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Bloom-forming macroalgae (*Ulva* spp.) inhibit the growth of co-occurring macroalgae and decrease eastern oyster larval survival

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Abstract

Macroalgal blooms have increased in frequency worldwide due to anthropogenic activities. Algal blooms can disrupt recreational activities, interfere with fisheries, and deplete oxygen during decomposition. Narragansett Bay has experienced macroalgal blooms dominated by blade-forming *Ulva* for over a century. Evidence from other systems has suggested that *Ulva* can negatively impact other organisms. The first objective of this study was to determine whether bloom-forming *Ulva compressa* and *U. rigida* inhibit the growth of co-occurring macroalgae, *Gracilaria vermiculophylla, Cystoclonium purpureum*, and *Chondrus crispus,* during co-culture via laboratory-based assays. We found that *U. compressa* and *U. rigida* significantly inhibited the growth of all three macroalgae. We were able to verify the negative effects of *Ulva compressa,* but not *U. rigida* on the growth of *G. vermiculophylla* in flow-through seawater tanks. Our second objective was to determine if *Ulva* exudate decreased the survival of eastern oyster larvae in laboratory challenge experiments. We documented a significant negative effect of *Ulva* exudate on oyster survival, which depended on both the *Ulva* species and the nutrient condition. The strongest effect on oyster larval survival was seen in larvae exposed to nutrient replete *Ulva compressa* exudate, which had less than 30% relative survival after one week. Our results indicate that bloom-forming *Ulva* has the potential to inhibit co-occurring macroalgae and cause oyster larval mortality.

KEY WORDS- *Ulva compressa*, *Ulva rigida*, macroalgal blooms, larval mortality.

Introduction

Macroalgal blooms, generally consisting of green ulvoid macroalgae (commonly referred to as “green tides”), have been increasing worldwide (Valiela et al. 1997, Nelson

1 et al. 2003, Teichberg et al. 2010, Liu et al. 2013, Smetacek & Zingone 2013) and are
2 common occurrences on the northeastern coast of the United States (Bricker et al. 2008).
3 Macroalgal blooms are typically driven by anthropogenic nutrient loading in shallow
4 estuaries and can result in declines in seagrass (Valiela et al. 1997, McGlathery 2001),
5 perennial algae, and overall community diversity (Worm & Lotze 2006). During bloom
6 decomposition, macroinvertebrate abundance declines (Cummins et al. 2004) and
7 dissolved organic nitrogen is released into the water column (Tyler et al. 2001) that can
8 fuel further primary production (reviewed by Raffaelli et al. 1998). Macroalgal blooms
9 are also costly to clean up (Atkins et al. 1993, Lapointe & Bedford 2007).

10 Narragansett Bay, Rhode Island, U.S.A is a 380 km^2 semi-diurnal, well mixed
11 tidal estuary (Deacutis et al. 2006). The northern part of the bay is heavily populated and
12 there are three major urban freshwater inflows that contribute anthropogenic nutrients to
13 the system (Deacutis et al. 2006, Thornber et al. 2008). Greenwich Bay, a small sub-
14 embayment on the western side of Narragansett Bay, has been plagued by persistent
15 macroalgal blooms during the summer months for more than a century, dominated by
16 *Ulva compressa* Linnaeus and *U. rigida* C. Agardh (Granger et al. 2000, Guidone et al.
17 2013, Thornber et al. 2017). For example, Granger et al. (2000) documented 100-400 g
18 dry mass/m^2 (1015-4060 g wet mass/m^2 based on the conversion factors of Angell et al.
19 2012) of *Ulva* in Greenwich Bay in 1996, while Guidone & Thornber (2013) observed a
20 maximum biomass of >1800 g wet mass/m^2 in 2010.

21 Although green macroalgal blooms can have significant deleterious impacts on
22 coastal ecosystems (see Fletcher 1996), they have historically been considered non-toxic
23 (Valiela & Cole 2002, Anderson 2009). Green macroalgae have been considered to be

less likely than red and brown macroalgae to inhibit or harm co-occurring organisms (Harlin 1987, Valiela et al. 1997), and therefore, competition between green macroalgae and co-occurring species has been investigated less than with red and brown macroalgae (Hurd et al. 2014). However, growing evidence has suggested that ulvoid species of green macroalgae (species in the Family Ulvophyceae) can inhibit the growth, germination, and/or development of co-occurring organisms (Nelson et al. 2003, Nan et al. 2008, Nelson & Gregg 2013, Van Alstyne et al. 2014, Van Alstyne et al. 2015; Table 1). Evidence of ulvoid species suppressing the growth of phytoplankton, especially species that cause harmful algal blooms, has been especially strong (*e.g.* Jin & Dong 2003, Nan et al. 2008, Wang et al. 2012, Accoroni et al. 2015).

While several researchers have reported positive and negative effects of ulvoid species on invertebrates (Nelson & Gregg 2013, Van Alstyne et al. 2014), to date there have been no reports of the potential effects of *Ulva* spp. on the economically important eastern oyster, *Crassostrea virginica*. Muñoz et al. (2012) showed that the presence of young *Ulva* thalli improved the post-larval growth rate of the commercially produced red abalone *Haliotis rufescens,* while Huggett et al. (2005) reported high settlement of the abalone *H. rubra* on two ulvoid species. Lamb (2015) noted that the presence of *Ulva* thalli in aquaculture bags resulted in slower growth of adult Pacific oysters, *Crassostrea gigas.* Currently there are 315 aquaculture farms that cultivate the eastern oyster *C. virginica* in the U.S. (USDA 2014), many of them in areas where *Ulva* is present. Therefore, it is important to understand the interactions between bloom-forming *Ulva* and the eastern oyster.

1 Given the mounting evidence from other systems dominated by ulvoid
2 macroalgae, we hypothesized that blade-forming species of *Ulva,* namely the bloom-
3 forming *U. compressa* and *U. rigida,* inhibit the growth of co-occurring organisms in
4 Narragansett Bay. The first objective of this study was to determine if *U. compressa* or *U.*
5 *rigida* negatively affect the growth of co-occurring macroalgae. Our second objective
6 was to determine if exudate from *U. compressa* or *U. rigida* affected the survival of
7 eastern oyster larvae. Testing the impacts of *Ulva* exudate on oyster larvae was important
8 for two reasons. First, *Ulva* blooms form in coastal ponds where eastern oyster
9 populations co-occur and oyster cultivation is present (Thorne-Miller et al. 1983, Beutel
10 2017). Second, larvae should be included in assays because they are generally more
11 sensitive to heavy metals and pollutants than adults (Connor 1972, His et al. 1999). We
12 discuss our findings in light of increased coastal development and eutrophication, which
13 will likely fuel increasing macroalgal blooms in the future.
14
15 **Materials and Methods**

16 **Genetic identification of *Ulva***

17 The genus *Ulva* contains many blade-forming species that appear
18 morphologically similar, however, the cell shape and numbers of pyrenoids can be used
19 to distinguish between *U. compressa* and *U. rigida* (Guidone et al. 2013). We examined
20 each blade of *Ulva* and determined its identity based on the morphological characteristics
21 detailed by Guidone et al. (2013). *Ulva compressa* cells are polygonal with rounded
22 corners and contain a single pyrenoid, while *U. rigida* cells are polygonal with angular
23 corners and contain 2-4 pyrenoids. However, *Ulva* morphology can be highly variable

1 (Hofmann et al. 2010), so we also used DNA barcoding to verify the accuracy of our
2 morphological identifications. We amplified a 678 bp segment from the *rbc*L gene of
3 specimens used in these experiments following the methods of Guidone et al. (2013)
4 except that we used a modified CTAB plant DNA extraction protocol based on Doyle and
5 Doyle (1987). The raw sequence chromatograms were trimmed and proofread in 4Peaks
6 (v.1.8, Nucleobytes) and sequences were aligned and assembled in Seq Man Pro (v.12,
7 DNA Star Inc.).
8
9 **Genetic identification of *Gracilaria***
10 Two species of *Gracilaria* occur in Narragansett Bay, the native *Gracilaria*
11 *tikvahiae* McLachlan and the introduced *Gracilaria vermiculophylla* (Ohmi) Papenfuss
12 (Nettleton et al. 2013). These two species have morphological characteristics that
13 overlap, and therefore restricted fragment length polymorphism (RFLP) and selected
14 DNA barcoding was performed to determine the species identification of material from
15 the laboratory-based mesocosm trials.
16 DNA was extracted using the modified CTAB plant DNA extraction protocol
17 based on Doyle and Doyle (1987). Polymerase chain reaction was performed in 50 µL
18 volumes containing 10 µL of 5X GoTaq® Flexi DNA Polymerase (Promega
19 Corporation), 7 µL of 25 mM Mg^{2+}, 1 µL of 2.5 mM dNTP, and 4 µL of extracted DNA
20 template (10-50 ng). A 307 bp segment from the mitochondrial gene COX1 was used for
21 species identification and was amplified with the forward primer CO1F328 and the
22 reverse primer CO1R634 (Nettleton et al. 2013). The PCR profile consisted of an initial
23 denaturation at 95°C for 2 minutes, followed by 30 cycles of 57°C for 1 minute, 73°C for

1 minute, and 95°C for 1 minute followed by a final minute at 57°C and a final extension at 73°C for 6 minutes. RFLP analysis was performed on the PCR samples after amplification following the protocol of Nettleton (2012). In addition to the RFLP analysis, three samples were chosen at random to be sequenced. PCR purification, sample preparation, sequencing, and sequence analysis were performed as described above (see "Genetic Identification of *Ulva*").

Effects of *Ulva* on co-occurring macroalgae

In order to determine whether *Ulva compressa* or *U. rigida* suppress the growth of other macroalgae, we performed a series of co-culture experiments. We co-cultured isolated tips of three species that are common in *Ulva* blooms in Narragansett Bay (Thornber, *unpub. data*), *Gracilaria vermiculophylla*, *Cystoclonium purpureum* (Hudson) Batters, and *Chondrus crispus* Stackhouse from adult thalli, in separate trials, with the bloom-forming *U. compressa* and *U. rigida.* We then conducted a series of semi-controlled trials with *U. compressa, U. rigida*, and *G. vermiculophylla* in outdoor flow-through seawater tanks.

All macroalgal material was collected in Narragansett Bay, Rhode Island during low tide in the intertidal or shallow subtidal zone and transported to the laboratory on ice for processing. Upon arrival at the laboratory, all material was cleaned with sterile seawater to remove epiphytes. Following epiphyte removal, tips of *G. vermiculophylla*, *C. purpureum,* and *C. crispus* were excised using sterile razor blades, rinsed three times with sterile seawater (30-32 psu), and placed in 250 mL flasks with sterile Von Stosch Enriched (VSE) natural seawater (Ott 1966) under acclimation conditions (20-23°C, 100

1 μmol photons $m^{-2} s^{-1}$, and a 16:8 Light: Dark photoperiod with constant aeration); total
2 acclimation to laboratory conditions occurred for at least 3 days, with at least 24 hours
3 allowed for wound healing following tip cutting. Natural seawater was obtained from the
4 Marine Science Research Center (MSRC) at the University of Rhode Island's
5 Narragansett Bay Campus, filtered to 0.2 μM, and autoclaved prior to use.

6 After the acclimation period, the blotted-dry wet mass of tips of *G.*
7 *vermiculophylla* were taken and the tips were placed in individual 1 L mesocosms that
8 were divided in half with mesh with 1 mm^2 openings and filled with 400 mL of sterile
9 VSE seawater. On the other side of the mesh, 0.4 g of either *U. compressa* or *U. rigida*
10 (=1 g/L) was added. Experimental culture conditions were equivalent to those provided
11 during the acclimation period and light was supplied from the top to ensure no
12 interspecific shading. In total, there were 21 mesocosms with seven replicates each of the
13 *U. compressa* treatment, *U. rigida* treatment, and mesocosm control (*G. vermiculophylla*
14 in mesh-divided mesocosm without *Ulva*) per trial. In order to prevent nutrient limitation,
15 NO_3^- was measured daily as a proxy for nutrient concentrations, and all VSE nutrients
16 ($NaNO_3$, $Na_2HPO_4 \cdot 12H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $Na_2EDTA \cdot 2H_2O$, Thiamine-
17 HCl, Biotin, Vitamin B_{12}) were replenished based on nitrate depletion. Nitrate was
18 measured using an API Nitrate Test Kit modified for a 1 mL sample. Nitrate was
19 considered depleted if it was below 40 ppm (*i.e.* full VSE enrichment) and was
20 replenished to this level daily. On average, we replenished nutrients in the *U. compressa*
21 treatment every other day and the *U. rigida* treatment every 2.6 days.

22 On Days 2, 4, 6 and 8 of each trial, the blotted-dry wet mass of *G.*
23 *vermiculophylla* tips was measured. Relative growth rate (RGR) was calculated using the

1 following equation: RGR (%) = $100 \times [\ln (L_2/L_1)/(t_2-t_1)]$, where L_2 and L_1 were the blade
2 weight at times t_2 and t_1, respectively. A total of two *G. vermiculophylla* trials were
3 performed. The same experimental design was used to conduct separate trials with
4 *Cystoclonium purpureum* (2 trials) and *Chondrus crispus* (2 trials). Daily pH levels were
5 determined for the first *Chondrus crispus* trial only using an EcoTestr™ pH meter
6 (Oakton ®).
7
8 ***Gracilaria vermiculophylla* control trials**
9 To confirm that the observed results were due to the presence of *Ulva* and not
10 simply due to the presence of another macroalga, two *G. vermiculophylla* control trials
11 with the same experimental design as the co-culture trials described above were
12 performed. Seven replicates each of two treatments, *G. vermiculophylla* and mesocosm
13 control, were included in each trial. The *G. vermiculophylla* treatment had 0.4 g (=1 g/L)
14 of *G. vermiculophylla* on one side of the mesh and a tip of *G. vermiculophylla* on the
15 other side.
16
17 **Semi-controlled outdoor flow-through seawater tank trials**
18 In order to determine whether *Ulva* suppressed the growth of co-occurring
19 macroalgae, semi-controlled trials in outdoor flow-through seawater tanks were
20 conducted at the MSRC during July 2015 (n=4). *G. vermiculophylla* (0.85 ±0.04 g) was
21 co-cultured with 1.5 g of either *U. compressa, U. rigida,* or *G. vermiculophylla* (control)
22 in separate flow-through tanks (n=3). *Ulva compressa, U. rigida,* or *G. vermiculophylla*
23 (1 g/L) were placed in individual mesocosms (16 x 11.9 x 7.62 cm) covered with mesh on

1 all sides (mesh size=1.6 cm^2) that was connected with cable ties to a mesocosm
2 containing *G. vermiculophylla*. The mesocosm pairs were arranged so that *Ulva* was
3 upstream of *G. vermiculophylla*. The mass of *G. vermiculophylla* was measured on Day 0
4 and Day 7 and relative growth rate (RGR) was calculated. Due to space limitations, four
5 individual trials were conducted with a single replicate from each treatment in each trial.
6 Water temperature was measured using HOBO Tidbit v2 water temperature loggers
7 (Onset Computer Corporation) and averaged 23.6°C (individual tanks ranged from
8 23.16°C ± 0.09 to 27.28°C ± 0.51; mean ± SE).
9

10 **Effects of *Ulva* on oyster larvae**

11 In order to determine whether exudate from *U. compressa* or *U. rigida* affected
12 the survival of eastern oyster larvae, a series of challenge experiments were conducted.
13 *Ulva compressa* and *U. rigida* (5 g/L) were cultured in nutrient replete (*i.e.* supplied full
14 VSE nutrients) or nutrient deplete (*i.e.* no nutrients supplied) seawater for 2-3 days, under
15 the same conditions outlined above, to produce *Ulva* exudate. This concentration of *Ulva*
16 was chosen to reflect those present in *Ulva* blooms. Bloom biomass can exceed 8,000
17 g/m^3 in the subtidal and 3,000 g/m^2 in the intertidal (Thornber et al. 2017). In the nutrient
18 replete cultures, NO_3^- was measured daily as a proxy for nutrient concentrations, and all
19 VSE nutrients were replenished based on nitrate depletion. However, exudate was not
20 collected for use in the challenge experiments until all NO_3^- was depleted in the nutrient
21 replete cultures, since nitrate can be toxic to juvenile and adult shellfish (Epifanio & Srna
22 1975).

23 At the end of the culture period, *Ulva* material was removed from the seawater

1 and the pH of exudate was adjusted to 7.9-8.0. The exudate was then filter sterilized (0.2
2 μM). Oyster larvae were obtained from the Blount Shellfish Hatchery at Roger Williams
3 University and acclimated to laboratory conditions in sterile natural seawater on a shaker
4 plate (40 rpm). Larvae were fed 2 mL/L of Shellfish Diet 1800® (Reed Mariculture,
5 Campbell, CA) every other day while in the laboratory. At the start of the experiments
6 oyster larvae were between 3 and 9 days old.

7 Challenge experiments (3 trials) were conducted in 6-well culture plates following
8 a slight modification of previously developed protocols (Karim et al. 2013, Sohn et al.
9 2016). Oyster larvae (~50-100) were collected onto 45 μM nylon mesh, washed with
10 filtered sterile seawater and placed into each well with 5 mL of the assigned treatment
11 water. Treatments included *U. compressa* + nutrients, *U. compressa* – nutrients, *U. rigida*
12 + nutrients, and *U. rigida* – nutrients. Each well plate contained three wells of a treatment
13 and three wells of control (sterile seawater). Larval survival was assessed on Days 3, 5,
14 and 7 by counting dead larvae (*i.e.* empty shells) in each well using an inverted
15 microscope. At the end of the experiment, larvae were fixed by adding 70% ethanol to
16 each well to obtain a total count. Percent survival of oyster larvae was calculated for each
17 day using the following equation: % survival = (total - dead) ÷ total × 100. In instances
18 where % survival was less than 0, due to human error in counting, survival was adjusted
19 to 0% (8 out of 141 observations). The relative percent survival (of control) was
20 calculated by randomly pairing each treatment well with a control well from the same
21 plate using the following equation: relative percent survival = (% survival of treatment ÷
22 % survival of control) × 100.

23

Statistical Analysis

We used separate split-plot analysis of variance (ANOVA) tests to determine the effect of co-culture with *U. compressa* and *U. rigida* on the growth rate of *G. vermiculophylla*, *C. purpureum,* and *C. crispus* with treatment as the main plot (3 levels) and time as the sub-plot (4 levels); Trial (n=2) was included as a blocking factor. We also used a split-plot ANOVA to test the effect of co-culture with *G. vermiculophylla* on the growth of *G. vermiculophylla* tips (*G. vermiculophylla* control trial). We used a one-way ANOVA to test the effect of treatment (3 levels) on the growth rate of *G. vermiculophylla* in semi-controlled outdoor flow-through seawater tank trials, with Trial (n=4) included as a blocking factor. We used a two-way split-split-plot ANOVA to determine the effect of *Ulva* species (main plot, 2 levels), nutrients (sub-plot, 2 levels), and day (sub-sub plot, 3 levels) on the percent survival (of control) of oyster larvae from the challenge experiment. Trial (n=3) was used as a blocking factor.

Prior to analyses, all data were examined for normality and homogeneity of variances and transformed where appropriate; *G. vermiculophylla* growth rate was log transformed to ensure homogeneity of variances. Our growth and percent survival data did not meet the assumption of normality, even after transformation; however ANOVA is robust to deviations from normality when experiments have balanced designs and reasonable sample sizes (Underwood 1997). Post-hoc comparisons were made using Tukey's Honestly Significant Differences tests. All statistical analyses were conducted using JMP (v.12.0.1, SAS Institute Inc.).

Results

Genetic identification of *Ulva*

All *U. compressa* specimens identified using morphological characteristics (n=14) in this study were verified by DNA barcoding using MegaAlign (v.12, DNA Star Inc.) to match *U. compressa* from the Northwest Atlantic (GenBank® Accession: KC582355.1). Although the holotype sequence for *U. compressa* is not currently available, our sequences agreed with the *U. compressa* concept identified by Guidone et al. (2013).

The topotype of *U. rigida* is labeled on GenBank® as *U. armorica* (Shimada et al. 2003; Guidone et al. 2013), which has since been synonymized with *U. rigida.* All *U. rigida* specimens identified using morphological characteristics in this study (n=14) were verified to match *U. rigida* from the Northwest Atlantic (GenBank® Accession: EU484395.1) and were 99% identical to the *U. rigida* topotype (GenBank® Accession: AB097630).

Genetic identification of *Gracilaria*

All *Gracilaria* specimens used in the laboratory co-culture experiments (n=6 for *Ulva* experiments and n=8 for *G. vermiculophylla* control) were identified through RFLP analysis as *G. vermiculophylla.* The three samples that were sequenced were identical to *G. vermiculophylla* from the Northwest Atlantic (GenBank® Accession: JQ675712.1) based on Nettleton et al. (2013).

Effects of *Ulva* on co-occurring macroalgae

The effect of treatment (*Ulva compressa, U. rigida,* and mesocosm control) on the relative growth rate of *Gracilaria vermiculophylla* was dependent on Day (Treatment x

Day: $F_{6,153} = 2.2$, $p = 0.048$; Figure 1a; Table S1). After eight days of co-culture, the RGR of *G. vermiculophylla* without *Ulva* (7.44 ± 1.35 % d^{-1}) was more than three times higher than *G. vermiculophylla* co-cultured with *U. rigida* (2.31 ± 0.69 % d^{-1}). *G. vermiculophylla* co-cultured with *U. compressa* had virtually no change in mass on Day 8 and grew significantly slower than *G. vermiculophylla* tips in the mesocosm control ($p=0.004$; Figure 1a).

Day significantly affected the relative growth rate of *Cystoclonium purpureum* ($F_{3,151} = 8.2$, $p < 0.001$) and was dependent on Treatment (Treatment x Day: $F_{6,151} = 3.9$, $p = 0.001$; Figure 1b; Table S2). There was no significant difference between the RGR of *C. purpureum* tips co-cultured with *U. rigida* (0.94 ± 1.28 % d^{-1}) and the mesocosm control (6.37 ± 0.96 % d^{-1}), after 8 days of co-culture. However, after 8 days of co-culture tips co-cultured with *U. compressa* grew significantly slower (-5.39 ± 1.22 % $d^{-}1$) than tips co-cultured with *U. rigida* ($p=0.024$) or alone ($p<0.001$; Figure 1b; Table 2).

The relative growth rate of *Chondrus crispus* was significantly affected by Treatment (*U. compressa, U. rigida,* and mesocosm control; $F_{2,152} = 39.3$, $p = 0.025$) with the effect of Treatment dependent on Day (Day: $F_{3,151} = 8.7$, $p < 0.001$; Treatment x Day: $F_{6,151} = 2.6$, $p = 0.018$; Figure 1c; Table S3). *C. crispus* thalli grown without *Ulva* grew significantly faster than thalli grown with *U. rigida* ($p=0.025$) or *U. compressa* ($p=0.019$) after six days of co-culture (Figure 1c; Table 3). On Day 8, *C. crispus* thalli in both *Ulva* treatments were losing mass while *C. crispus* cultured without *Ulva* was growing at a RGR of 5.7 ± 0.5 % d^{-1} (Figure 1c, Table 3). In the first *C. crispus* trial, the pH levels were 8.1 ±0.03, 9.2±0.2, and 8.9±0.2 in the mesocosm control, *U. compressa* treatment, and *U. rigida* treatment, respectively.

1 There was no effect of co-culture with *G. vermiculophylla* on the relative growth
2 rate of tips of *G. vermiculophylla* in the control trials (Treatment: $F_{1,101} = 3.2$, $p = 0.157$;
3 data not shown). The average RGR of *G. vermiculophylla* cultured alone was 6.29 ± 0.52
4 % d^{-1}, while tips co-cultured with *G. vermiculophylla* had an average RGR of 3.79 ± 0.90
5 % d^{-1}.
6 The overall relative growth rate of *G. vermiculophylla* in outdoor flow-through
7 seawater tank trials was significantly different among treatments ($F_{2,6} = 5.8$, $p = 0.0393$).
8 There was no significant difference in the RGR of *G. vermiculophylla* between the
9 mesocosm control (8.5 ± 1.7 % d^{-1}) and *U. rigida* (7.7 ± 3.0 % d^{-1}) treatments or between
10 *U. rigida* and *U. compressa* (3.9 ± 0.9 % d^{-1}) treatments. However, *G. vermiculophylla* in
11 the mesocosm control grew significantly faster than *G. vermiculophylla* co-cultured with
12 *U. compressa* (p=0.043).
13
14 **Effects of *Ulva* on oyster larvae**
15 Survival in the control oyster larvae wells was good throughout the 7-day
16 challenge experiment. Mean survival in the controls was 97.8% ±1.3% on Day 3, 89.4%
17 ±2.5% on Day 5 and 71.9% ±3.8% on Day 7 (mean ± SE, n=48).
18 Relative percent survival of oyster larvae was significantly lower when larvae
19 were cultured in exudate from *U. compressa* (79.5 ± 1.9 %) than from *U. rigida* (98.1 ±
20 1.9 %; $F_{1,2} = 47.4$, $p = 0.008$; Figure 2; Table S4). The effect of *Ulva* species on oyster
21 larval survival was dependent on nutrients and time (*Ulva* species × nutrients × day=
22 $F_{2,122} = 6.7$, $p = 0.002$; Table S4). Post hoc analysis revealed no difference between the
23 treatments after 3 days of culture. However, oyster survivorship was significantly lower

when cultured in *U. compressa* + nutrients than *U. compressa* – nutrients ($p<0.001$) and *U. rigida* + nutrients ($p<0.001$) after 5 days of culture (Figure 2). This pattern was consistent after 7 days, when oyster survival in the *U. compressa* + nutrients treatment was less than 30% (Figure 2).

Discussion

While green macroalgae have been traditionally thought of as non-toxic, increasing evidence has shown that species of ulvoid macroalgae can inhibit co-occurring phytoplankton (Nan et al. 2008; Tang & Gobler 2011), macroalgae (Gao et al. 2014), and invertebrates (Nelson & Gregg 2013, Van Alstyne et al. 2014, Peckol & Putnam 2017). Here, we found that two dominant bloom-forming ulvoid species, *Ulva compressa* and *U. rigida*, inhibit the growth of the co-occurring red macroalgae, *Gracilaria vermiculophylla, Cystoclonium purpureum*, and *Chondrus crispus* at *Ulva* concentrations that are observed during blooms (Thornber et al. 2017). Thornber et al. (2017) documented blooms dominated by *Ulva* that reached a biomass of >3000g/m^2 in the intertidal and >8000g/m^3 in the subtidal zone; blooms with over 12,000 g/m^3 were recently documented in a coastal salt pond (Green-Gavrielidis et al. 2017). We were able to validate the negative effect of *U. compressa* on the growth rate of *G. vermiculophylla* through trials in outdoor flow-through seawater tanks.

Previous studies have reported that species of *Ulva* (e.g. *Ulva linza;* Gao et al. 2014) inhibited the growth and photosynthesis of *G. lemaneiformis* in co-culture experiments, through a combination of chemical and nutrient competition. In our study, we attempted to eliminate the effects of nutrient competition by replenishing nutrients

daily. It should be noted, however, that nitrate was used as a proxy for all nutrients in the seawater media and concentrations of other essential nutrients (e.g. phosphorus, trace minerals) were not measured. Despite this, we believe that nutrient limitation was unlikely since the uptake rate of nitrogen is generally several times higher than the uptake of other nutrients in macroalgae (Wallentinus 1984). Additionally, although previous studies have indicated that *Ulva* and *Gracilaria* have similar nitrogen uptake rates (Wallentinus 1984, Naldi & Wheeler 2002), we saw no negative effect on the growth rate of *G. vermiculophylla* in our control trials, which suggests that nitrogen limitation did not occur. However, we cannot completely eliminate the possibility that nutrient competition played a role in our study. Future studies should test the concentrations of all nutrients to eliminate nutrient competition as a mechanism.

We found that nutrient replete *U. compressa* caused significant mortality in oyster larvae, while nutrient deplete *Ulva* extract had no significant effect on larval mortality. Other studies have shown that bryozoan and hydroid larvae can be negatively impacted by brown algae (Schmitt et al. 1998), red algae can cause necrosis in soft corals (de Nys et al. 1991), and green algae can negatively affect the development of Pacific oyster larvae (Nelson & Gregg 2013), growth rate of adult Pacific oysters (Nelson et al. 2003, Nelson & Gregg 2013, Van Alstyne et al. 2014), and metamorphosis of crab larvae (Van Alstyne et al. 2014). Interestingly, several studies have reported that the toxicity of phytoplankton increased under nutrient limitation. For example, the haptophyte *Prymesium parvum* causes significant mortality in other phytoplankton species and the toxicity of *P. parvum* was enhanced under nutrient limited conditions (Granéli & Johansson 2003, Uronen et al. 2005, reviewed by Granéli et al. 2008). Ribalet et al.

1 (2007) reported that production of toxic polyunsaturated aldehydes (PUAs) by marine
2 diatoms increased under nutrient limitation. Our results indicate that *U. rigida* grown
3 under nutrient deplete conditions had a stronger negative effect on oyster larval survival,
4 although this trend was not statistically significant. Contrastingly, Nan et al. (2008)
5 showed that *Ulva lactuca* caused mortality in microalgae under nutrient replete
6 conditions, similar to our findings for *U. compressa*.

7 Previous researchers have also demonstrated that the effect of macroalgae on co-
8 occurring species is dependent on species-specific characteristics. For example, Accoroni
9 et al. (2015) showed that co-culture with fresh thalli of the brown alga *Dictyota*
10 *dichotoma* had a stronger negative effect on the growth of the benthic diatom *Ostreopsis*
11 cf. *ovata* than co-culture with *U. rigida.* There are also species-specific effects within the
12 Ulvales. Nelson et al. (2003) showed that extract from *U. obscura* more strongly
13 inhibited the germination of *Fucus gardneri* than extract from *U. fenestrata*.
14 In our laboratory-based mesocosms studies, both *U. compressa* and *U. rigida* inhibited
15 the growth of *G. vermiculophylla* and there was no significant difference in the growth of
16 *G. vermiculophylla* between the *Ulva* treatments. However, our results from outdoor
17 flow-through seawater tank trials showed that only *U. compressa* significantly suppressed
18 the growth of *G. vermiculophylla.* Although there was a trend of reduced *G.*
19 *vermiculophylla* growth in the *U. rigida* treatment, there was no significant effect of co-
20 culture with *U. rigida*, likely due to low replication. Furthermore, we documented
21 consistent, contrasting responses of oyster larvae to exudate of *U. compressa* and *U.*
22 *rigida.* Therefore, we hypothesize that the mechanisms responsible for the negative
23 effects of *U. compressa* and *U. rigida* on co-occurring organisms are species specific.

1 The three co-occurring macroalgae tested here also responded differently to *U.*
2 *compressa* and *U. rigida.* For example, the native *C. purpureum* began to lose mass or
3 show negligible growth in the presence of *U. compressa* and *U. rigida* after 4 and 6 days
4 of co-culture, respectively. *Chondrus crispus* grown in the presence of both *Ulva* species
5 had lower growth rates, but only began to lose mass after 8 days of co-culture with *U.*
6 *compressa*. Interestingly, the non-native *G. vermiculophylla* appeared to be the least
7 affected of the three macroalgae tested; *G. vermiculophylla* did not experience a
8 significant reduction in growth rate in either *Ulva* treatment until 8 days of co-culture had
9 passed and never began to lose mass. Differences in the response of species to ulvoids
10 have also been documented in phytoplankton (Tang & Gobler 2011) and could have
11 ecological consequences for species presence and abundance in or near macroalgal
12 blooms. In Narragansett Bay, *Gracilaria* spp. is very common in blooms (Thornber et al.
13 2017), perhaps owing to its ability to coexist with *U. compressa* and *U. rigida*. Species
14 interactions shape ecological communities and the impacts of *U. compressa* and *U. rigida*
15 on community composition require further research.
16 The responses documented here could be the result of allelopathy (*i.e*. chemical
17 inhibition) by *U. compressa* and *U. rigida.* However, identifying chemically mediated
18 interactions depends on detection of chemicals at or near the alga surface (Steinberg and
19 de Nys 2002). Therefore, this hypothesis cannot be validated until allelochemicals are
20 detected, isolated, and identified from *U. compressa* and *U. rigida*, and the effect of those
21 isolated allelochemicals on target species is tested. Furthermore, it is important to note
22 that *Ulva* can also compete with co-occurring macroalgae through other mechanisms
23 such as nutrient competition (discussed above) or through pH alteration. For example,

Ulva intestinalis has been shown to raise the pH of rockpools to a level (>10) where seaweeds cannot utilize external carbonic anhydrase (CA) to covert HCO_3- to CO_2 for use in photosynthesis, and therefore become carbon limited (Bjork et al. 2004). *Chondrus crispus* utilizes HCO_3- only through external CA and becomes bleached when growing in rockpools dominated by *U. intestinalis* due to high pH (Bjork et al. 2004). Although pH was only measured in the first *Chondrus crispus* trial, we did document pH levels that were potentially high enough to interrupt external CA activity. Alterations in pH, however, cannot explain all of the results documented here. In particular, research has shown that species of *Gracilaria* and closely related *Gracilariopsis* use both external CA (sensitive to high pH) and a direct HCO_3- transporter (not sensitive to pH) simultaneously to take up inorganic carbon (Andría et al. 1999, Pérez-Lloréns et al. 2004), yet we documented a negative effect of *U. compressa* on the relative growth rate of *Gracilaria vermiculophylla* in closed mesocosms. Additionally, in the oyster larval survival assays, we adjusted the pH of the *Ulva* exudate to match control seawater (7.9-8.0) prior to use. If pH were responsible for the negative effects of *Ulva* on oyster larvae, we should have seen no difference in the survival between treatments.

We have demonstrated for the first time that *U. compressa* has a significant negative effect on the survival of eastern oyster larvae, an important aquaculture crop in the U.S. (USDA 2014), when cultured under eutrophic conditions. Approximately two-thirds of U.S. coastal waterways, including Narragansett Bay, are considered degraded by an excess of nitrogen (N) from anthropogenic influences (Howarth & Marino 2006). Excess nutrients are known to cause blooms of ulvoid macroalgae (Teichberg et al. 2010) and our results suggest that *U. compressa* can cause mortality in oyster larvae in these

1 systems especially when oyster spawning coincides with the occurrence of *Ulva* blooms.
2 Interestingly, *U. rigida* did not cause significant mortality of oyster larvae, although it did
3 inhibit the growth of co-occurring macroalgae. One important caveat of this study is the
4 lack of validation of these effects *in situ.* While we did use ecologically relevant
5 concentrations of *Ulva* in our study, these results are likely to change as a result of
6 hydrodynamics (Steinberg et al. 2002). Further research is required to examine the effects
7 of *Ulva* on other economically important bivalves (e.g. clams and scallops) and on post-
8 larval eastern oysters and to verify these effects *in situ*.
9

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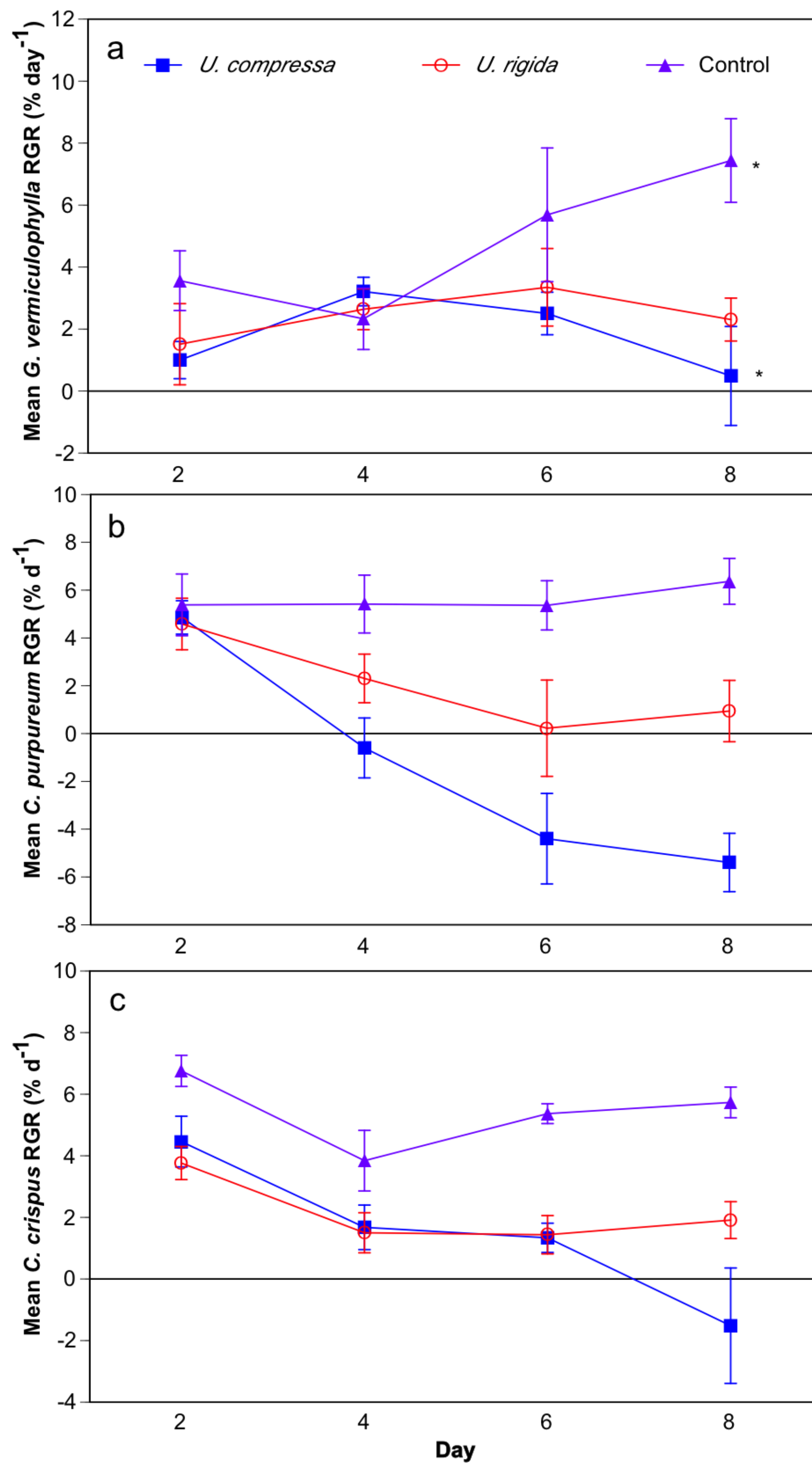
3

1 **Figure Legend**

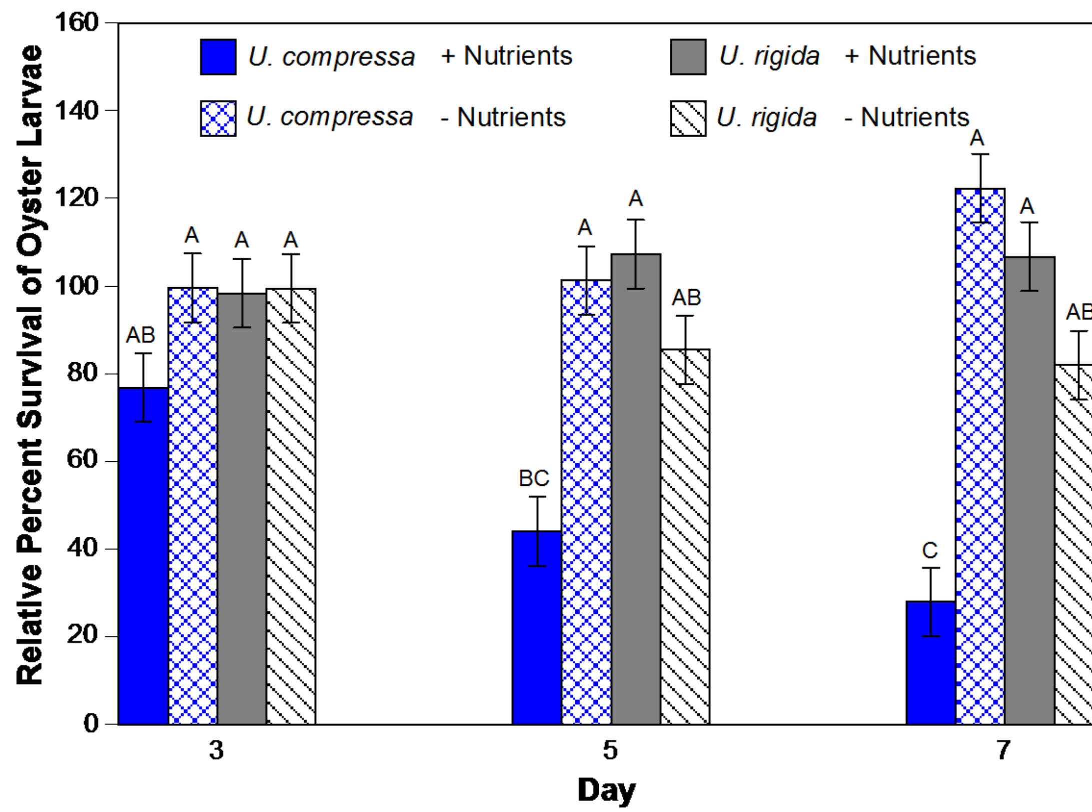
2 Figure 1. Mean relative growth rate (% d^{-1}) of a) *Gracilaria vermiculophylla* b)
3 *Cystoclonium purpureum* and c) *Chondrus crispus* co-cultured with *U. compressa, U.*
4 *rigida*, or alone (control). Error bars represent ±1SE. Asterisks (*) denote a statistically
5 significant difference based on Tukey's HSD post hoc comparisons. The results of
6 posthoc comparisons for *C. purpureum* and *C. crispus* are available in Table 2 and 3,
7 respectively.

8 Figure 2. Relative percent survival of oyster larvae exposed to exudate from *U.*
9 *compressa* and *U. rigida* grown under nutrient replete (+ Nutrients) or nutrient deplete (-
10 Nutrients) conditions. Bars with a letter in common are not statistically different based on
11 Tukey's HSD post hoc comparisons. Error bars represent ±1SE.

12



1
2 Figure 1.



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2 Figure 2.

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1 Table 1: Selected examples of studies documenting the effects of ulvoid macroalgae
2 (Family Ulvophyceae) on co-occurring organisms.
3

Location	Macroalgal Taxa	Documented Effects	Reference(s)
Washington, U.S.A	*U. obscura*	Inhibited development of *Fucus* zygotes and crab larvae, growth of *Ulva lactuca*	Van Alstyne et al. 2014
Washington, U.S.A	*U. lactuca, U. obscura,* and/or *U. fenestrata*	Inhibited development of *Fucus* zygotes, growth of *Ulva, Ulvaria*, and epiphytic macroalgae, inhibited/killed oyster larvae	Nelson et al. 2003, Nelson & Gregg 2013
Hawaii, U.S.A.	*U. reticulata*	Inhibited/killed fouling invertebrates	Walters et al. 1996
New York, U.S.A.	*U. lactuca*	Inhibited feeding of amphipod; inhibited growth of multiple harmful microalgal species	Borowsky & Borowsky 1990, Tang & Gobler 2011
Connecticut, U.S.A.	*U. lactuca*	Killed barnacles; killed zoeae crab larvae	Magre 1974, Johnson & Welsh 1985
Sirolo, Italy	*U. rigida*	Inhibited growth of toxic benthic dinoflagellate	Accoroni et al. 2015
Qingdao, China	*U. pertusa, U. linza, U. intestinalis,* and/or *U. lactuca*	Inhibited growth of red tide microalgae	Jin & Dong 2003, Nan et al. 2008, Wang et al. 2012

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1 Table 2. Mean relative growth rate (RGR; % d^{-1}) of *Cystoclonium purpureum* tips
2 cultured with *U. compressa, U. rigida,* or alone (mesocosm control). Means without a
3 common superscript letter differ significantly ($p<0.05$) based on Tukey's HSD post hoc
4 comparisons.
5

Days of Co-culture	**Treatment**	**RGR (% d^{-1}) ±1SE**
2	*U. compressa*	4.89 ± 0.70^{abc}
	U. rigida	4.58 ± 1.08^{abc}
	Mesocosm control	5.38 ± 1.29^{ab}
4	*U. compressa*	-0.60 ± 1.25^{cde}
	U. rigida	2.31 ± 1.02^{abc}
	Mesocosm control	5.42 ± 1.21^{ab}
6	*U. compressa*	-4.40 ± 1.89^{de}
	U. rigida	0.22 ± 2.01^{bcde}
	Mesocosm control	5.36 ± 1.03^{ab}
8	*U. compressa*	-5.39 ± 1.22^{e}
	U. rigida	0.94 ± 1.28^{abcd}
	Mesocosm control	6.37 ± 0.96^{a}

6
7 Table 3. Mean relative growth rate (RGR; % day^{-1}) of *Chondrus crispus* tips cultured
8 with *U. compressa, U. rigida,* or alone (mesocosm control). Means without a common
9 superscript letter differ significantly ($p<0.05$) based on Tukey's HSD post hoc
10 comparisons.
11

Days of Co-culture	**Treatment**	**RGR (mg d^{-1}) ±1SE**
2	*U. compressa*	4.46 ± 0.82^{abc}
	U. rigida	3.76 ± 0.54^{abc}
	Mesocosm control	6.76 ± 0.50^{a}
4	*U. compressa*	1.68 ± 0.72^{cd}
	U. rigida	1.50 ± 0.65^{cd}
	Mesocosm control	3.84 ± 0.98^{abc}
6	*U. compressa*	1.34 ± 0.47^{cd}
	U. rigida	1.44 ± 0.62^{cd}
	Mesocosm control	5.37 ± 0.32^{ab}
8	*U. compressa*	-1.52 ± 1.87^{d}
	U. rigida	1.91 ± 0.60^{bcd}
	Mesocosm control	5.73 ± 0.50^{a}

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