationship between changes in aquatic environmental factors and allelopathy. There have been some studies focused on examining the influence of pH and temperature on allelopathy of aquatic organisms (Ray & Bagchi, 2001; Schmidt & Hansen, 2001; Valenti et al., 2010; Valenti, 2010).

In our previous experiments (Jin & Dong, 2003), we found that the macroalga Ulva pertusa (both non-sexual and sexual strains), which is commonly found within HABs, secretes molecules that have allelopathic growth inhibition and algicidal effects on Heterosigma akashiwo, a harmful microalgae species in HABs. We investigated whether changes in nonnutrient environmental factors could affect the growth inhibitory influence of U. pertusa on H. akashiwo and monitored their growth rates.

MATERIALS AND METHODS

Algae

Because the sexual strain of U. pertusa would release spores during experimental cultures and might interfere with the results of this study, we decided to use an axenic non-sexual strain of U. pertusa, which could also inhibit the growth of the microalgae (Jin & Dong, 2003) and an axenic strain of H. akashiwo so that the effects of bacteria could be precluded as well.

An axenic strain of H. akashiwo was obtained from the Microalga Research Laboratory of the Ocean University of China in Qingdao, China. An axenic non-sexual strain of U. pertusa was provided courtesy of Professor Akira Taniguchi at Tohoku University in Japan. All algae were cultured aseptically in f/2 medium (Guillard & Ryther, 1962) in illuminating incubators with an irradiance level, measured in photosynthetic photon flux density (PPFD), of 60 μmol m^{-2} s^{-1} and a 12:12-h light/dark cycle at 20°C. Cultures were refreshed with fresh f/2 medium every 4 days to ensure nutrient sufficiency. All flasks containing microalgae were shaken twice daily at set times to prevent algal growth on the flask walls.

Seawater for experiments

Aged natural seawater was filtered through glass-fibre papers (Whatman GF/C, 0.22-μm pore size) to eliminate bacteria, organic particles and debris. The pH and salinity of the seawater were adjusted to 8.5 and 35 g NaCl per kg water (salinity unit abbreviated as g kg^{-1} from here forward), respectively; seawater was used as the basic culture medium for algae in all experiments.

Culture experiments with single-factor and two-factor incubations

Before we tested two-factor combinations, we examined how the individual manipulation of temperature, salinity, irradiance and pH affects macro- and microalgal growth and macroalgal inhibition of microalgal growth to establish appropriate levels of these four factors for the two-factor combination experiments. Natural seawater pH ranges generally from 7 to 9, but this pH range might not encompass the limits of macro- and microalgal growth and inhibition. Therefore we used a pH range that extends beyond that found in natural seawater in this study. Various levels of the single factors were tested in parallel as follows: temperature (10, 15, 25 and 30°C), salinity (10, 20, 30 and 40 g kg^{-1}), irradiance (20, 100, 200 and 400 μmol m^{-2} s^{-1}), and pH (5.5, 7, 8.5 and 10). While one factor was varied, the other culture conditions were kept at baseline levels (i.e. temperature, 20°C; salinity, 35 g kg^{-1}; irradiance, 60 μmol m^{-2} s^{-1}; and pH, 8.5) with a 12:12-h light/dark cycle.

Temperature was controlled by way of culture-illuminating incubators. Medium with a salinity of <35 g kg^{-1} was prepared by diluting the stock culture medium (35 g kg^{-1}) with distilled water. Medium with salinity adjusted to 40 g kg^{-1} was prepared by monitored evaporation of the basic culture medium in an oven at 60°C. Irradiance was varied by adjusting the lights in the culture-illuminating incubators. Medium pH was altered by titrating stock culture medium with 0.1 M HCl or 0.1 M NaOH. Because algae can change the pH of their culture medium through photosynthesis and respiration, pH was tested and adjusted as needed every 12 h to reset levels using the aforementioned reagents.

Microalgae and macroalgae were co-cultured in 100-mL conical glass flasks containing 40-mL of 2f culture medium. Nutrient concentrations were four times that of the stock f/2 medium to ensure that there were sufficient nutrients for the growth of both the microalgae and the macroalgae. Flask mouths were covered with kraft paper. Jin & Dong (2003) found that when they combined H. akashiwo cells (initial cell density 1×10^5 cells mL^{-1}) with fresh U. pertusa (initial fresh tissue weight, 0.025 g) in 40-mL of culture medium, growth of H. akashiwo was inhibited. Therefore, in the present study, we elected to use a lower initial fresh tissue weight of U. pertusa. Exponentially growing H. akashiwo cells (initial cell density 1×10^5 cells mL^{-1}) were placed in medium with fresh U. pertusa tissue (initial fresh tissue weight, 0.01 g). Monocultures of each alga served as controls. All experiments were performed separately for a period of 8 days in (at least) triplicate. Aseptic techniques were employed in all experimental steps.

At the end of the experiment, 1-mL samples were collected from each flask and preserved in Lugol's solution. Microalgal cells were counted under an Olympus optical microscope by a

hemocytometer and microalgal cell densities were calculated for all groups. We also determined the wet weights of macroalgal fresh tissue in the control groups using an analytical balance. The procedures and culture conditions in the following experiments were the same as described above unless otherwise stated.

Six two-factor culture experiments (Temperature × Salinity, Temperature × pH, Irradiance × Salinity, Irradiance × pH, Salinity × pH and Temperature × Irradiance) were conducted with the same procedures and parameter combinations indicated above. For each of the six experiments, two of the four factors were combined (i.e. Temperature × Salinity) and the parameter levels were varied systematically across parallel trials. Only the two factors being tested varied between trials in each experiment, while the other culture conditions were at baseline levels. One of the test variables was varied between side-by-side incubators; and the other was varied between the four compartments within each incubator.

Data processing and statistical analysis

Mean *U. pertusa* and *H. akashiwo* growth rates are represented as μ_U and μ_H, respectively. Mean growth rate μ (% d^{-1}) was calculated as:

$$\mu = \frac{100 \ln(N_t/N_o)}{t}$$

where N_o and N_t are the microalgal cell density or wet weight of macroalgal fresh tissue in the control groups at the beginning and designated end point of these experiments, respectively. The parameter t represents the duration of these experiments in days.

The growth inhibitory effects of the macroalgae on the microalgae were represented as the growth inhibition rate I of the microalgae, reported as a percentage, and calculated as:

$$I = \frac{100(H_c - H_t)}{H_c}$$

where H_c and H_t are the microalgal cell density of the control (microalga monoculture) and treatment (micro- and macroalgae co-culture) groups at the end of these experiments, respectively.

Single-factor culture experimental data were compared with t-tests. After normality and variance homogeneity tests were applied, two-factor culture experimental data were analysed by analyses of variance (ANOVAs) and Tukey's *post hoc* tests. A significance criterion of $P < 0.05$ was used in all cases.

RESULTS

Effects of individual environmental factors on algal growth and macroalgal inhibition of microalgal growth

Under our baseline factor conditions, maximal mean growth rates of *U. pertusa* (MGU) and *H. akashiwo* (MGH) were observed at 25°C, with macroalgal inhibition of microalgal growth (MIMG) also being maximal at 25°C (Figure 1A). MGU and MGH were maximal when the medium salinity
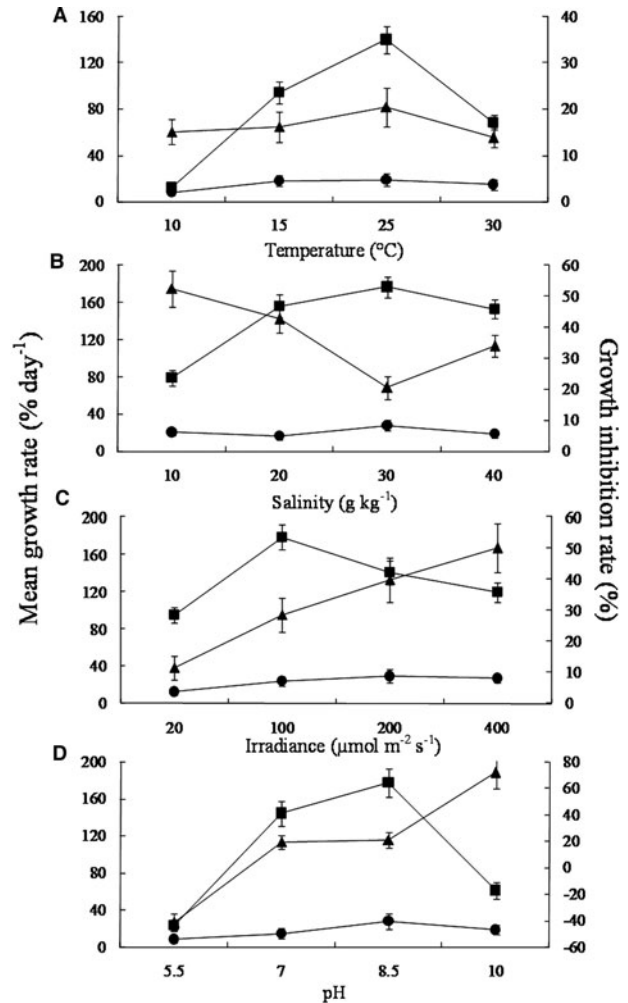

Fig. 1. Mean growth rates of *Ulva pertusa* (●) and *Heterosigma akashiwo* (■), and *Heterosigma akashiwo* growth inhibition rates (▲) by *Ulva pertusa* in single-factor incubations in which temperature (A, °C), salinity (B, g kg^{-1}), irradiance (C, μmol m^{-2} s^{-1}) and pH (D) were varied systematically. Mean values are shown with standard errors (N = 3 replicates per group).

was 30 g kg^{-1}, whereas MIMG had the opposite response to salinity, with the MIMG nadir occurring at 30 g kg^{-1} (Figure 1B). MGU and MGH were maximal with irradiance PPFDs of 200 and 100 μmol m^{-2} s^{-1}, respectively. MIMG increased with increasing irradiance, such that maximal inhibition was observed when the irradiance PPFD was at 400 μmol m^{-2} s^{-1} (Figure 1C). MGU and MGH were maximal at a pH of 8.5. MIMG increased with increasing pH, with the strongest inhibition occurring at a pH of 10 (Figure 1D).

Effects of temperature on algal growth in two-factor combination experiments

As summarized in Table 1, significant group variances in MGU were observed in three of the two-factor combination experiments: temperature-salinity, temperature-pH and temperature-irradiance. There was a significant temperature × irradiance interaction, but not significant temperature × pH or temperature × salinity interactions. The optimal temperature range for growth of *U. pertusa* was 20–25°C (Figure 2A, B, F). MGH also differed significantly with temperature changes, and significant interactions with

Table 1. Effects of varying two-factor combinations of temperature (T), salinity (S), irradiance (L) and pH (P) on mean growth rates of *U. pertusa*.

Varied factors	Source of variation	Sum of squares	Degrees of freedom	Mean square	ANOVA F value	ANOVA P value
T and S	T	449.056	3	149.685	18.846	0.0001
	S	15.723	3	5.241	0.660	0.583
	$T \times S$	73.991	9	8.221	1.035	0.435
	Error	254.167	32	7.943		
	Total	7710.938	48			
T and P	T	701.660	3	233.887	24.522	0.0001
	P	484.212	3	161.404	16.923	0.0001
	$T \times P$	121.387	9	13.487	1.414	0.223
	Error	305.208	32	9.538		
	Total	7885.938	48			
L and S	L	3447.233	3	1149.078	111.708	0.0001
	S	82.389	3	27.463	2.670	0.064
	$L \times S$	137.272	9	15.252	1.483	0.197
	Error	329.167	32	10.286		
	Total	24,985.938	48			
L and P	L	594.531	3	198.177	35.812	0.0001
	P	2715.625	3	905.208	163.576	0.0001
	$L \times P$	273.177	9	30.353	5.485	0.0001
	Error	177.083	32	5.534		
	Total	18,112.500	48			
S and P	S	217.188	3	72.396	18.533	0.0001
	P	1399.740	3	466.580	119.444	0.0001
	$S \times P$	141.927	9	15.770	4.037	0.002
	Error	125.000	32	3.906		
	Total	12,534.375	48			
T and L	T	449.056	3	149.685	37.083	0.0001
	L	1012.858	3	337.619	83.642	0.0001
	$T \times L$	254.199	9	28.244	6.997	0.0001
	Error	129.167	32	4.036		
	Total	5542.188	48			

temperature were observed in the MGH data (Table 2). The optimal temperature for growth of *H. akashiwo* was 25°C (Figure 3A, B, F).

Effects of salinity on algal growth rates in two-factor combination experiments

MGU differed significantly between the groups in the salinity-pH combination experiment, but not in the salinity-temperature or salinity-irradiance experiments. Similarly, a significant interaction between varied factors in the MGU data was observed only in the salinity-pH combination experiment (Table 1). The macroalgae grew in all levels of salinity, but the optimal salinity for *U. pertusa* growth was 30 g kg^{-1} (Figure 2A, C, E). In contrast to MGU, MGH varied significantly in all two-factor experiments in which salinity was one of the varied factors (Table 2). The optimal salinity for *H. akashiwo* growth was 30 g kg^{-1} (Figure 3A, C, E).

Effects of irradiance on algal growth in two-factor combination experiments

MGU differed significantly across groups in three of the two-factor combination experiments involving irradiance. Significant irradiance × pH and irradiance × temperature interactions were observed (Table 1). Optimal growth of *U. pertusa* was observed when irradiance was in the PPFD

range of 200–400 μmol m^{-2} s^{-1} (Figure 2C, D, F). Similarly, MGH differed significantly in relation to irradiance (Table 2). Optimal growth of *H. akashiwo* was observed with an irradiance PPFD of 100 μmol m^{-2} s^{-1} (Figure 3C, D, F).

Effects of pH on the algal growth in two-factor combination experiments

MGU differed significantly across groups when pH was one of the two varied factors (Table 1). Significant pH × irradiance and pH × salinity interactions were observed in the MGU data ($P < 0.001$). Optimal *U. pertusa* growth occurred when pH was in the range of 8.5–10 (Figure 2B, D, E). MGH responsivity to pH in the two-factor experiments was similar to that of MGU, except that a pH × temperature interaction was also observed (Table 2). The optimal pH for *H. akashiwo* growth was 8.5 (Figure 3B, D, E).

Effects of non-nutrient factors on macroalgal inhibition of microalgal growth in two-factor combination experiments

With the exception of the interactions in the irradiance-salinity manipulations, all treatment effects and all interaction effects were highly significant (Table 3; $P < 0.001$). Optimal MIMG was associated, generally, with a temperature of 25°C, salinity of 10 g kg^{-1} (except for pH-salinity, in
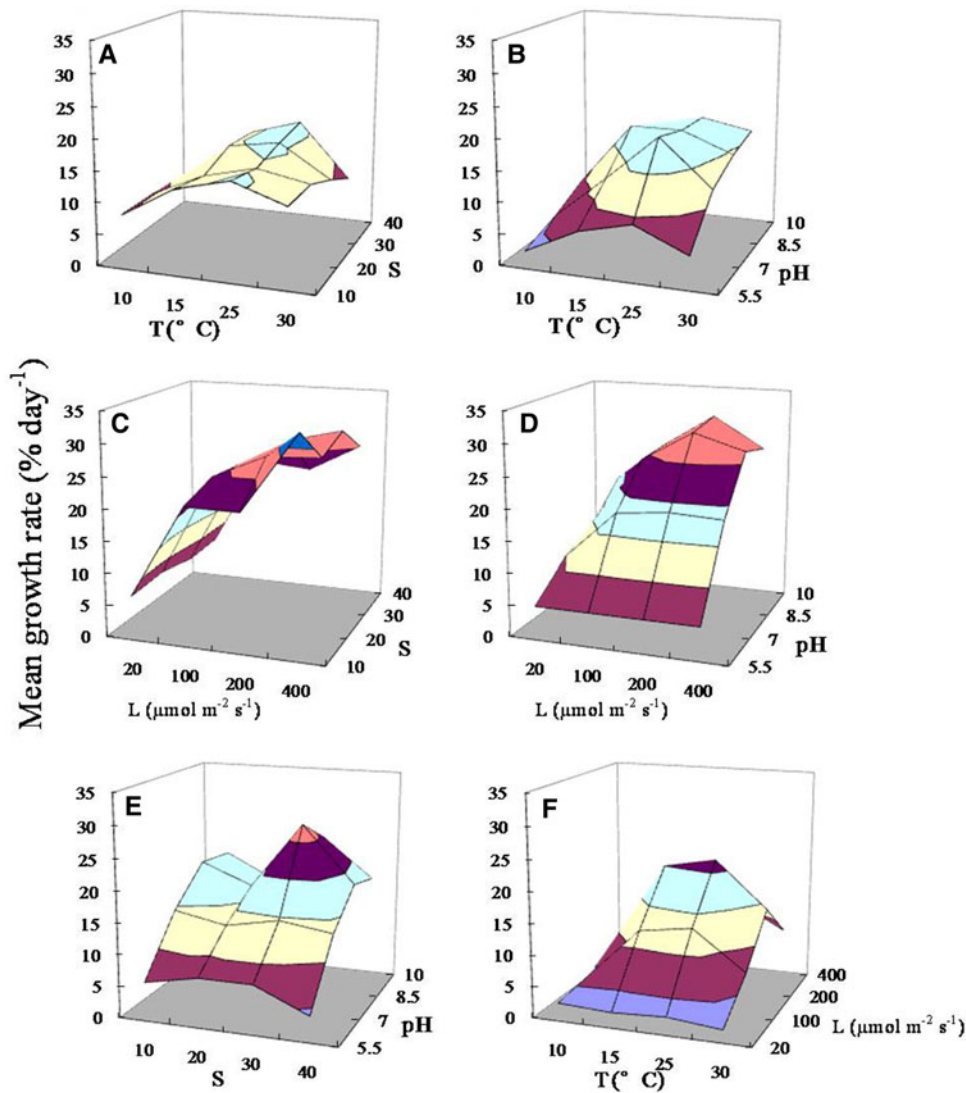
Fig. 2. Mean growth rates of *Ulva pertusa* in two-factor combination experiments wherein two of the following variables were changed across groups in each experiment: temperature (T), salinity (S, g kg^{-1}), irradiance (L), and pH. Mean values are plotted (N = 3 replicates per group).

which optimal salinity was 40 g kg^{-1}), an irradiance level of 400 μmol m^{-2} s^{-1} and a pH of 10 (Figure 4). It is noteworthy that the inhibition rate was negative when the pH was 5.5 in three experiments involving pH manipulation, indicating that the macroalgae had a stimulating effect (of ∼30–90%) on microalgal growth under acidic conditions (Figure 4B, D, E).

DISCUSSION

In the present study, we conducted laboratory experiments under controlled environmental conditions, in which nutrient competition, light competition and possible bacterial effects were mitigated, to investigate the impact of non-nutrient environmental factors on the growth inhibitory effects of *U. pertusa* on *H. akashiwo*. We found that the growth inhibitory efficacy of the macroalgae on the microalgae was modulated by temperature, salinity, irradiance and pH. The present two-factor combination experiments pointed to the following parameters as optimal for *U. pertusa* inhibition of *H. akashiwo*

growth: moderate temperature (25°C); low salinity (10 g kg^{-1}); high irradiance (400 μmol m^{-2} s^{-1}); and a highly alkaline pH (10).

Ulva pertusa can reduce eutrophication of mariculture waters and improve water quality; and polyculture with *U. pertusa* can improve the productivity, survival rate, and feeding coefficient of culture species, such as prawn and shrimp (Jin & Dong, 2003; Tang & Gobler, 2011; Ye *et al.*, 2014). Our results demonstrate that harmful microalgae blooms in confined mariculture areas can be mitigated with *U. pertusa* polyculture. Moreover, when people use the macroalgae in marine polycultures in aquaculture factories, non-nutrient environmental factors of the culture water can be adjusted to optimize the HAB mitigating effects of the macroalgae.

Our findings are consistent with recent studies demonstrating that *Ulva* species, including *U. fasciata, U. pertusa* and *U. linza*, can have strong growth-inhibiting effects on *H. akashiwo, Alexandrium tamarense* and *Prorocentrum micans* (Jin & Dong, 2003; Jin *et al.*, 2005; Wang *et al.*, 2007; Nan *et al.*, 2008). Additionally, polyunsaturated fatty acids

Table 2. Effects of varying two-factor combinations of temperature (T), salinity (S), irradiance (L) and pH (P) on mean growth rates of *H. akashiwo*.

Varied factors	Source of variation	Sum of squares	Degrees of freedom	Mean square	ANOVA F value	ANOVA P value
T and *S*	*T*	77,559.417	3	25,853.139	608.948	0.0001
	S	21,857.970	3	7285.990	171.615	0.0001
	T × S	11,644.855	9	1293.873	30.476	0.0001
	Error	1358.574	32	42.455		
	Total	261,453.986	48			
T and *P*	*T*	34,377.515	3	11,459.172	268.147	0.0001
	P	37,839.842	3	12,613.281	295.154	0.0001
	T × P	14,794.690	9	1643.854	38.467	0.0001
	Error	1367.507	32	42.735		
	Total	18,3585.587	48			
L and *S*	*L*	29,867.258	3	9955.753	197.237	0.0001
	S	51,345.896	3	17,115.299	339.078	0.0001
	L × S	3545.899	9	393.989	7.805	0.0001
	Error	1615.234	32	50.476		
	Total	46,5603.916	48			
L and *P*	*L*	10,812.460	3	3604.153	100.737	0.0001
	P	97,056.555	3	32,352.185	904.250	0.0001
	L × P	6089.369	9	676.597	18.911	0.0001
	Error	1144.893	32	35.778		
	Total	349,222.323	48			
S and *P*	*S*	28,462.276	3	9487.425	369.375	0.0001
	P	119,768.767	3	39,922.922	1554.322	0.0001
	S × P	14,512.290	9	1612.477	62.779	0.0001
	Error	821.923	32	25.685		
	Total	428,633.485	48			
T and *L*	*T*	50,071.782	3	16,690.594	867.323	0.0001
	L	44,973.849	3	14,991.283	779.019	0.0001
	T × L	19,572.292	9	2174.699	113.008	0.0001
	Error	615.801	32	19.244		
	Total	18,1181.255	48			

(PUFAs) and organosulphur compounds have been implicated as potential allelopathic agents for *Ulva* species in three studies (Alamsjah *et al.*, 2005, 2008; Tang & Gobler, 2011). Importantly, in natural ecosystems, the mechanisms by which *Ulva* inhibit HABs may include allelopathy, nutrient scavenging, algicidal bacteria, and pH changes (Schmidt & Hansen, 2001; Tang & Gobler, 2011).

It should be noted that the effectiveness of allelopathic chemicals does not necessarily coincide with the optimal growth conditions for the allelochemical donor organism. Rather, the allelopathic effects of aquatic organisms are generally enhanced under stressful conditions (e.g. disadvantageous environmental factors or nutrient limitations). There are two explanations for this phenomenon: (1) the allelochemical donor organism may augment productivity of allelochemicals when stressed; and/or (2) target organisms may be more sensitive when stressed (Gross, 2003; Gross *et al.*, 2007).

Temperature and salinity

Intertidal and coastal algae often experience extreme temperature and rapid salinity changes (Kakinuma *et al.*, 2006; Cade-Menun & Paytan, 2010; Martinez *et al.*, 2010; Butron *et al.*, 2012). Temperature is a key factor in the growth and physiology of all aquatic organisms, and salinity is very important for the growth of the macroalgae *U. pertusa* and the microalgae *H. akashiwo* (Kakinuma *et al.*, 2006; Shikata *et al.*, 2007; Martinez *et al.*, 2010; Butron *et al.*, 2012).

Our results showed that maximal inhibition of *H. akashiwo* growth by *U. pertusa* occurred at 25°C, which was also the optimal growth temperature for both species. Temperature affects enzyme activity, thereby potentially modulating a variety of biochemical processes including nitrogen absorption, which is essential for photosynthesis and respiration in algae (Lomas & Glibert, 1999). Kakinuma *et al.* (2006) observed that thermal stress influenced protein and amino acid (AA) metabolism in sterile *U. pertusa*, suggesting that carbon and nitrogen metabolism in the macroalgae may be temperature-sensitive. The observed changes in metabolism could also be due to the formation of dormant *H. akashiwo* cells at extreme temperatures (Han *et al.*, 2002; Martinez *et al.*, 2010; Butron *et al.*, 2012), and such dormant cells may be more tolerant of allelochemicals secreted by *U. pertusa*.

Our observations of strong microalgal growth inhibition under low-salinity conditions may be due to the loss of cell walls around *H. akashiwo* cells (Guo, 1994; Shikata *et al.*, 2007), and/or a general weakening of the structure and physiology of microalgal cells under low-salinity conditions that renders them highly sensitive to allelopathic influences. Secondly, the photosynthetic activity of *U. pertusa* may be suppressed under high-salinity conditions (Kakinuma *et al.*, 2006), which would make the macroalgae reduce allelochemical secretion. Finally, it is also possible that high salinity has severe effects on the carbon and nitrogen metabolism of the macroalgae (Kakinuma *et al.*, 2006), such that synthesis of allelochemicals in *U. pertusa* is disturbed.
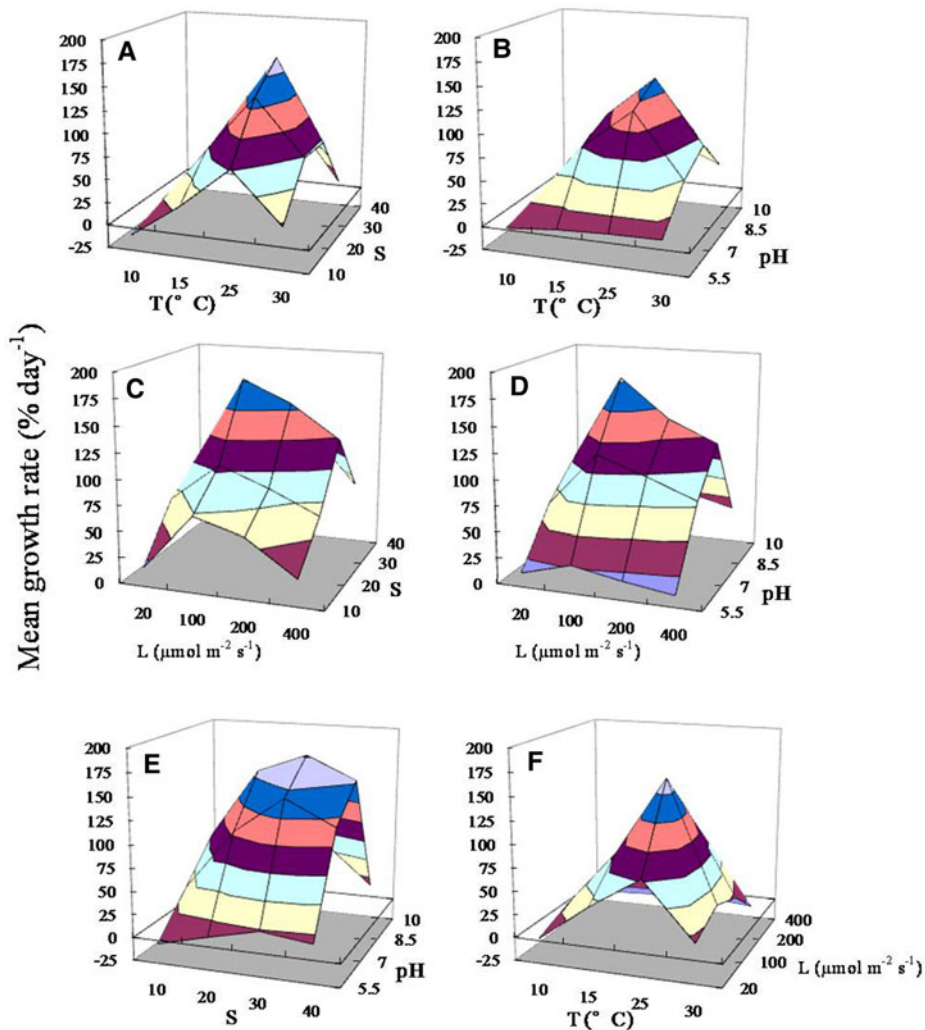
Fig. 3. Mean growth rates of *Heterosigma akashiwo* in two-factor combination experiments wherein two of the following variables were changed across groups in each experiment: temperature (T), salinity (S, g kg^{-1}), irradiance (L) and pH. Mean values are plotted (N = 3 replicates per group).

Temperature and pH

Marine plants need CO_2 or HCO_3^- to sustain photosynthesis, and pH affects the dynamic equilibrium of CO_2 and HCO_3^- in seawater (Menéndez *et al.*, 2001; Valenti *et al.*, 2010; Kim *et al.*, 2013). Over at least the past 250 years, increasing atmospheric CO_2 has resulted in a net uptake of CO_2 by the surface waters of the oceans and increasing ocean temperature. The dissolution of anthropogenic CO_2 leads to increasing carbonic acid (H_2CO_3) and carbonate (CO_3^{2-}) ions with a decreasing pH, which is a phenomenon termed ocean acidification (Hofmann *et al.*, 2010; Kim *et al.*, 2013).

In this study, the macroalgae *U. pertusa* tolerated all temperature and pH levels tested (5.5 – 10), similar to previous results (Menéndez *et al.*, 2001). However, *H. akashiwo* showed a relatively narrower temperature and pH tolerance, with dramatic decreases in its growth rate being observed under low pH (5.5) and high pH (10) conditions.

Strong macroalgal inhibition of microalgal growth was observed under highly alkaline conditions (pH 10). This phenomenon could be related to the increasing influence of high pH on the cellular carbon and nitrogen quotas of *H. akashiwo* (Kim *et al.*, 2013). We also hypothesized that *U. pertusa*

increased productivity of allelochemicals, or there was an enhanced effectiveness of the allelochemicals, at high pHs. That is, alkalinity may disrupt the physiology of microalgal cells such that they are rendered highly sensitive to macroalgal influences. Valenti (2010) proposed that pH can change the ionization of allelochemicals, and thereby influence the lipophilicity, bioavailability, bioaccumulation and aquatic toxicity of allelochemicals. Further research is needed to test this hypothesis.

Regardless of the mechanisms underlying the effects of pH on macroalgal inhibition of microalgal growth, our observation that alkalinity favours allelopathic inhibition of *H. akashiwo* growth is consistent with prior research. Schmidt & Hansen (2001) investigated the impact of pH on the effectiveness of the allelopathic chemicals of *Chrysochromulina polylepis* on *Heterocapsa triquetra*. They found that the allelopathic effects of *C. polylepis* were strongest under alkaline conditions (pH, 8.9 – 9.6) but not detectable at a near-neutral pH (7.7). Likewise, Ray & Bagchi (2001) reported that the productivity of allelochemicals secreted by the cyanobacteria *Oscillatoria laetevirens* was increased under highly alkaline conditions. Recently, Valenti *et al.* (2010) found that both temperature

Table 3. Effects of varying two-factor combinations of temperature (T), salinity (S), irradiance (L) and pH (P) on *U. pertusa* inhibition of *H. akashiwo* growth.

Varied factors	Source of variation	Sum of squares	Degrees of freedom	Mean square	ANOVA F value	ANOVA P value
T and S	T	9467.764	3	3155.921	17.539	0.0001
	S	14,722.219	3	4907.406	27.273	0.0001
	T × S	8096.046	9	899.561	4.999	0.0001
	Error	5757.940	32	179.936		
	Total	61,171.806	48			
T and P	T	1412.523	3	470.841	22.688	0.0001
	P	32,590.833	3	10,863.611	523.487	0.0001
	T × P	6462.007	9	718.001	34.598	0.0001
	Error	664.077	32	20.752		
	Total	54,959.991	48			
L and S	L	14,372.848	3	4790.949	187.802	0.0001
	S	8121.033	3	2707.011	106.113	0.0001
	L × S	485.359	9	53.929	2.114	0.058
	Error	816.340	32	25.511		
	Total	96,775.503	48			
L and P	L	7201.103	3	24,000.368	167.481	0.0001
	P	51,955.695	3	17,318.565	1208.372	0.0001
	L × P	5526.587	9	614.065	42.845	0.0001
	Error	458.629	32	14.332		
	Total	101,060.770	48			
S and P	S	3636.866	3	1212.289	84.629	0.0001
	P	85,355.006	3	28,451.669	1986.196	0.0001
	S × P	15,516.914	9	1724.102	120.359	0.0001
	Error	458.391	32	14.325		
	Total	12,3347.712	48			
T and L	T	4844.789	3	1614.930	190.776	0.0001
	L	9485.942	3	3161.981	373.533	0.0001
	T × L	2339.429	9	259.937	30.707	0.0001
	Error	270.882	32	8.465		
	Total	64,243.889	48			

and pH influenced the toxicity of *Prymnesium parvum*. Toxins released by *P. parvum* were more potent when exposure occurred at a higher (e.g. 8.5) *vs* a lower pH. They suggested that the presence of a higher proportion of prymnesins in non-ionized forms at a pH of 8.5 could explain the greater toxicity of *P. parvum*; the same principle might explain the efficacy of *U. pertusa*'s allelochemicals under alkaline conditions as well.

It is noteworthy that under acidic conditions (pH 5.5), the presence of macroalgae stimulated microalgal growth, because photosynthesizing macroalgae increased the pH of the culture medium, making it more hospitable to the microalgae (Tang & Gobler, 2011). Additionally, it is possible that a low pH lowers the allelochemical productivity of *U. pertusa* or reduces the effectiveness of its allelochemicals.

Irradiance and salinity

Light has a complex distribution pattern in natural aquatic systems. Both low and high light intensities can limit algal growth due to their effects on photosynthesis. At low light intensities, algae may not receive sufficient energy to meet their physiological needs, whereas high light intensities can cause photoinhibition (Cade-Menun & Paytan, 2010).

In this work, interactions between irradiance and salinity had a significant impact on the growth rates of the microalgae *H. akashiwo*, but not on the growth rates of the macroalgae *U. pertusa* or the macroalgae's allelopathic inhibition of *H. akashiwo* growth. A high level of inhibition of microalgal growth was observed under high-irradiance, low-salinity conditions. Light intensity is expected to affect the abundance of proteins and lipids in algae (Cade-Menun & Paytan, 2010). Given the hypothesis that PUFAs are active allelopathic agents of *Ulva* (Alamsjah *et al.*, 2005, 2008; Tang & Gobler, 2011), an increased abundance of lipids in *Ulva* under high light intensities may elevate PUFA production. Moreover, *Ulva* can osmoregulate by altering tissue water content and K^+ concentration (Cohen & Fong, 2004), but *H. akashiwo* cannot maintain normal growth under low-salinity conditions (Martinez *et al.*, 2010), making the microalgae more susceptible to macroalgal influences.

We also hypothesized that photochemical reactions of the allelochemicals under high levels of irradiance might enhance their effectiveness. Consistent with this possibility, Sun *et al.* (1989) demonstrated that the productivity and secretion of algicides by *Eichhornia crassipes* were inhibited when its roots were exposed to high levels of irradiance. However, this hypothesis should be tested directly.

Irradiance and pH

Under low-irradiance and low-pH conditions, *U. pertusa* sustained good growth whereas the growth of *H. akashiwo* was significantly inhibited. As discussed above, diminished
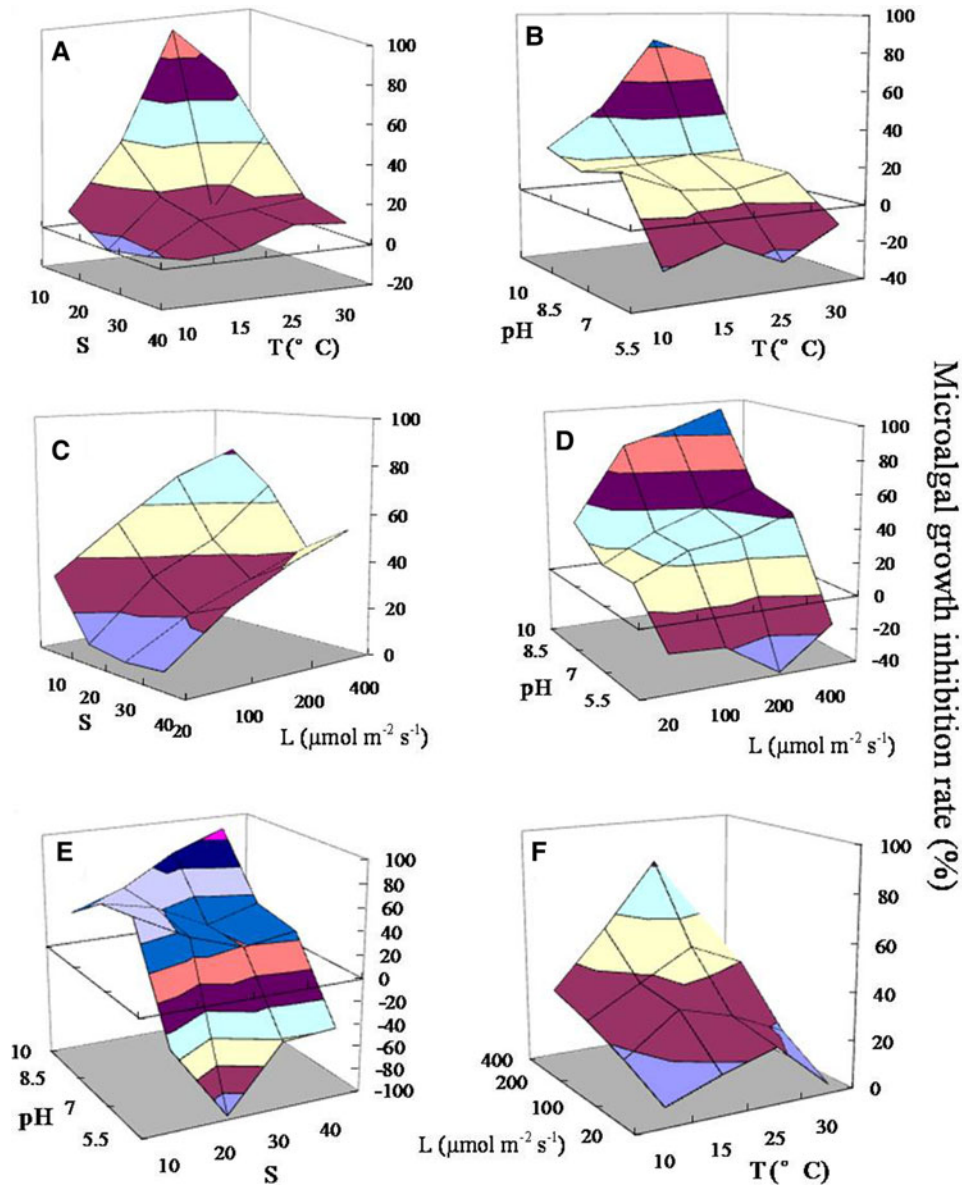
Fig. 4. Inhibition of *Heterosigma akashiwo* growth by *Ulva pertusa* in two-factor combination experiments wherein two of the following variables were changed across groups in each experiment: temperature (T), salinity (S, g kg^{-1}), irradiance (L) and pH. Mean values are plotted (N = 3 replicates per group).

microalgal growth under low-irradiance conditions could be due to reduced photosynthesis (Yan *et al.*, 2002; Shikata *et al.*, 2007; Butron *et al.*, 2012); and acidic conditions could produce structural compromise (Guo, 1994; Shikata *et al.*, 2007).

The most pronounced inhibition of microalgal growth occurred under high-irradiance (400 μmol m^{-2} s^{-1}), highly alkaline (pH 10) conditions. Butron *et al.* (2012) found that when the irradiance level reached 300 μmol m^{-2} s^{-1}, *H. akashiwo* would develop a photoprotective response that was associated with slowed growth. However, *Ulva* demonstrated robust growth in the presence of high irradiance up to ~2000 μmol m^{-2} s^{-1} (Henley *et al.*, 1992). Different photoinhibition thresholds of *U. pertusa* and *H. akashiwo* may account for this phenomenon. Moreover, it is possible that the mechanisms of the microalgal growth inhibition by the macroalgae are strengthened in high pH conditions, as discussed above.

Salinity and pH

Maintenance of cell turgor by alteration of osmotic potentials, which are determined by concentrations of internal inorganic ions and organic osmolytes, is a typical salinity tolerance mechanism in marine algae (Kakinuma *et al.*, 2006). To our knowledge, experiments combining salinity and pH manipulations in aquatic ecosystems are rare. In this study, the microalgal cells likely could not sustain sufficient osmotic pressure under low- and high-salinity conditions to thrive (Yan *et al.*, 2002; Martinez *et al.*, 2010). Microalgal growth was inhibited most strongly by the macroalgae under high-salinity (40 g kg^{-1}), highly alkaline (pH 10) conditions. It is reasonable to suppose that the structural integrity of the microalgal cells was compromised under these conditions, making them more susceptible to allelochemicals (Zhou *et al.*, 2008; Valenti *et al.*, 2010). Valenti *et al.* (2010) concluded that increasing pH increases the potency of *P. parvum*-excreted

toxins over a wide range of salinities. In a study examining interactions between plankton and the cyanobacterium *Anabaena*, Engstr *et al.* (2011) found that the growth and toxin production of *Anabaena* varied with salinity, suggesting that the salinity of open water areas might affect the allelopathy of aquatic organisms.

Temperature and irradiance

It has been demonstrated that allelopathic inhibition of terrestrial plant growth is enhanced in environments with a high temperature and high light intensity (Lobon *et al.*, 2002). The allelopathic effect has been reported to be temperature sensitive, and to be further enhanced when a high temperature is combined with high light intensity (Lobon *et al.*, 2002).

Our results demonstrated that the macroalgae *U. pertusa* grew under all conditions of the two-factor combination experiments, whereas the growth of the microalgae *H. akashiwo* was inhibited dramatically (near zero growth) under extreme temperature and irradiance conditions. Inhibition of photosynthesis in microalgal cells might explain this phenomenon to some extent (Yan *et al.*, 2002; Zhou *et al.*, 2008; Cade-Menun & Paytan, 2010; Butron *et al.*, 2012).

Microalgal growth was inhibited most strongly by macroalgae under moderate-temperature ($25°C$), high-irradiance ($400\ \mu mol\ m^{-2}\ s^{-1}$) conditions. There are several possible explanations for this finding. Firstly, photoinhibition at high light intensities will limit the total C that can be fixed by microalgae, thus affecting the abundance of proteins and lipids (Cade-Menun & Paytan, 2010). Decreased levels of internal proteins and lipids in *H. akashiwo* may damage the physiological functions of the microalgae, resulting in less growth. Secondly, high temperature stress can alter lipid composition in algae, particularly the degree of fatty acid saturation (Cade-Menun & Paytan, 2010). As discussed above, if PUFAs were potential active allelopathic agents of *U. pertusa*, increments in temperature may decrease PUFA saturation, thereby elevating the algicidal activities of PUFAs derived from the macroalgae (Alamsjah *et al.*, 2005, 2008). Finally, (relatively) high temperature and high irradiance acted together to suppress the photosynthetic efficiency and capacity of *H. akashiwo*, much more than temperature or irradiance alone, resulting in microalgal growth inhibition (Henley *et al.*, 1992).

CONCLUSIONS

The present results demonstrate that the environmental factors of temperature, salinity, irradiance, and pH affect the growth of *U. pertusa* and *H. akashiwo* and the magnitude of macroalgal effects on microalgae. *Ulva pertusa*'s inhibition of *H. akashiwo* growth was favoured under extreme conditions, including low salinity, high irradiance and high pH. These findings demonstrate that non-nutrient environmental factors have a pronounced importance in determining the growth inhibitory effects of macroalgae upon microalgae, and contribute to the understanding of the allelopathic potential of *Ulva*. Maintenance of appropriate levels of non-nutrient environmental factors in confined areas should be helpful in mitigating HABs by facilitating the allelopathic effects of aquatic macrophytes and macroalgae.

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