

**The Need for Balance: The Impact of Nitrogenous Waste from Deposit Feeders on the  
Primary Productivity of Microalgae on Heron Island**

**Jayne Campbell**

**University of Queensland**

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## **ABSTRACT**

**Context:** Microalgae are beneficial to coral reef ecosystems as long as their abundance is balanced out by the ecosystem. Deposit feeders recycle nutrients in these ecosystems, which impacts the conditions for microalgal growth and their primary productivity.

**Aims:** We provide an investigation of the importance of species that recycle nutrients to the health of Heron Island's coral reef ecosystems and their effects on primary production by microalgal assemblages.

**Methods:** Flow-through tanks were used to determine how the influx of nitrogenous waste from the deposit feeder species, *Holothuria atra*, affected the growth of microalgae by measuring their photosynthetic rate in terms of oxygen production and cell density.

**Key results:** There was a positive linear correlation between the levels of primary production of microalgae and the number of days exposed to the waste of deposit feeders based on changes in oxygen production levels and cell density of microalgae.

**Conclusions:** The addition of nitrogenous waste by *Holothuria atra* influenced the primary productivity of benthic microalgae. Different adaptations of various microalgae resulted in certain genera having higher proportions than others when exposed to nitrogenous waste.

**Implications:** Sea cucumbers must be protected from overfishing to prevent the balance of nutrients in coral reef ecosystems from being disturbed.

**Keywords:** *Holothuria atra*, microalgae, nitrogenous waste, primary productivity, oxygen production, haemacytometer, Heron Island, Great Barrier Reef

## INTRODUCTION

Coral reef ecosystems are acclaimed for their high biodiversity and productivity, which allows them to house 35,000 to 60,000 different species of marine plants and animals (Hoegh-Guldberg *et al.* 2017, El-Naggar 2020). This attribute contributes to their ability to create ecological services, including providing food and habitats for an extensive range of marine organisms (Hoegh-Guldberg *et al.* 2017, Coker *et al.* 2013). These ecosystems are typically dominated by scleractinian corals, which are considered the primary habitat-forming organisms and influence the reef fish assemblages and communities (Stoddart 1969, Coker *et al.* 2013). The physical structure of coral reefs is known for its complexity since the stony (scleractinian) corals provide refuge areas and form specific niches that help moderate levels of both competition and predation (Graham and Nash 2012, Coker *et al.* 2013). This trait directly results in high diversity and abundance of reef fishes (Luckhurst and Luckhurst 1978).

The high degrees of reliance on corals by reef fish means that when corals die, negative consequences for biodiversity and production travel up trophic webs beginning with grazer fish species (Jones *et al.* 2004, Coker *et al.* 2013). Worldwide, corals have declined in response to the impacts of living during the Anthropocene; the current time period characterized by humans directly and indirectly affecting ecosystems through their activities and being a force of change (Jones *et al.* 2004, Williams *et al.* 2019, Polapa *et al.* 2021). Humans directly impact coral reefs negatively through pollution and overfishing, which decreases biodiversity and production while upsetting the balance of nutrients (El-Naggar 2020). Indirect effects include anthropogenic climate change, mainly from carbon dioxide emissions, which leads to ocean acidification and rising temperatures (Polapa *et al.* 2021). These issues upset the natural balance of nutrients and lead to coral bleaching events. Corals expel the symbiotic microalgae that live within them,

known as zooxanthellae, and consequently lose color and turn white (Smith *et al.* 2004). In addition, pollution can cause eutrophication, which increases phytoplankton biomass and the nutrient load in the water column. This problem makes it even more difficult for corals to survive because their zooxanthellae's ability to photosynthesize becomes compromised (D'Angelo and Wiedenmann 2014). The zooxanthellae create high rates of primary production in coral reefs, but they are not the only group of important microalgae in this regard (Heil *et al.* 2004).

Benthic microalgae exist at the interface between sediment and water in the top few millimeters of sediment due to most sediments having shallow photic depths (Underwood 2001). Their omnipresent distribution and role in nutrient recycling demonstrates how relevant their contribution is to the primary productivity of coral reef ecosystems (Uthicke and Klumpp 1998, Heil *et al.* 2004, Hopes and Mock 2015). Benthic microalgae assemblages are known as microphytobenthos and tend to be diverse, consisting mainly of diatoms in addition to cyanobacteria, flagellates, and green algae (Uthicke and Klumpp 1998, Heil *et al.* 2004). These assemblages can live within reef sediments in various environments, such as being attached to sand grains, macroalgae, or vertically migrating through sediments (Heil *et al.* 2004). Due to their diverse habitats, these communities serve many ecological functions, including carbon fixation, where they produce organic molecules that enter the trophic web through consumption by primary consumers such as sediment feeders (Uthicke and Klumpp 1998, Heil *et al.* 2004, Gupta 2023). Understanding the contribution of benthic microalgae to primary productivity is important because photosynthesis from algae makes up more than half of the net global primary productivity and supplies aquatic food webs (Chen *et al.* 2021).

Microalgae directly benefit the corals in the Great Barrier Reef by providing them with additional nutrients in the form of nitrogen and carbon (Uthicke and Klumpp 1998). Corals also

indirectly benefit from microalgae due to their relationship with meiofauna (Buffan-Dabau and Carman 2000). The meiofauna consume benthic microalgae when conducting diurnal vertical migrations, which involve surfacing at night, and then the corals take in the meiofauna (Buffan-Dabau and Carman 2000). These benefits for coral are based on there being a certain nutrient balance combined with top-down control by grazers, so in certain circumstances, microalgae can also have negative effects on coral reefs (McManus and Polsenberg 2004, Adam *et al.* 2022). An overabundance of algae can limit coral recruitment and growth by taking over available space and blocking corals' ability to photosynthesize (McManus *et al.* 2018). Another issue is the phenomenon known as “coral-algal phase shift,” which refers to the decline in the biodiversity and ecosystem services of coral reefs due to a decrease in coral cover and an increase in algae (McManus and Polsenberg 2004). An overabundance of algae can also shallow the redox layer (Hallmann *et al.* 2021).

Deposit feeders are essential to coral reefs for many reasons, including their ability to counteract the shallowing of the redox layer by benthic microalgae through bioturbation (MacTavish *et al.* 2012, Williamson 2021). Marine ecosystems benefit from this bioturbation because when sediments are shifted, food and nutrients are redistributed (MacTavish *et al.* 2012). Deposit feeders also introduce nutrients by producing nitrogenous waste after consuming sediment, which stimulates benthic microalgae productivity (MacTavish *et al.* 2012). Research has shown that benthic microalgae increased their productivity, measured by oxygen production, after being exposed to water from tanks with holothurians (Uthicke 2001a). Uthicke's research also indicated that this was due to the excretion of ammonium from holothurian's nitrogenous waste (Uthicke 2001a). To explore the relationship between deposit feeders and microalgae in the context of the Great Barrier Reef, we will be studying the species *Holothuria astra* on Heron

Island. Heron Island is known to have a high biomass of benthic microalgae and a large population of *Holothuria atra* (Roelfsema *et al.* 2002, Heil *et al.* 2004). We hypothesize that the influx of nitrogenous waste produced by the *Holothuria atra* species of sea cucumber will have an effect on the primary productivity of microalgae in terms of its cell density and oxygen production.

## MATERIALS AND METHODS:

This research study was conducted over a period of five days where the dependent variable, the productivity of microalgae, was measured in terms of biomass growth and photosynthetic rate. This was done by conducting cell density counts and examining oxygen production respectively (Nguyen *et al.* 2022). A control and experimental group were used to determine how the presence of sea cucumbers and their production of nitrogenous waste affected microalgae production. Flow tanks were used to keep the sea cucumbers and sediment tanks separated from the ones with the microalgae as well as control the amount of water that entered the tanks. Before the study commenced, two sets of flow tanks were set up for the control and the experimental group. This consisted of a 71 L tank connected to a 27 L tank by a clear tube that allowed seawater to constantly flow from the larger tank into the smaller one. The seawater filter was adjusted so that water poured into both tanks at a flow velocity of approximately 800 mL per minute.

After this initial setup was completed, the microalgae samples were collected at low tide from an area adjacent to North Beach. This was done by scooping microalgae off the top of sediment into a container with holes on the sides that allowed water to flow out when it was lifted out of the water. The amount of sediment taken with it was limited as much as possible. The sediment was relatively coarse and the microalgae was a brown-tinted green color and

visible throughout the sediment. This mixture was transferred to a tray (42 cm by 29 cm) and 500 mL of filtered seawater from the research station was added to homogenize it. The tray was monitored for bubble production to ensure there was photosynthetic activity (Uthicke 2001a). To set up the individual microalgae samples, eight shallow glass dishes were labeled 1C-4C and 1E-4E for the control and experimental groups. Each dish was filled with 75 mL of the microalgae sediment from the tray and each set of four was placed in the smaller tanks. The remaining microalgae sediment was set aside to be analyzed later using the haemacytometer.

To finish prepping the experiment, sea cucumbers were obtained at low tide during a reef walk in the same area as the microalgae were collected. The specimens obtained were of the species *Holothuria atra* and close to 17 cm in length since that had been documented as their average size on Heron Island (Table 1, Buccheri 2019). The number of specimens collected was based on research methods used by Sven Uthicke who recommended 1.7 sea cucumbers per one meter squared (Uthicke 2001a). Four specimens were collected to replicate this method for a high density. The specimens were treated with caution to prevent unnecessary stress that could result in injury or in the worst case, evisceration (Okada and Kondo 2019). 30 L of sediment was obtained from around the same area as the sea cucumbers were collected to ensure the sediment was ideal for them to consume. This was done by scooping the top five mm of sediment with a small shovel and transferring it into a bucket. The sediment was then evenly distributed into the two large tanks and allowed to settle before the sea cucumbers were placed in.

### **Haemacytometer Procedure**

The final procedure before beginning the experiment was to collect approximately four mL of microalgae and seawater from the extra microalgae sediment in the tray and transfer it into a five mL beaker. After the microalgae settled to the bottom of the beaker, approximately three

mL of seawater was removed using a disposable pipette in order to concentrate the sample. The pipette was used again to resuspend the microalgae and create a homogenous mixture. The proper procedure would indicate adding 10 drops of 1-2% Lugol's solution (100g KI dissolved in 1 L distilled water with 50 g crystalline iodine added to 100 mL anhydrous acetic acid) per 200 mL of suspended microalgae in order to fix motile cells in solution (ANACC 2020a). Due to the restraints on chemical use at the Heron Island Research Station, this exact procedure could not be done thus certain estimates were made.

The haemacytometer and coverglass slips were then sterilized with 80% ethanol and dried. A cover glass slip was placed over the haemacytometer and the chamber was filled by pipetting 10 µL of the microalgae sample at a 45-degree angle (20 µL pipette) (ANACC 2020a). Capillary action was allowed to draw the solution into the chamber as it filled completely. This was done quickly so cells did not settle in the pipette before going into the grids (ANACC 2020a). The haemacytometer was then visualized on an Olympus CX23 compound microscope. At 100x magnification, a photo was taken of the center large square (1mm x 1mm) and the number of large motile microalgal cells was counted from that photo (Fig. 1). This was done to increase accuracy since we could not fix the motile cells. Then, at 400x magnification, the number of cells was recorded in each of the 25 medium squares (0.23mm x 0.23 mm) (Fig. 2).

Classification diagrams were referred to during this process to determine the genera of each microalgal cell (Fig. 3, Fig. 4, Fig. 5, Fig. 6). Cells were only counted if they were within the square or touched the top or left edge of the square (Janta et al. 2013, ANACC 2020b). The main distinguishing factors were green pigmentation and distinct perimeters ( Janta et al. 2013). There were many “potential” microalgae of extremely minuscule size, but we were unable to count them while maintaining accuracy. This was due to the limitation of not being able to zoom

past 400x. The cell counts were added to the counts of the large motile microalgae cells from earlier to determine the approximate cell count for the center squares. Analyses were recorded for all four grids of the two haemacytometer plates (Fig. 7). The cell counts for each were averaged since the experimental group had not yet been exposed to nutrient supplementation. The average cell count was multiplied by the conversion factor for the Bright-line hemacytometer (10000) and the dilution ratio (1). Images of the different microalgae genera were taken through the microscope. Three collections of images were made to visualize the microalgae assemblages for day zero, day five control group, and day five experimental group (Fig. 8, Fig. 9, Fig. 10).

The haemacytometer procedure from day one was repeated on day 5 and the collection of images was utilized to speed up genera identification. The difference was that the final average cell counts were determined for the control and experimental microalgae samples separately based on four cell counts per sample. This resulted in two values for the cell densities that represented the control and experimental groups (Table 2). A table of the fifteen genera observed was created based on the cell counts for each during day zero, the day five control group, and the day five experimental group (Table 2). A stacked column chart was created to visualize this data and view the proportions of genera (Fig. 11).

### Oxygen Production

In order to test the photosynthetic rate of the microalgae, oxygen production was tested each day. Only one set of microalgae samples was measured each day because the research station only had two glass funnels. The oxygen-capturing apparatus consisted of a 10 cm in diameter inverted funnel attached to a 10 mL falcon tube (Fig. 12). On day one, the two apparatuses were submerged and secured over dishes 1E and 1C. Over time, oxygen production displaced water at the top of the falcon tube. After six hours, the volume of oxygen in the top of

the falcon tube was measured and then the apparatuses were removed to allow microalgae growth to continue undisturbed. This procedure was repeated for days two through five by testing oxygen production on samples 2E and 2C, 3E and 3C, 4E and 4C, and 1E and 1C respectively. Day five was the final oxygen production analysis where the same samples from the first day were measured again. This data was visualized by creating a scatterplot and linear regressions for the control and experimental data (Fig. 13).

## RESULTS

### Haemacytometer Cell Analysis

For the beginning of the experiment, the average cell count for all four grids was calculated to be 44.25 cells per  $1\text{ mm}^2$  and the cell density was found to be  $4.425 \times 10^5$  cells/mL. This fell within the ideal density range for the Bright-Line haemacytometer and represents both samples (ANACC 2020). The average cell counts and microalgal cell densities were much larger on day five than on day zero for both groups (Table 2). The cell density was also larger for the experimental group than for the control group on day five. Over the course of the study, the control cell density increased by 4.24 times and the experimental cell density by 5.38 times.

### Genera Cell Counts

A stacked column chart was created to show the proportions of microalgae genera observed on day zero, day five for the control group, and day five for the experimental group (Fig. 11). The figure showed that *Chlorella spp.* was consistently the most abundant genus throughout the experiment.

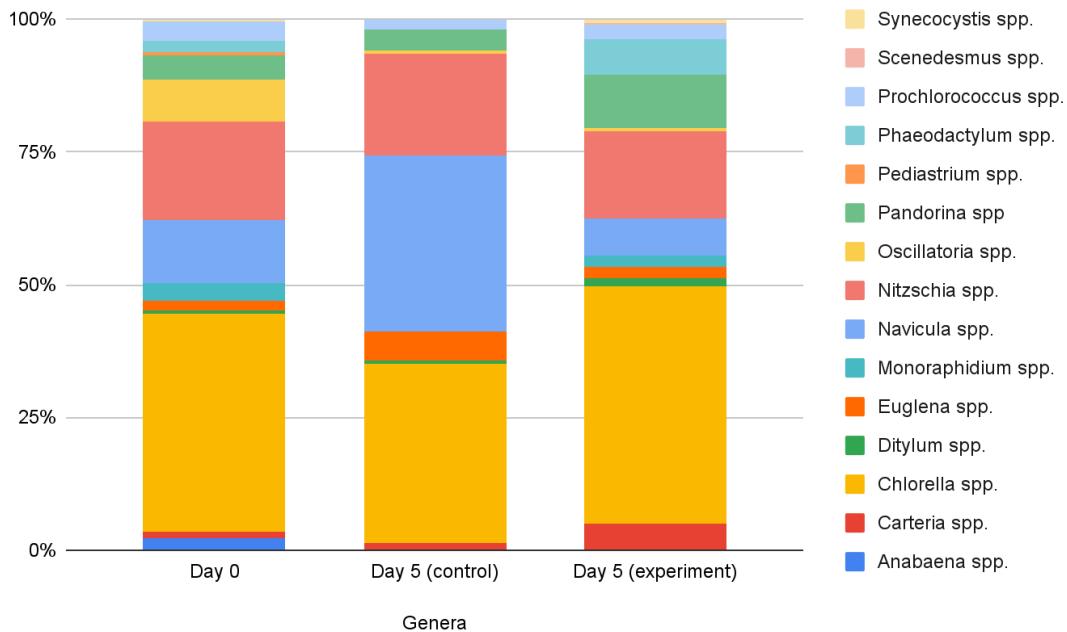


Figure 11: Stacked column chart comparing the proportions of each microalgal genera observed with the haemacytometer initially and on day five for control and experimental groups.

### Oxygen Production

On day one, the volume of oxygen production measured for samples 1E and 1C was the same. This was logical since the experimental group had not had prolonged exposure to nutrient supplementation yet. On days two and three, the oxygen production was higher for the experimental group and the same for both groups on the fourth day. The oxygen production was much higher for the experimental group on the fifth day. A scatterplot was created for this data with lines of best fit.

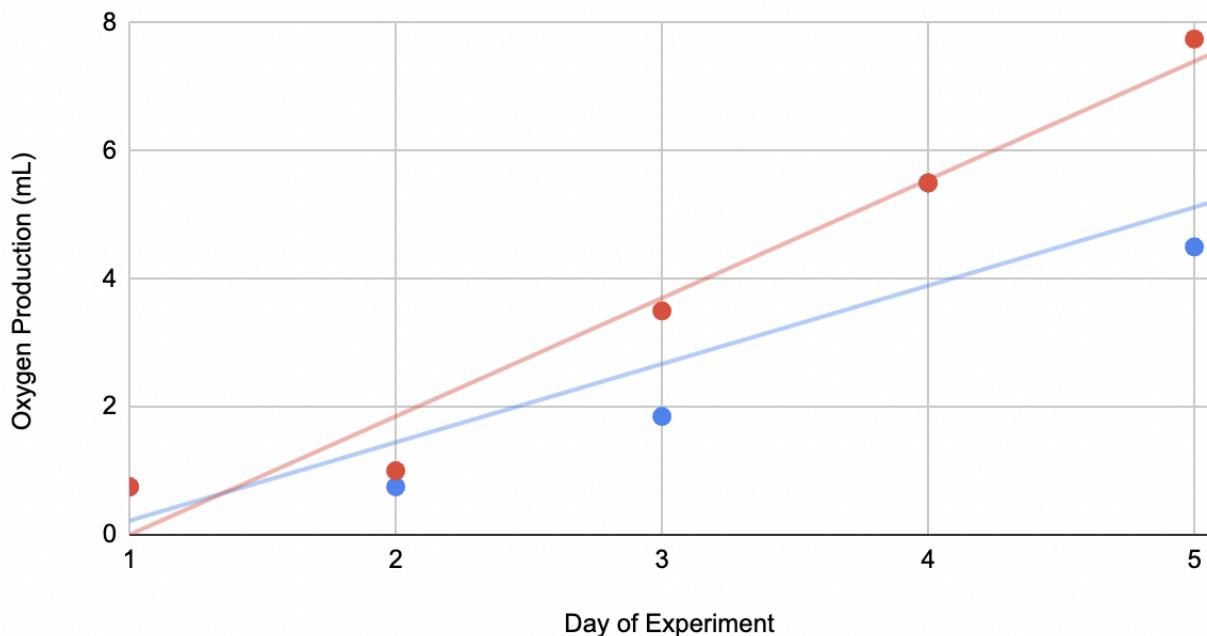


Figure 13: Scatterplot showing oxygen production for control [blue] and experimental [red] groups over five days with linear regressions. The equation of the control trendline is  $y = 1.23x - 1.01$  with  $R^2 = 0.773$ . The equation of the experimental trendline is  $y = 1.85x - 1.85$  with  $R^2 = 0.959$ .

## DISCUSSION

The haemacytometer cell analysis proved that the microalgal cell density increased over the course of the five days with and without the addition of nitrogenous waste from the sea cucumbers. It is likely that the nitrogenous waste helped the microalgae grow because the average cells and density were higher for the experimental group than for the control group (Table 2). The proportions of microalgae genera observed were also different for the control and experimental groups, which suggests that the nitrogenous waste created conditions that favored certain genera more than others.

The control and experimental groups also differed in terms of oxygen production (Fig. 12). Based on the high  $R^2$  value, the linear regression for the experimental group showed a

strong, direct, positive, and linear correlation between the days of the experiment and oxygen production in mL. This suggests that the addition of nitrogenous waste by deposit feeders had a direct effect on oxygen production and consequently on primary productivity. For the control group linear regression, there was a low R<sup>2</sup> value indicating a weak positive correlation making it unclear which factors that affected primary productivity were the most important.

The cell density and oxygen production both measured primary productivity since the microalgae produced oxygen as they photosynthesized, which allowed this study to have two ways of understanding the effects on microalgal growth. Taken together, the cell density and oxygen production data supported the hypothesis that the influx of nitrogenous waste would have an impact on the primary productivity of microalgae. To further understand the effects of the nitrogenous waste, it was relevant to examine the changes in the proportions of the microalgae genera at the beginning and end of the study.

The stacked column chart highlighted which microalgae genera dominated during each part of the study (Fig. 12). *Chlorella spp.* had a very high growth rate and was consistently the most abundant genera recorded. This data aligns with findings from research on this genus that discuss its rapid growth and common widespread distribution (Masojídek and Torzillo 2008, Najm *et al.* 2017). The fast growth can be attributed to this genus's mode of asexual reproduction through autosporulation where four daughter cells form inside the parent's cell wall (Safi *et al.* 2014). In terms of proportions, *Chlorella spp.* did decrease from 41.1% to 33.9% in the control while it increased from 41.1% to 44.9% in the experimental group. The increase in proportions for the experimental group signifies that this genus benefited from exposure to the nitrogenous waste and thrived more with it than without. However, its dominance overall suggested that this genus is well adapted and can thrive regardless of exposure to nutrients from the sea cucumber

waste. This could be partly due to its resistant cell walls; a trait that has helped them to be dominant and common microalgae (Safi et al. 2014).

The next most abundant genera was *Nitzschia spp.* This supports past research that has shown these microalgae are r-selected diatoms, which means they have fast growth rates and high cell yields (Loureiro et al. 2008). This genus was less abundant than *Navocula spp.* for the control group, suggesting that it thrived better in the higher nutrient environment. A unique feature of this genus is that it contains many potentially toxic species with the ability to form harmful algal blooms (Loureiro et al. 2008). In certain instances, these blooms result in the production of domoic acid, which is a known neurological toxin (Fryxell et al. 1997).

The third most abundant genus, *Navicula spp.*, are also diatoms, but thrived more in the control group. These two genera likely have different ideal conditions with *Nitzschia spp.* preferring higher nutrient levels. Their high proportions support the fact that diatoms are very common in aquatic environments. Marine diatoms like those in these two genera are known as extremely important carbon dioxide fixers and contribute heavily to carbon fixation (Falciatore et al. 2019, Li and Zhang 2021). Microalgae overall are extremely important generators of primary productivity and vital to the stability of trophic food webs (Uthicke and Klumpp 1998, Heil et al. 2004, Hopes and Mock 2015). Research on microalgae has shown that productivity in terms of oxygen production increases when more ammonia is available (Najm et al. 2017). This is because microalgae utilize inorganic nutrients to conduct photosynthesis, which includes those made available by sea cucumbers in their nitrogenous waste (Moriarty 1982, Uthicke 2001b, Vidal-Ramirez and Dove 2016, Najm et al. 2017).

While the results indicated that the nitrogenous waste increased the primary productivity of microalgae, there were several limitations that should have been avoided. The most evident

being that only one microalgae sample was measured from each group each day because the research station only had two clear funnels. Ideally, the oxygen production of all eight samples would have been measured every day because in order to conduct statistical tests and determine significance, more data points are needed (Schwerna *et al.* 2016). The other main limitation of this study was the fact that it was very difficult to replicate the natural environment for the microalgae since coral reefs are very complex ecosystems. Future research should attempt to better mirror the conditions of coral reef ecosystems while also conducting more replicates to reduce error and increase the statistical significance of the results.

The impact of deposit feeders on the primary productivity of microalgae in the Great Barrier Reef is a topic worth more exploration because the relationship is very important and delicate. Sea cucumbers help to maintain the balance needed in coral reefs through bioturbation, which counteracts the shallowing of the redox layer by benthic microalgae (MacTavish *et al.* 2012, Hallmann 2021). This balance optimizes biodiversity while the sea cucumbers also help primary productivity by expelling nitrogenous waste. Without these benefits, there is a danger of either too little algae which could result in a decrease in primary productivity, or too much, which could lead to a decrease in coral cover and a decline in biodiversity (McManus and Polsonberg 2004). Sea cucumber species have experienced reductions in both diversity and density, which can often be attributed to factors resulting from the influence of humans including climate change, development, and overfishing. For the health of the Great Barrier Reef, future research must be done to determine how to best protect sea cucumber populations and maintain the important relationships between them and benthic microalgae and the rest of the reef.

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## APPENDIX

Table 1: Sizes of the sea cucumbers collected in terms of length and width and the average lengths and widths

<i>Holothurian atra</i>	Length (cm)	Width (cm)
1	20.6	4.3
2	18.1	6.0
3	21.5	6.1
4	19.9	5.8
Average:	20.0	5.6

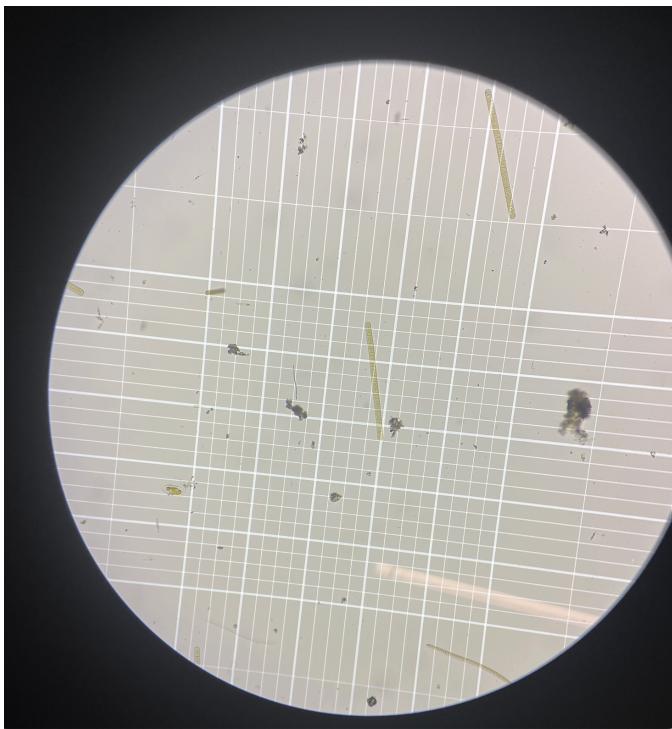
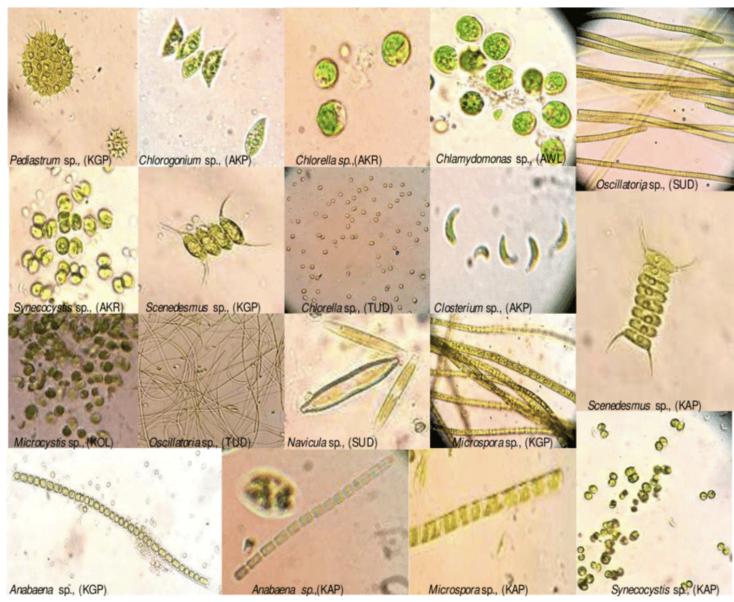


Figure 1: Image of microscope view showing large center square (1 mm by 1mm)

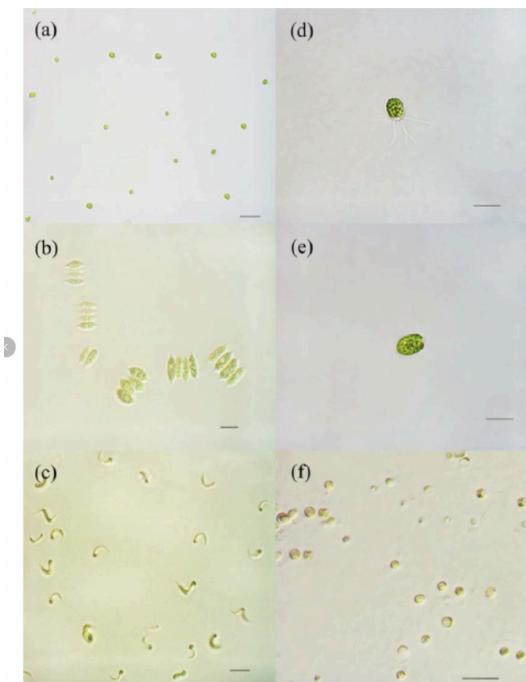


Figure 2: Image showing examples of microalgae genera



Microscopic images of microalgae isolated from the different water samples

Figure 3: Image used to identify microalgae genera (Ayele *et al.* 2019)



The shape of all microalgae under compound microscope (a) Chlorella sp. AARL G008 (b) Scenedesmus sp. AARL G022 (c) Monoraphidium sp. AARL G044 (d) Carteria sp. AARL G045 (e) Carteria sp. AARL G046 (f) Nannochloropsis limnetica SAG 18-99. Scale bar = 10  $\mu$ m. 2.2 Growth of Native Thai Strains soxhlet extraction [11]. The growth and lipid

Figure 4: Image used to identify microalgae genera (Janta et al. 2013)

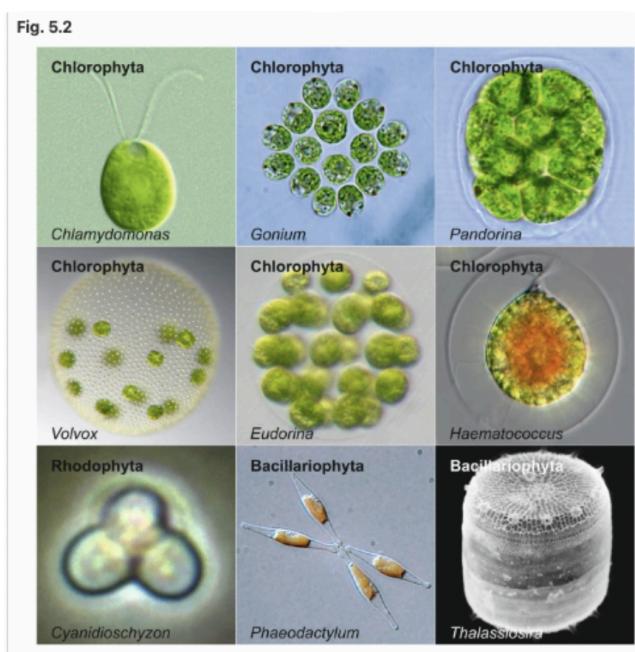


Figure 5: Image used to identify microalgae genera (Hallmann 2020)



Figure 6: Image used to identify microalgae genera (Institute of Oceanology)

## Day 0 Sample Cell Count

Age; 0 days

<b>1</b>	2	1	4	3	3	<b>2</b>	3	1	0	2	2
4	3	1	1	0			3	1	1	0	1
2	2	2	2	5			4	3	0	2	1
2	2	1	0	0			4	3	1	2	1
2	0	2	1	1			1	1	0	0	1
Large motile: 2						Large motile: 2					
<b>Total no. = 48</b>						<b>Total no. = 40</b>					
<b>3</b>	2	0	2	0	1	<b>4</b>	2	0	2	0	1
3	0	6	5	6			3	0	6	5	6
1	4	2	2	0			1	4	2	2	0
0	2	1	0	0			0	2	1	0	0
0	2	1	2	2			0	2	1	2	2
Large motile: 0						Large motile: 3					
<b>Total no. = 44</b>						<b>Total no. = 45</b>					

Figure 7: Grid used to count microalgae cells for day zero

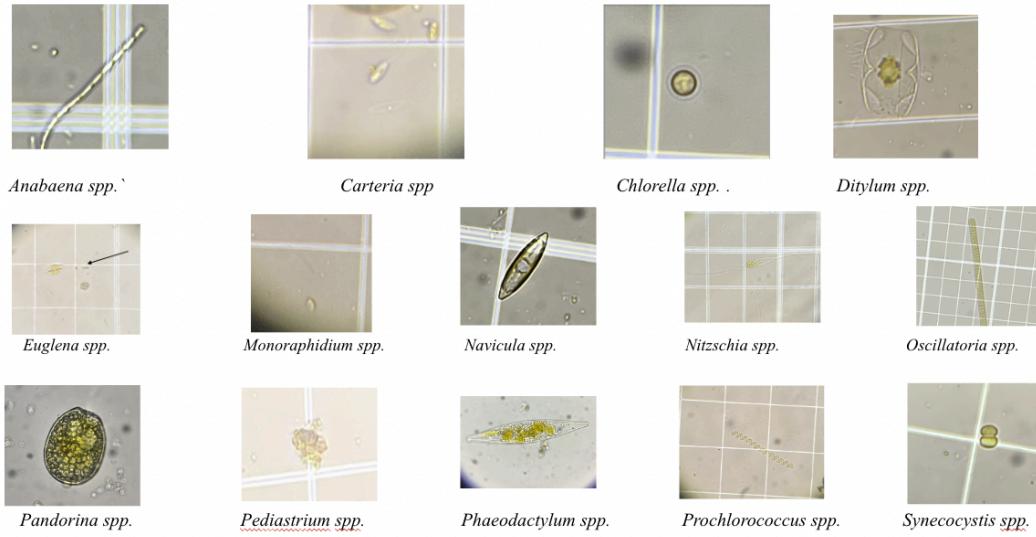


Figure 8: Collection of images taken through the microscope of the different microalgae genera labeled based on the classifications made when counting cells on day zero

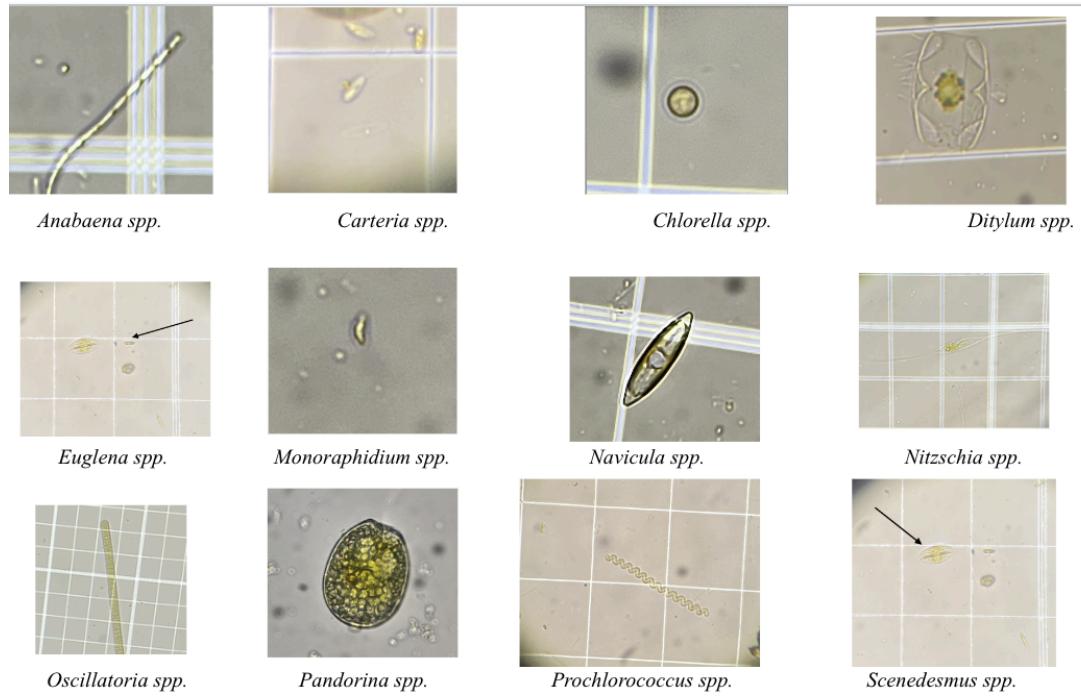


Figure 9: Collection of images taken through the microscope of the different microalgae genera labeled based on the classifications made when counting cells on day five for control group

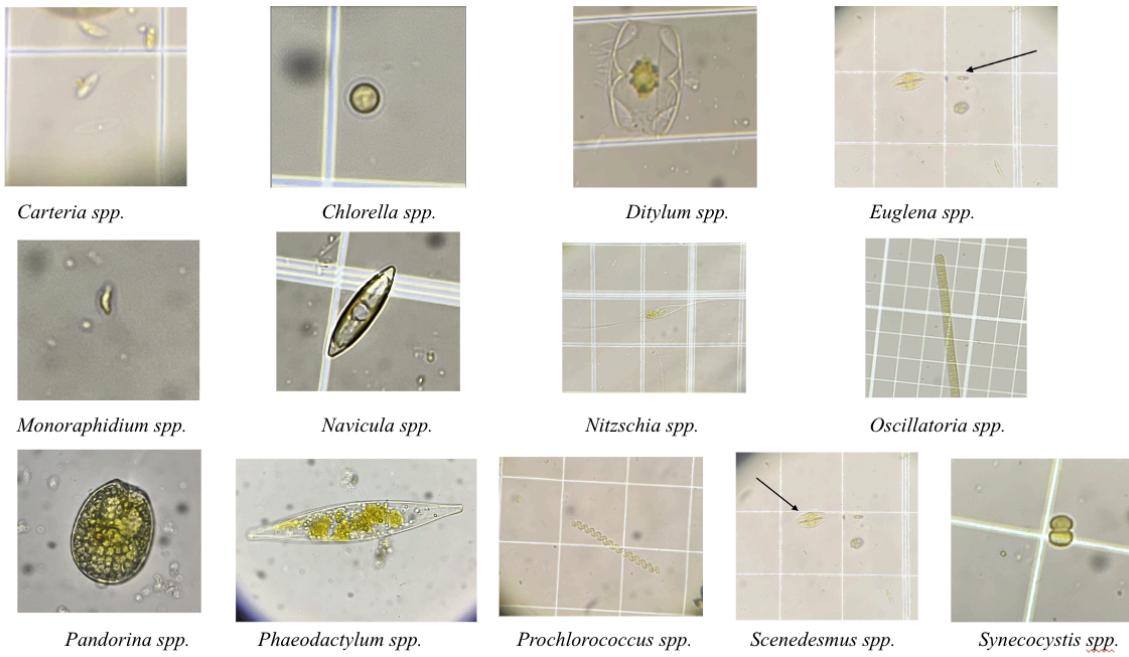


Figure 10: Collection of images taken through the microscope of the different microalgae genera labeled based on the classifications made when counting cells on day five for experimental group

Table 2: Counts of microalgae genera overall initially and finally for control and experimental groups

Genera	Day 0	Day 5 (control)	Day 5 (experiment)
<i>Anabaena</i> spp.	4	1	0
<i>Carteria</i> spp.	2	8	48
<i>Chlorella</i> spp.	72	250	438
<i>Ditylum</i> spp.	1	5	14
<i>Euglena</i> spp.	3	40	22
<i>Monoraphidium</i> spp.	6	1	20
<i>Navicula</i> spp.	21	244	67
<i>Nitzschia</i> spp.	32	140	161
<i>Oscillatoria</i> spp.	14	6	7

Genera	Day 0	Day 5 (control)	Day 5 (experiment)
<i>Anabaena spp.</i>	4	1	0
<i>Pandorina spp</i>	8	29	97
<i>Pediastrum spp.</i>	1	0	0
<i>Phaeodactylum spp.</i>	4	0	65
<i>Prochlorococcus spp.</i>	6	13	28
<i>Scenedesmus spp.</i>	0	1	3
<i>Synechocystis spp.</i>	1	0	6

Table 3: Haemocytometer cell analysis in terms of average cells per 1 mm<sup>2</sup> and calculated microalgal cell density calculated by multiplying the average cells by the Bright-line conversion factor

Haemocytometer Cell Analysis		
	Average cells (cells/1 mm <sup>2</sup> )	Microalgal cell density (cells/mL)
Day 0	44.25	442500
Day 5 (control)	183	1830000
Day 5 (experimental)	238	2380000

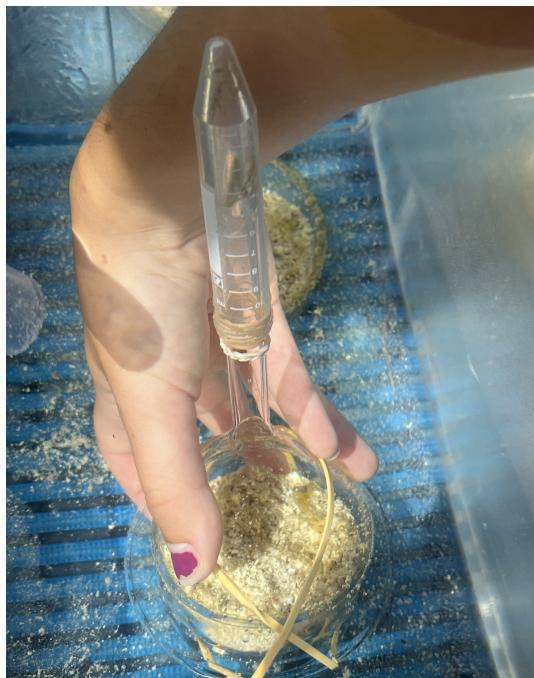


Figure 12: Image of the oxygen-capturing apparatus consisted of a 10 cm in diameter inverted funnel attached to a 10 mL falcon tube