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The Effect of Naphthalene-Acetic Acid on Biomass Productivity and Chlorophyll Content of Green Algae, Coccolithophore, Diatom, and Cyanobacterium Cultures

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Abstract The application of biochemical stimulants to enhance biomass and metabolite productivity is being investigated here and may be a simpler approach to achieve our goals of higher productivity and lower costs than methods such as genetic modification. The research builds on prior work of screening various biochemical stimulants representing different types of plant growth regulators with the green alga, *Chlorella sorokiniana.* Here, we report the impact on biomass and chlorophyll productivity by comparing the delivery method of a previously identified superior stimulant, the synthetic auxin naphthalene-acetic acid (NAA), solubilized in ethanol or methanol. Algae evaluated included the green alga, *C. sorokiniana*, as well as a mixed consortium that includes *C. sorokiniana* along with two other wild-isolated green algae, *Scenedesmus bijuga* and *Chlorella minutissima.* It was found that NAA dissolved in ethanol was more effective in enhancing biomass productivity of *C. sorokiniana.* However, no differences were observed with the mixed consortia. The most effective treatment from this step, $EtOH_{500ppm}$+NAA_{5ppm}, along with two other NAA concentrations ($EtOH_{500ppm}$+$NAA_{2.5ppm}$ and $EtOH_{500ppm}$+NAA_{10ppm}), was then applied to six diverse species of microalgae to determine if the treatment dosage was effective for other freshwater and marine green algae, cyanobacteria, coccolithophore, and diatoms. It was found that three of the species bioassayed, *Pleurochrysis carterae*, *C. sorokiniana*, and *Haematococcus pluvialis* exhibited a substantial boost in biomass productivity over the 10-day growth period. The use of ethanol and NAA at a combined dosage of $EtOH_{500ppm}$+NAA_{5ppm} was found to generate the highest biomass productivity for each of the species that responded positively to the treatments. If scalable, NAA and ethanol may have the potential to lower production costs by increasing biomass yields for commercial microalgae cultivation.

Keywords Plant growth regulators · Phytohormones · Auxins · Naphthalene-acetic acid · Bioenergy · Biofuels · Biomass · Biostimulants · Microalgae · Phytohormones · *Chlorella sorokiniana* · *Haematococcus pluvialis* · *Phaeodactylum tricornutum* · *Pleurochrysis carterae* · *Dunaliella bardawil* · *Nostoc*

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Introduction

The production of microalgae on a commercial scale for bioremediation of wastewaters and production of bioenergy and biomaterials is being pursued with great interest in the recent past. One of the key bottlenecks with commercial microalgae production for biofuels is the high cost of production that is estimated in excess of \$3,000 ton^{-1} today [1]. One way to reduce cost is by increasing biomass productivity within the production cycle. Use of biochemical stimulants can be an effective way to achieve this because they are relatively low cost, easy to incorporate, and can have significant productivity enhancements. Past studies have shown that the auxin family of phytohormones is particularly effective at inducing a growth response from plants and algae. The research presented here builds upon prior results from biochemical stimulation studies that investigated many types of growth promoters ranging from phytohormones to elemental micronutrients with the green alga, *Chlorella sorokiniana* [2]. That study identified the synthetic auxin, naphthalene-acetic acid (NAA), as the most effective growth promoter at a concentration ranging from 2.5 to 10 ppm with the green alga, *C. sorokiniana*.

Research in the field of plant hormones began in the late 1800s, and over the course of 50 years, scientists identified several compounds responsible for affecting plant growth and development. It was observed that plant hormones were active at small concentrations, usually in the nanomolar range, although sometimes even at lower picomolar concentrations. The identification of many auxins, particularly indole-acetic acid (IAA), as plant growth regulator occurred in 1933 [3]. One of the other auxins identified was naphthalene acetamide (NAM), and it was thought that the molecularly similar synthetic compound, NAA, may be able to induce growth response in plants as well. Mitchell and Stewart compared the effect of these two compounds and found that the synthetic auxin, NAA at a concentration of 5 ppm, stimulated the top growth of bean plants and, furthermore, that NAA was associated with cellular proliferation of various tissues in the stem [4]. The same year, Brannon and Bartsch began investigating the impact of various natural and synthetic auxins on the growth of green algae and found that the application of NAA dissolved in ethanol at a concentration of 3.3 ppm increased the growth of *Chlorella vulgaris* by 172% compared to control. Additionally, it was observed that the naturally occurring auxin, phenyl-acetic acid at a concentration of 30 ppm, increased growth in *C. vulgaris* by 261% over control [5]. Decades of research has shown that auxins are involved in the regulation of cell division, cell growth, apical dominance, responses to directional stimuli, and fruit setting. However, it is known that not all responses to auxin are stimulatory and that auxins can become inhibitory at higher concentrations [3].

The synthetic auxins, such as NAA, act by increasing the endogenous IAA concentrations either by promoting new synthesis or by inhibiting IAA conjugation or breakdown [3]. Among the synthetic auxins, NAA is commonly used at relatively low concentrations to elicit auxin-type responses in cell growth, cell division, fruit setting, rooting, etc., and are found to be relatively stable in plant tissues and are metabolized very slowly in plant tissues [3]. Recent research has discovered the existence of mutant phenotypes that exhibit an altered response to auxins in terrestrial plants, such as tomato, soybean, and tobacco. The genes identified provide genetic evidence that the encoded proteins of several *Aux/IAA* genes are involved and regulate distinct aspects of the auxin response. However, other genes exist which are related to physiological responses from the presence of auxins. These genes encode proteins that are related to known auxin functions, such as cell wall loosening, ethylene biosynthesis, production of proteins associated with the cell walls, production of calcium-binding proteins that modulate activities of protein kinases or phosphatases, and induction of cell cycle regulatory proteins [3].

It is recognized that the research on the effect of plant growth regulators with algae lags far behind work with other terrestrial plants. Traditionally, plant hormones and synthetic plant growth regulators are used as valuable research tools to elucidate physiological responses of plants or to probe biochemical control mechanisms. However, their use could also be extended to the field of algae production to enhance the potential viability of commercial applications of alga-based renewable biomass production [6]. By identifying optimal stimulant dosages that enhances biomass productivity in commercially attractive strains of microalgae, it may be possible to lower production costs per ton of biomass produced, and increase the profitability of industries producing algae for food, feed, fuel, biomaterials, nutraceuticals, and pharmaceuticals.

Materials and Methods

The research presented here evaluated whether ethanol or methanol was a more suitable solvent to dissolve the auxin, NAA, for dosing algal cultures. The use of ethanol or methanol as a solvent may introduce inhibitory effects on the algal growth; therefore, a study designed to compare 500 ppm of each solvent with NAA was performed to determine which solvent is preferred. This was conducted with a monoculture and a consortia culture containing three species of green algae to evaluate whether a mixed culture would respond in a similar manner as a single species. The results from this experiment established the most effective treatment combinations and were then applied to six diverse species of microalgae to determine if the treatment dosage was effective for other freshwater and marine green algae, cyanobacteria, coccolithophore, and diatoms. These species were selected for this multispecies screening for various reasons. The freshwater green alga, *C. sorokiniana*, was selected because of its use in prior experiments as being a fast growing strain that thrives in wastewater conditions. It has also been shown to contain considerable amounts of lutein [7]. The freshwater green alga, *Haematococcus pluvialis*, was selected due to the high value pigment, astaxanthin, it produces in the highest concentrations found in nature [8]. The marine diatom, *Phaeodactylum tricornutum*, was selected to diversify the study to include diatoms, since they are known to have high oil content, and because this specific strain is well studied in the literature [9]. The marine coccolithophore, *Pleurochrysis carterae* (also known as *Cricosphaera carterae*), was selected due to its high oil content and its ability to grow on wastewater and dominate outdoor raceway cultivation [10, 11]. The halotolerant green alga, *Dunaliella bardawil* (also known as *Dunaliella salina*), was selected due to its commercially viable cultivation for beta-carotene and its high glycerol content [12]. The wild isolated cyanobacterium, *Nostoc* sp., was selected as a nitrogen-fixing organism that showed promise for cultivation in wastewater [10].

Strain and Culture Maintenance

The freshwater green algae, *C. sorokiniana* (UTEX 2805) and *H. pluvialis* (UTEX 2505), were obtained from the UTEX Culture Collections, while the mixed consortia, which comprised *C. sorokiniana* (UTEX 2805), *Chlorella minutissima* (wild isolate), and *Scenedesmus bijuga* (wild isolate), was created from previously isolated organisms in our laboratory [10] and maintained in BG11 growth medium using the formulation described by Stanier et al. [13]. The diatom, *P. tricornutum* (UTEX 640), and coccolithophore, *P. carterae* (UTEX LB 1014), were obtained from UTEX Culture Collections and maintained in a modified BG11 saline growth medium that comprised standard BG11 with the addition of

Oceanic marine salt mix (Oceanic Systems, Dallas, TX, USA) at a concentration of 35 g L^{-1}. The hypersaline green alga, *D. bardawil* (UTEX LB 2538), was obtained from UTEX Culture Collections and maintained in the modified BG11 saline growth medium which was further supplemented with additional NaCl (23 g L^{-1}) and $MgCl_2$ (5 g L^{-1}) for a hypersaline media. The cyanobacteria, *Nostoc* species, were previously isolated in our laboratory from a carpet industry wastewater [10] and maintained in a nitrogen-free BG11 growth media. The pH of all BG11 culture mediums was adjusted to 7.5±0.2 before inoculation, and the algae were maintained in a temperature-controlled growth chamber at 25±1 °C and 100± 10 μmol m^{-2} s^{-1} light intensity provided by cool white fluorescent (6,500 K) T-8 bulbs with light:dark cycles of 12:12 h.

Experimental Conditions and Experimental Plan

The experimental conditions and protocol used for these studies were the same as those reported by Hunt et al. [2]. The biochemical stimulant, 1-naphthalene-acetic acid (NAA), was used for all treatments, and dosages were selected based upon the experimental results from previous investigations [2]. The pure compound of NAA was obtained from Super-Grow Plant Care, Montreal, Canada (www.super-grow.biz). The solvents, ethanol and methanol, were both supplied by Thermo Fisher Scientific Inc, Waltham, MA. All experiments were conducted in 250-mL Erlenmeyer flasks with 100-mL BG11 growth medium supplemented with the biochemical stimulants to be tested. Growth studies were performed in a temperature-controlled growth chamber as previously mentioned. For the purpose of screening, previously reported dosages that demonstrated growth-enhancing effects were used [2]. The experimental results presented here are the result of two 10-day static flask experiments each investigating a different aspect of the biochemical stimulant treatments.

The first experiment evaluated the impact of the solvent used to dissolve NAA, by comparing ethanol (EtOH) and methanol (MeOH) at 500 ppm as the solvent containing NAA at 5 ppm ($EtOH_{500ppm}$+NAA_{5ppm}, $MeOH_{500ppm}$+NAA_{5ppm}). Each treatment was prepared with a known quantity of biostimulant dissolved in 500 μL of ethanol and added to 1 L of the respective BG11 growth media for a final concentration of 500 ppm for ethanol and the desired concentrations of NAA and were autoclaved for sterility at 121 °C at 103 kPa (15 psi). These treatments were evaluated for their impact on growth and chlorophyll concentration of *C. sorokiniana* and the mixed algal consortia described earlier.

The second experiment was a multispecies study investigating the impact of three dosages of NAA ($EtOH_{500ppm}$+$NAA_{2.5ppm}$, $EtOH_{500ppm}$+NAA_{5ppm}, $EtOH_{500ppm}$+NAA_{10ppm}) using ethanol as the solvent (500 ppm). An additional delayed dosage treatment was added to *C. sorokiniana*, where three previously un-dosed *C. sorokiniana* cultures were removed from the growth chamber on the fifth day and a filter-sterilized dosage of $EtOH_{500ppm}$+NAA_{5ppm} was applied to each flask and returned to the growth chamber for the remaining 5 days of growth. The goal was to determine whether previously identified effects were more universally applicable for other species. The study investigated the two freshwater green algae *C. sorokiniana* and *H. pluvialis*, the hypersaline green alga *D. bardawil*, the saltwater diatom *P. tricornutum*, the coccolithophore *P. carterae*, and the cyanobacteria *Nostoc* sp. for their response to NAA.

All media preparations were autoclaved after adding the biochemical stimulant. An exponentially growing culture of *C. sorokiniana* at a cell concentration of 0.06 g L^{-1} was used as inoculum in experiment I, while exponentially growing cultures at cell densities of 0.07 g L^{-1} (*C. sorokiniana*), 0.26 g L^{-1} (*H. pluvialis*), 0.26 g L^{-1} (*P. tricornutum*),

0.32 g L^{-1} (*P. carterae*), 0.33 g L^{-1} (*D. bardwil*), and 0.05 g L^{-1} (*Nostoc sp.*) were used in experiment II. After inoculation, the flasks were incubated for 10 days in the growth chamber. Details of culture and treatment dosages used in both experiments are summarized in Table 1.

Cultures were sampled on day 5 and 10, where day 5 sampling represented initial exponential phase while day 10 sampling represented the culture entering the late exponential phase. The productivity values presented in this manuscript were calculated by taking the biomass density (g L^{-1}) on the sampling day (day 5 and 10) and dividing by 5, which represents the number of days between sampling, to provide productivity values (g L^{-1} day^{-1}) in the windows of day 0–5 and day 5–10, respectively. Each treatment was performed in triplicate, and the parameters measured are reported as the mean with respective standard deviations. Due to variations in initial cell densities resulting from the addition of inoculum, which can have a significant impact on measured productivity over time, comparisons across species with different initial cell densities are reported as increases in productivity relative to their respective control within each experiment. The data for both experiments were analyzed using SAS for ANOVA and Tukey analysis

Table 1 Experimental treatments and dosages for each culture tested

Experiment	Organism	Treatment	Dosage (ppm)
Experiment I: comparison of ethanol and methanol as a solvent for NAA	*C. sorokiniana*	EtOH	500
		MeOH	500
		EtOH + NAA	500+5
		MeOH + NAA	500+5
	Mixed consortia (*C. sorokiniana, C. minutissima, S. bijuga*)	EtOH	500
		MeOH	500
		EtOH + NAA	500+5
		MeOH + NAA	500+5
Experiment II: effect of NAA with ethanol on the growth and chlorophyll productivities of six different algae species	*C. sorokiniana*	EtOH + NAA	500+2.5
			500+5
			500+10
	H. pluvialis	EtOH + NAA	500+2.5
			500+5
			500+10
	P. tricornutum	EtOH + NAA	500+2.5
			500+5
			500+10
	P. carterae	EtOH + NAA	500+2.5
			500+5
			500+10
	D. bardawil	EtOH + NAA	500+2.5
			500+5
			500+10
	Nostoc sp.	EtOH + NAA	500+2.5
			500+5
			500+10

EtOH ethanol, *MeOH* methanol, *NAA* 1-naphthalene-acetic acid

comparing the phytohormone treatments with each individual day. The Tukey analysis was evaluated at the $p<0.05$ confidence interval.

Analyses

Biomass was determined by filtering 25 mL of algal culture through a preweighed Whatman GF/C filter (4.7 cm diameter; 1.2 μm pore size). The filter was washed with 10 mL of 0.65 M ammonium formate solution to remove excess salts and dried overnight at 60 °C in a forced air oven. The dried filter with biomass was cooled in a desiccator and weighed again to estimate the final dry weight. For chlorophyll *a* estimation, 10 mL of homogenized algal culture was centrifuged at 5,000 rpm for 10 min, and the algal pellet was exhaustively extracted with hot methanol (95% *v*/*v*) until it was colorless. The amount of chlorophyll *a* extracted in the methanol was determined spectrophotometrically according to the method described by Porra et al. [14] using the following equation:

$$\text{Chlorophyll } a \ (\mu\text{g mL}^{-1}) = 16.29 \text{ x } \text{OD}_{665} - 8.54 \text{ x } \text{OD}_{652}$$

Results and Discussion

Comparison of Ethanol or Methanol as Solvent for Biochemical Stimulant

Results from C. sorokiniana

The choice of solvent applied to the cultures was found to dramatically affect the growth and chlorophyll dynamics for *C. sorokiniana* (Fig. 1a, b). The highest biomass density attained by *C. sorokiniana* was (0.576 g L^{-1}) by the treatment of $EtOH_{500ppm}$+NAA_{5ppm} representing a 120% increase versus the control (0.263 g L^{-1}) over the 10-day experiment. These results are similar to the results that were previously reported for a mixture of NAA in EtOH [2].

It was found that the biomass productivity between day 0 and 5 with ethanol alone ($EtOH_{500ppm}$) was statistically the same as the treatment $EtOH_{500ppm}$+NAA_{5ppm} ($p<0.05$) and induced almost the same increase in biomass productivity of 0.042 g L^{-1} day^{-1} and was found to be approximately 150% over control (Fig. 1a). This suggests that the initial boost in growth seen in both treatments may be the result of the ethanol during the initial lag and early exponential phases of *C. sorokiniana*. In the second phase of growth (days 5 to 10), biomass productivity of the $EtOH_{500ppm}$ treatment reduced to values closer to the control, and the difference was not statistically significant, whereas the $EtOH_{500ppm}$+NAA_{5ppm} treatment was statistically different from ethanol alone ($p<0.05$) and exhibited a second boost in growth productivity to 0.062 g L^{-1} day^{-1} representing 138% higher than control, where the control was at 0.026 g L^{-1} day^{-1} (Fig. 1a). The decrease in biomass productivity in the $EtOH_{500ppm}$ treatment and the boost in productivity for $EtOH_{500ppm}$+NAA_{5ppm} after day 5 gave the $EtOH_{500ppm}$+NAA_{5ppm} treatment the highest overall growth for *C. sorokiniana* compared to the other treatments.

In the first 5 days of growth, $MeOH_{500ppm}$+NAA_{5ppm} showed no statistical differences from that of the control ($p<0.05$); however, with NAA ($MeOH_{500ppm}$+NAA_{5ppm}), the second growth phase (days 5 to 10) was significantly higher than control ($p<0.05$) with an increase in biomass productivity of 129% over that of the control. In contrast, the treatment with methanol alone ($MeOH_{500ppm}$) dropped in productivity to 87% below control and was found to

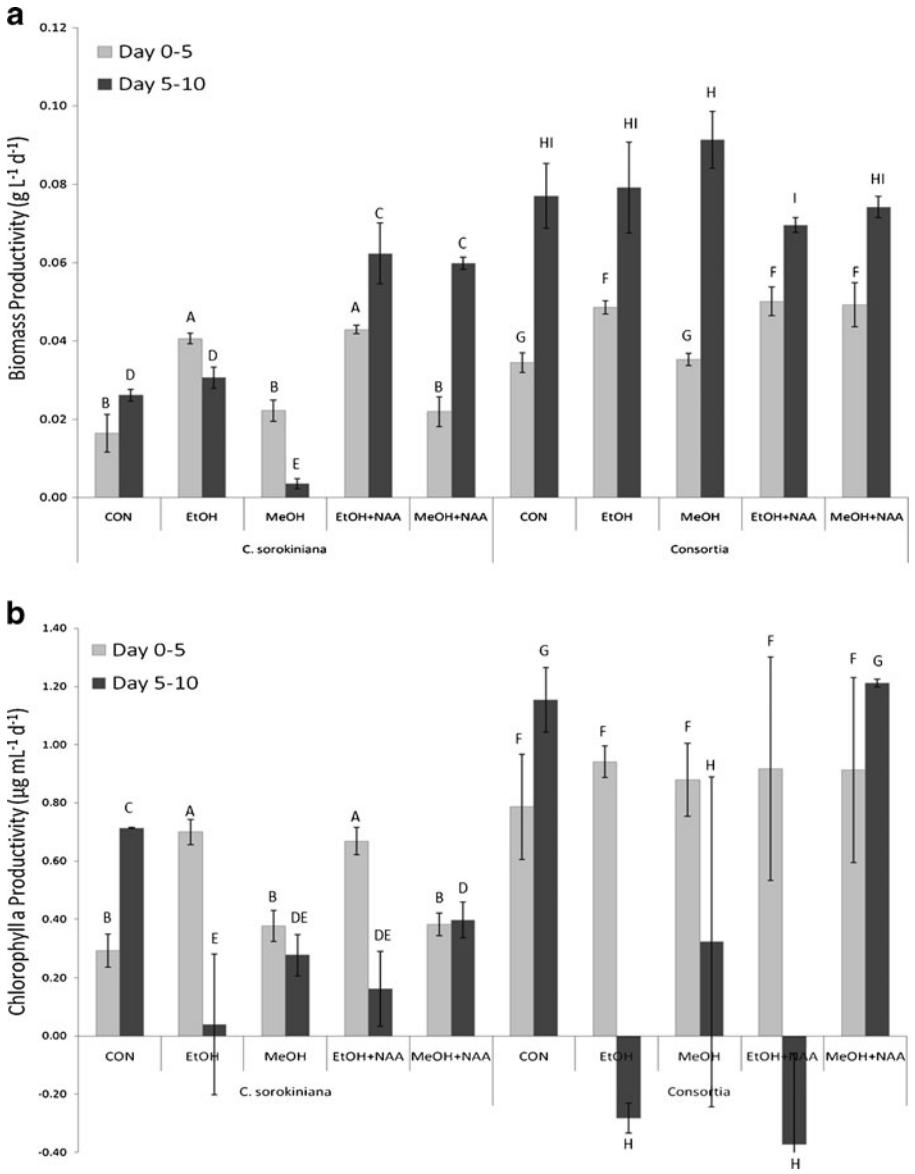


Fig. 1 **a** Biomass productivity responses of *C. sorokiniana* (*left*) and mixed consortia (*right*) to solvent $_{500\ ppm}$ and NAA_{5ppm}. Solvents compared are methanol (*MeOH*) and ethanol (*EtOH*) either by themselves or with biochemical stimulant naphthalene-acetic acid (*NAA*). The control (*CON*) did not receive any biochemical stimulant. *Individual bars* are means (n=3), and *error bars* represent two standard deviations. *Day 0–5* and *Day 0–10 bars* represent average productivities over the first 5 days and between fifth and tenth day, respectively. Statistical comparison is only valid for each individual species during the same time interval. **b** Chlorophyll *a* productivity responses of *C. sorokiniana* (*left*) and mixed consortia (*right*) to solvent $_{500\ ppm}$ and NAA_{5ppm}. Solvents compared are methanol (*MeOH*) and ethanol (*EtOH*) either by themselves or with biochemical stimulant naphthalene acetic acid (*NAA*). The control (*CON*) did not receive any biochemical stimulant. *Individual bars* are means (n=3), and *error bars* represent two standard deviations. *Day 0–5* and *Day 0–10 bars* represent average productivities over the first five days and between fifth and tenth day, respectively. Statistical comparison is only valid for each individual species during the same time interval

be statistically different from all other treatments by day 10. The final biomass density of 0.463 g L^{-1} for the $MeOH_{500ppm}$+NAA_{5ppm} treatment, however, was less than that for $EtOH_{500ppm}$+NAA_{5ppm} (0.576 g L^{-1}). These results seem to suggest that independent of the solvent used for delivering NAA, the effectiveness of NAA appears to be in the 5–10-day period. Additionally, in contrast to methanol that had no observable effect of its own, ethanol seemed to provide a growth stimulus in the 0–5-day growth period.

Results of chlorophyll productivity of *C. sorokiniana* mirrored the biomass productivity in the case of ethanol treatments ($EtOH_{500ppm}$ and $EtOH_{500ppm}$+NAA_{5ppm}) where these treatments were significantly different from the control ($p<0.05$). Large increases in their chlorophyll productivity by day 5 of 139% and 128% (0.700 and 0.668 μg ml^{-1} day^{-1}) relative to control (0.293 μg ml^{-1} day^{-1}), respectively, were observed (Fig. 1b). However, between day 5 and 10, these two treatments showed a decrease in chlorophyll productivity of 70% to 90% below the control. There was no statistical difference between the treatments $EtOH_{500ppm}$+NAA_{5ppm}, $MeOH_{500ppm}$, or $MeOH_{500ppm}$+NAA_{5ppm}; however, $EtOH_{500ppm}$, $EtOH_{500ppm}$+NAA_{5ppm}, and $MeOH_{500ppm}$ were all statistically different from the control ($p<0.05$). The observed chlorophyll inhibition was correlated with biomass productivity inhibition in the case of the $EtOH_{500ppm}$ treatment. However when NAA_{5ppm} was present with ethanol, the biomass productivity increased despite low chlorophyll productivities. These results seem to suggest a mechanism of hormone-induced cell division or proliferation rather than growth stimulation from enhancing the photosynthetic efficiency or apparatus. The enhancement of the growth rate of this green alga despite inhibition of chlorophyll productivity is a key result of this study in which future investigations should examine in more detail the potential for growth stimulation without proportional chlorophyll synthesis. The treatment of methanol ($MeOH_{500ppm}$ and $MeOH_{500ppm}$+NAA_{5ppm}) on *C. sorokiniana* demonstrated a small increase in chlorophyll productivity during the first 5 days but was not statistically significant ($p<0.05$).

Results from the Mixed Consortium

The response on biomass productivity to NAA and solvents from the mixed consortia of green algae relative to control was much lower than that of *C. sorokiniana* (Fig. 1a). The treatments $EtOH_{500ppm}$+NAA_{5ppm}, $MeOH_{500ppm}$+NAA_{5ppm}, and $EtOH_{500ppm}$ were found to be statistically different from $MeOH_{500ppm}$ and the control on day 5 ($p<0.05$). The treatment $EtOH_{500ppm}$ showed increase of biomass productivity to 0.049 g L^{-1} day^{-1} representing a 41% increase over the control, where the control had a value of 0.034 g L^{-1} day^{-1}. These productivities were comparable to that of $EtOH_{500ppm}$+NAA_{5ppm} which was found to be 0.050 g L^{-1} day^{-1}. The biomass productivity for treatments containing ethanol was all reduced to the control level by day 10 (Fig. 1a).

The application of methanol alone ($MeOH_{500ppm}$) had no statistical difference on biomass productivity during the first or last 5 days of growth compared to the control, while all other treatments were statistically different to the control after the first 5 days of growth ($p<0.05$). When NAA was added ($MeOH_{500ppm}$+NAA_{5ppm}), a 43% increase in biomass productivity (0.049 g L^{-1} day^{-1}) was observed relative to control (0.034 g L^{-1} day^{-1}). This suggests that there is a positive growth stimulation impact with NAA; however, the toxicity or inhibition of methanol at the levels tested prevented a growth response analogous to using ethanol as a solvent. By day 10, only the treatment $MeOH_{500ppm}$ and $EtOH_{500ppm}$+NAA_{5ppm} were found to be statistically different from each other ($p<0.05$). The methanol treatment ($MeOH_{500ppm}$) showed a slight increase to 19% higher average biomass

productivity, but was not statistically significant from the control ($p<0.05$). The $MeOH_{500ppm}+NAA_{5ppm}$ was observed to be at the same biomass productivity as the control (0.075 g L^{-1} day^{-1}).

The response of chlorophyll productivities from the mixed consortium to the treatments demonstrated marginal variation by the end of day 5, where all treatments had average chlorophyll productivities less than 20% below control (approximately 0.8 μg mL^{-1} day^{-1}) and were not found to be statistically different from each other ($p<0.05$). However, the inhibition of ethanol on chlorophyll synthesis becomes evident by day 10 as both treatments with ethanol ($EtOH_{500ppm}$ and $EtOH_{500ppm}+NAA_{5ppm}$) demonstrated a reduction in chlorophyll productivity of 124% and 132% compared to control, and these two treatments were statistically different from the control and $MeOH_{500ppm}+NAA_{5ppm}$, but was not different from $MeOH_{500ppm}$ alone ($p<0.05$). This implies that chlorophyll concentrations for $EtOH_{500ppm}$ and $EtOH_{500ppm}+NAA_{5ppm}$ at the tenth day were lower than that at the fifth day. These treatments were the only ones in this experiment that exhibited a negative productivity value suggesting strong chlorophyll *a* inhibition, although the biomass productivity was comparable to the control (0.075 g L^{-1} day^{-1}) during this same period. The results suggest that using these treatment combinations is not ideal for stimulating the growth of the mixed consortia tested here and, furthermore, that the use of ethanol appears to be toxic and inhibitory to chlorophyll synthesis at the 500-ppm concentration tested.

The ethanol and NAA treatments with the mixed consortia demonstrated a different growth response than *C. sorokiniana.* During the first 5 days of growth, the ethanol treatments exhibited the largest increase in biomass productivity for this culture which corresponded with marginal increases in chlorophyll productivity. However by day 10, this enhancement in biomass productivity waned and subsequently demonstrated a strong inhibition in chlorophyll *a* productivity which was the highest inhibition observed in any treatment from this experiment. The cultures that experienced this inhibition had visible differences in the flasks, where the cultures had turned a light yellowish-green color compared to their control, which was dark green. Despite the decreased chlorophyll content measured in the cells, the biomass productivity and final density were very similar to their control.

This study demonstrates that using ethanol as a solvent for NAA is better than methanol for *C. sorokiniana* and that a NAA dosage of 5 ppm was able to stimulate the growth response substantially above the control. The application of NAA with either solvent in the dosages examined in this study was not supportive or synergistic to enhance the biomass productivity of the mixed green algae consortium despite the inclusion of *C. sorokiniana*, which comprised one third of the consortium. This could be due to the release of toxins or inhibitory compounds, such as reactive oxygen species, if one of the other two species (*S. bijuga* or *C. minutissima*) had an adverse reaction to the solvent dosage.

Effect of Ethanol and NAA on Different Species of Algae

Results from C. sorokiniana

The previous investigation demonstrated that ethanol was the preferred solvent to dissolve NAA for growth stimulation. The aim of this study was to confirm these previous results and evaluate different dosages of NAA with six different algae species. All references to NAA treatments assume an additional to $EtOH_{500ppm}$ for the delivery of NAA. During the first

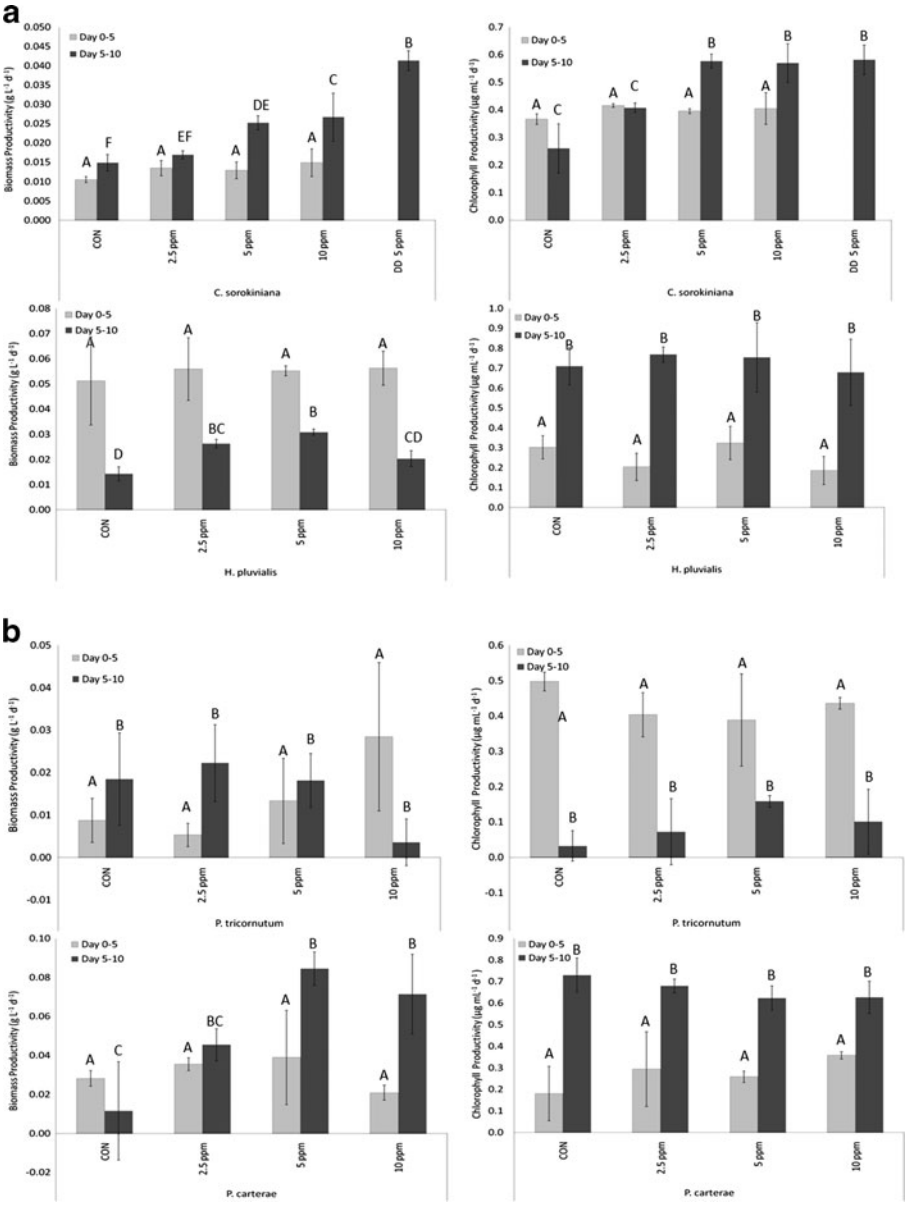
Fig. 2 **a** Biomass and chlorophyll *a* productivity for *C. sorokiniana* and *H. pluvialis* to ethanol $_{500\ ppm}$ and ▶ NAA $_{2.5ppm,\ 5ppm,\ 10ppm}$. The control (*CON*) did not receive any biochemical stimulant. *Individual bars* are means (n=3), and *error bars* represent two standard deviations. *Day 0–5* and *Day 0–10 bars* represent average productivities over the first 5 days and between fifth and tenth day, respectively. Statistical comparison is only valid for each individual species during the same time interval. **b** Biomass and chlorophyll *a* productivity for *P. tricornutum* and *P. carterae* to ethanol $_{500\ ppm}$ and NAA $_{2.5ppm,\ 5ppm,\ 10ppm}$. The control (*CON*) did not receive any biochemical stimulant. *Individual bars* are means (n=3), and *error bars* represent two standard deviations. *Day 0–5* and *Day 0–10 bars* represent average productivities over the first 5 days and between fifth and tenth day, respectively. Statistical comparison is only valid for each individual species during the same time interval. **c** Biomass and chlorophyll *a* productivity for *D. bardawil* and *Nostoc* sp. to ethanol $_{500\ ppm}$ and NAA $_{2.5ppm,\ 5ppm,\ 10ppm}$. The control (*CON*) did not receive any biochemical stimulant. *Individual bars* are means (n=3), and *error bars* represent two standard deviations. *Day 0–5* and *Day 0–10 bars* represent average productivities over the first 5 days and between fifth and tenth day, respectively. Statistical comparison is only valid for each individual species during the same time interval. **d** Biomass productivity between day 0 and 10 for *C. sorokiniana*, *H. pluvialis*, *P. tricornutum*, *P. carterae*, *D. bardawil*, and *Nostoc* sp. in experiment II under the treatments of ethanol $_{500\ ppm}$ and $NAA_{2.5ppm,\ 5ppm,\ 10ppm}$. The control (*CON*) did not receive any biochemical stimulant. *Individual bars* are means (n=3), and *error bars* represent two standard deviations. Statistical comparison is only valid for each individual species during the same time interval

5 days of growth, *C. sorokiniana* did not demonstrate any changes in biomass productivity compared to the control ($p<0.05$; Fig. 2a, d). However, the treatments of NAA_{5ppm}, $NAA_{10\ ppm}$, and the 5-day delayed dosage NAA_{5ppm} (DD-NAA_{5ppm}) were all found to be statistically different from the control ($p<0.05$). The growth stimulation, which was observed between day 5 and 10 under the treatments of NAA_{5ppm} and $NAA_{10\ ppm}$, showed 69% and 79% increase in biomass productivity relative to control, respectively ($p<0.05$). The delayed dosage treatment exhibited the highest increase in biomass productivity of 177% (0.0414 g L^{-1} day^{-1}) versus control (0.0149 g L^{-1} day^{-1}) compared to any other treatment of *C. sorokiniana* in this study. The standard treatments were dosed with NAA on day 0 at the initial cell concentration of 0.07 g L^{-1}, whereas the delayed dosage cultures were grown for an additional 5 days to a cell concentration of 0.12 g L^{-1} before receiving their respective 5 ppm dosage. The difference in biomass productivity between the NAA_{5ppm} and delayed dose NAA_{5ppm} was 64%±10%, whereas the difference in the concentration of the culture at time of dosage was 76%±6%. This suggests that future studies should investigate the potential for adding delayed dosages of EtOH+NAA after 5 days as well as extend the growth period and apply dosages at 10 days to *C. sorokiniana* and the other species that responded well to these treatments.

There was no statistically significant impact on chlorophyll productivity from the treatments using ethanol and NAA during the first 5 days with *C. sorokiniana* ($p<0.05$). However, between day 5 and 10, the chlorophyll productivity significantly increased ($p<0.05$) in the treatments NAA_{5ppm}, NAA_{10ppm}, and delayed dose NAA_{5ppm} exhibiting a 122%, 119%, and 124% increase compared to the control, respectively. The increase in chlorophyll was correlated with the respective increase in biomass productivity. In all treatments, the chlorophyll productivity reached a plateau from 0.4 to 0.6 μg ml^{-1} day^{-1} by day 10, indicating that chlorophyll synthesis or inhibition is not the most critical parameter for maximizing growth when dosing with the auxin, NAA.

Results from H. pluvialis

The freshwater green algae, *H. pluvialis*, showed positive responses to NAA with respect to its biomass and chlorophyll productivity over the 10-day growth period



(Fig. 2a, d). The first 5 days of growth showed no statistical difference compared to the control for all three treatments ($p<0.05$). The measured biomass productivity by day 10 showed more statistically significant differences between the treatments with NAA_{5ppm} and $NAA_{2.5ppm}$ compared to the control ($p<0.05$). The maximum increase observed in biomass productivity was from the NAA_{5ppm} treatment with a 117% (0.0308 g L^{-1} day^{-1}) increase over control (0.0142 g L^{-1} day^{-1}), which resulted in a final biomass density of 17%

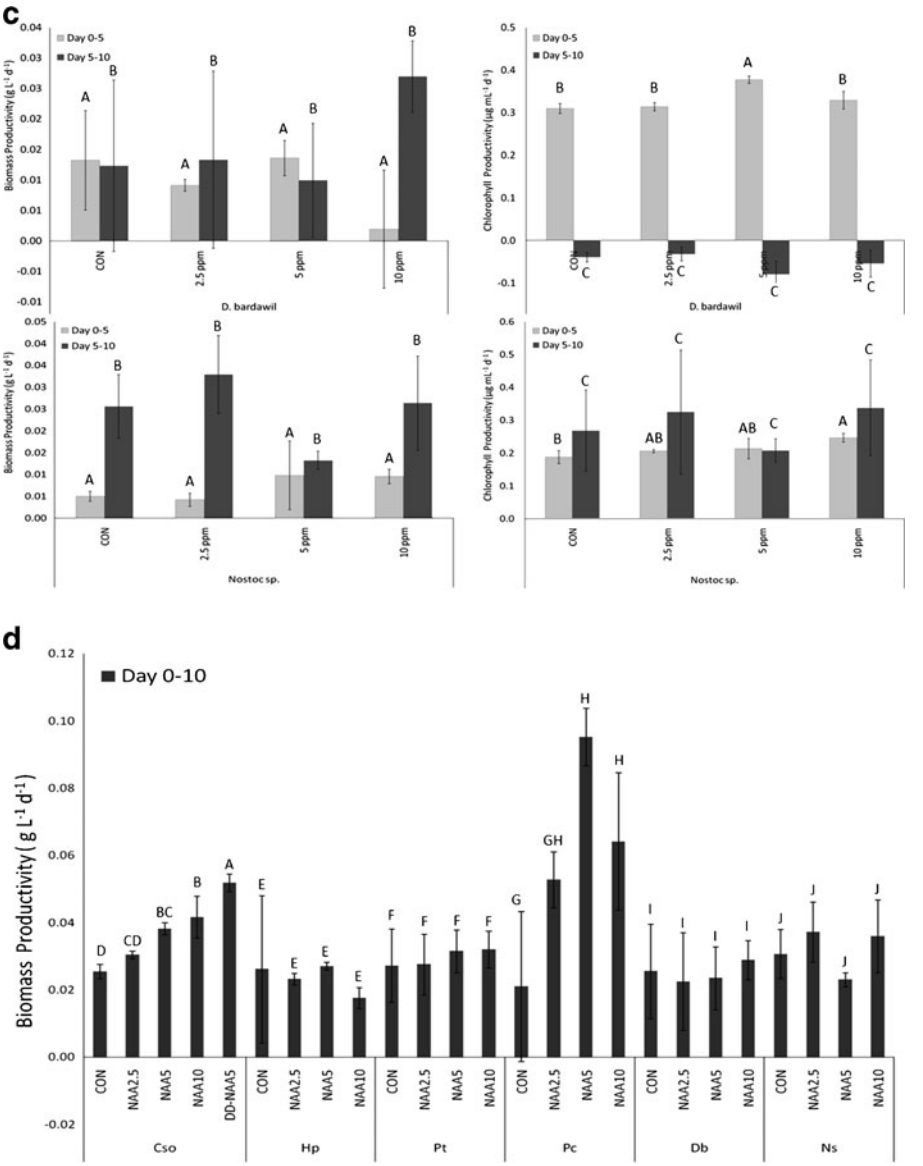


Fig. 2 (continued)

higher than control. The treatment $NAA_{2.5ppm}$ had a significant impact on growth by increasing biomass productivity by 85% (0.0263 g L^{-1} day^{-1}) compared to the control (0.0142 g L^{-1} day^{-1}). Higher concentrations of NAA (NAA_{10ppm}) did not produce further benefits and showed a tapering effect with a biomass productivity of 0.0203 g L^{-1} day^{-1} which was not statistically significant compared to control ($p<0.05$).

The impact on average chlorophyll productivity with *H. pluvialis* appeared to have a slight negative impact of 33% and 38% decrease compared to control on day 5 for the $NAA_{2.5ppm}$ and NAA_{10ppm}, respectively; however, these changes were not statistically

significant ($p<0.05$). The chlorophyll productivity was not inhibited as strongly for the NAA_{5ppm} treatment and was found to be similar to the control productivity despite the dramatic increase in biomass productivity observed by day 10. It was also noted that the color of most of the cultures treated with EtOH+NAA was different from that of the control, which appeared as a green-orange color, suggesting that this treatment may also induce stress factors which may accelerate the onset of astaxanthin production; however, more detailed studies are needed to confirm this effect.

Results from P. tricornutum

The diatom, *P. tricornutum*, exposed to EtOH+NAA showed only small variations in biomass productivity (Fig. 2b, d). The NAA_{10ppm} treatment had the largest impact over the first 5 days showing an increase in average biomass productivity of 225% over control, but was not statistically significant ($p<0.05$) because of high variability in this treatment and in the control. The biomass productivity data for the day-10 data showed that the treatments $NAA_{2.5ppm}$ and NAA_{5ppm} did not have a strong impact. However, it was observed that the NAA_{10ppm} produced an opposite effect past day 5 and measured an 81% (0.0036 g L^{-1} day^{-1}) decrease in biomass productivity compared to the control (0.0185 g L^{-1} day^{-1}; Fig. 2b). The high variability observed by the control affected any statistically significance in the results obtained ($p<0.05$).

The chlorophyll activity of *P. tricornutum* exhibited a slight depression in chlorophyll productivity for all NAA treatments, but these changes were small and not statistically significant ($p<0.05$). Although the NAA_{5ppm} treatment showed a marginal average decrease of 22% for day 5, the day-10 samples measured an increase in chlorophyll productivity compared to the control (0.032 μg ml^{-1} day^{-1}), but this increase was not statistically significant (Fig. 2b). This enhancement was not observed with the NAA_{10ppm} that showed a highly variable increase of 53% compared to control and was not statistically significant ($p<0.05$). In all *P. tricornutum* samples, it was noted that the chlorophyll productivity for day 5 was much higher than the day 10, suggesting that the cultures were able to synthesize enough chlorophyll in the early exponential phase to sustain continual growth through day 10.

Results from P. carterae

The coccolithophore, *P. carterae*, demonstrated a strong response to the EtOH+NAA treatments tested (Fig. 2b, d). For all treatments, the biomass productivity by day 5 was not significantly different than control ($p<0.05$) for the treatments $NAA_{2.5ppm}$, NAA_{5ppm}, and NAA_{10ppm}. However, by day 10, the treatments NAA_{5ppm} and NAA_{10ppm} were both significantly different from the control ($p<0.05$). The biomass productivity in the NAA treatments was dramatically increased by 293% (0.0454 g L^{-1} day^{-1}), 631% (0.0844 g L^{-1} day^{-1}), and 519% (0.0714 g L^{-1} day^{-1}) for $NAA_{2.5ppm}$, NAA_{5ppm}, and NAA_{10ppm}, respectively, compared to the control (0.0115 g L^{-1} day^{-1}). One reason that *P. carterae* had such extremely high increases in percent difference in productivities (293–631%) was because the control culture after day 5 decreased its biomass productivity by 59%, whereas the treated cultures continue to increase their biomass productivity by (28%, 117%, and 242%) for $NAA_{2.5ppm}$, NAA_{5ppm}, and NAA_{10ppm}, when comparing their day-10 to their day-5 biomass productivity values. The optimal dosage of the ones tested for *P. carterae* appears to be NAA_{5ppm} because as the dosage increases or decreases, the enhancement of biomass productivity decreases.

These significant increases in biomass productivity did not directly correlate to the chlorophyll productivities. The average chlorophyll productivity was enhanced sharply during the first 5 days of growth, providing 63% (0.294 μg ml^{-1} day^{-1}), 43% (0.258 μg ml^{-1} day^{-1}), and 98% (0.358 μg ml^{-1} day^{-1}) increases over control (0.180 μg ml^{-1} day^{-1}), but due to high variation in the control and $NAA_{2.5ppm}$, none of the treatments demonstrated a statistically significant difference ($p<0.05$). However, after the day-5 increase in chlorophyll productivity over control, the day-10 productivity values were similar to the control and not statistically significant.

Results from D. bardawil

The halo-tolerant green algae *D. bardawil* did not demonstrate a significant response to the treatments (Fig. 2c, d). The biomass productivity during the first 5 days was mostly inhibitory showing −31%, 3%, and −85% responses for $NAA_{2.5ppm}$, NAA_{5ppm}, and NAA_{10ppm}, respectively; however, these differences in the average biomass productivity were not statistically significant compared to the control ($p<0.05$). Interestingly, the most dramatic increase in biomass productivity was observed in the most inhibited samples of NAA_{10ppm}, which demonstrated a 119% (0.0270 g L^{-1} day^{-1}) increase compared to control (0.0132 g L^{-1} day^{-1}), but this difference did not meet the $p<0.05$ confidence level.

The response of EtOH+NAA to chlorophyll productivity showed little effect for $NAA_{2.5ppm}$, while the response for NAA_{5ppm} had a 22% increase over the control by day 5, which was statistically significant ($p<0.05$). The chlorophyll productivity of NAA_{5ppm} treatment dropped to −101% compared to control by day 10, but due to high variability, this was not statistically significant ($p<0.05$).

Results from Nostoc species

The wild isolated cyanobacteria, *Nostoc* sp., exhibited a response to the EtOH+NAA treatments examined (Fig. 2c, d). The treatment $NAA_{2.5ppm}$ had only a marginal effect on the average biomass productivity; however, the NAA_{5ppm} treatment was found to initially boost the growth by 96%, although these samples showed a high variability and were not statistically significant ($p<0.05$). The biomass productivity of the treatment NAA_{10ppm} showed an early increase of 90% (0.0095 g L^{-1} day^{-1}) by day 5 compared to control (0.0050 g L^{-1} day^{-1}); however, that enhancement is reversed as well by day 10 which was measured to have the same biomass productivity as the control. Despite the differences in biomass productivities for day 5 and 10, none of these treatments were found to be statistically significant ($p<0.05$).

Conclusions

The treatment of microalgae with NAA requires the use of a solvent to dissolve the NAA powder for dosing into water-based media. In this work, we compared ethanol and methanol as the two solvents in controlled experiments. It was found that for *C. sorokiniana*, ethanol was a better solvent choice than methanol, which caused inhibition over the 10 days of growth. The application of NAA at a concentration of 5 ppm with ethanol was found to sustain the accelerated biomass productivity through day 10 and was able to enhance productivity over twofold. Although it was observed that NAA_{10ppm} resulted in slightly higher biomass productivity, this treatment doubled the amount of

NAA applied for only a marginal increase in biomass productivity; thus, we conclude that the NAA_{5ppm} may be the preferred option for stimulating a growth response in *C. sorokiniana*. Furthermore, the delayed dosage of NAA_{5ppm} delivered at the fifth day of normal growth exhibited the largest impact on biomass productivity, suggesting that the timing and amount of dosage are critical parameters. Although it was determined that the combination of ethanol and NAA was ideal for *C. sorokiniana*, the response in the mixed consortia to treatments containing ethanol was observed to have an early boost in biomass productivity, but diminishing by day 10 and showing little enhancement over the control. The treatments tested in this study did not demonstrate substantial enhancements for the mixed consortia, and future studies can investigate a reduction in the solvent concentration, which appeared to be inhibitory, while also testing different ranges of phytohormones such as NAA.

The multispecies experiment showed that half of the cultures tested responded favorably to the treatment of ethanol and NAA at the dosages tested. The top responder was *P. carterae* which demonstrated significant increases in biomass productivity compared to the control and had the highest final day density of any species tested. The next species that responded best was *C. sorokiniana*, which demonstrated that even higher productivity values can be attained if the dosage is given at a delayed interval (NAA addition on the fifth day only). The delayed dosage aspect needs to be investigated further to determine what factors are involved in inducing the largest increase in biomass productivity, such as cell density, growth phase, etc. The third species that responded well to the treatment, *H. pluvialis*, has great potential for enhanced productivity and lowered costs associated with its cultivation for the high value pigment, astaxanthin and omega-3 fatty acids. Although the increase of 17% over the control is relatively small compared to the two other top performers, future research can optimize the dosage and timing for even greater impact. If the effect is scalable and adopted in commercial production, then it could result in a substantial improvement for cultivation of this species. The impact of ethanol and NAA on the other three species, *P. tricornutum*, *D. bardawil*, and *Nostoc* sp., did not show a statistically significant response to consider these dosages for future applications.

The results from this research show that combining ethanol with NAA is a viable approach for enhancing biomass productivity in diverse species of microalgae; however, future studies with other species of algae not tested are needed to evaluate their response to this treatment.

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