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Biochemical composition of the brown tide causative species *Aureococcus anophagefferens* cultivated in different nitrogen sources*

Jian GAO[1, #], Yuelei DONG[1, 3, #], Xiaoyu ZHOU[1], Lei CUI[1, 3], Songhui LÜ[1, 2, 3, **]

[1] *Research Center of Harmful Algae and Marine Biology, College of Life Science and Technology, Jinan University, Guangzhou 510632, China*

[2] *Southern Marine Science and Engineering Guangdong Laboratory, Zhuhai 519000, China*

[3] *Key Laboratory of Eutrophication and Red Tide Prevention of Guangdong Higher Education Institutes, Jinan University, Guangzhou 510632, China*

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Abstract A large-scale algal bloom, caused by *Aureococcus anophagefferens*, has plagued the coastal embayment of Qinhuangdao, China since 2009. The bay scallop agriculture industry in this area has been adversely affected. Researchers claimed that the poor nutritional value of brown tide cells might be responsible for the detrimental effects on bivalve mollusks. To verify whether brown tide cells are nutritionally inadequate food sources, the biochemical composition (total extractable lipids, amino acids, fatty acids, and monomeric carbohydrates) of the Chinese strain *A. anophagefferens* was determined during the late logarithmic growth phase when culturing in different nitrogen sources (nitrate, urea and nitrate-urea mixture). Cells cultured in nitrate contained 39.12% protein, 21.99% total extractable lipid, 10.25% total carbohydrates, and a relatively high amount of polyunsaturated fatty acid (PUFA) (51.98%, percentage of total fatty acids), including eicosapentaenoic acid (EPA) (4.81%) and docosahexaenoic acid (DHA) (14.56%). The gross biochemical composition and PUFA content in *A. anophagefferens* in nitrate cultivation are comparable with values found in the literature of frequently used species in bivalve feeding. Nine monomeric carbohydrates were significantly reduced when cultivated in urea and nitrate-urea mixture (P<0.05). The DHA, EPA, and PUFA contents significantly decreased when cultivated in urea (P<0.05). Although the nutritional value of *A. anophagefferens* dropped when cultured in urea, it is still comparable with certain favorably used algal species in bivalve feeding (i.e., *Skeletonema costatum*), indicating that *A. anophagefferens* is not a nutritionally inadequate food source.

Keyword: brown tide; *Aureococcus anophagefferens*; fatty acid; carbohydrate; nutritional value

1 INTRODUCTION

Summer algal blooms, caused by the rapid growth and proliferation of eukaryotic picoplankton *Aureococcus anophagefferens*, have become a recurring phenomenon in many coastal embayments, e.g., the east coast of the United States, since 1985 and Saldanha Bay, South Africa, since 1997 (Sieburth et al., 1988; Bricelj and Lonsdale, 1997; Probyn et al., 2001, 2010). In 2009, a large-scale bloom caused by a similar picoplankton (~2 μm), which was later confirmed to be *A. anophagefferens*, occurred along the coast of Qinhuangdao, China (Zhang et al., 2012). Since then, such blooms have recurred in this area, making China the third country to suffer from brown tides.

The maximum cell density of brown tide along the Qinhuangdao coast can reach over 10^6 cells/mL, covering more than 3 000 km^2 and normally lasting

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** Corresponding author: lusonghui1963@163.com
# Jian GAO and Yuelei DONG contributed equally to this work and should be regarded as co-first authors.

for 1–3 months (Zhang et al., 2012; Zhen et al., 2016). Both the feeding activities and growth of shellfish , especially the bay scallop *Argopecten irradians*, the main maricultural species cultivated in this region, were severely affected (Zhang et al., 2012). From 2009 to 2016, the annual economic loss caused by brown tide exceeded USD 14.5 million (Ou et al., 2018b).

Brown tide has recurred in coastal ecosystems of the United States for over three decades and has also significantly damaged the agriculture industry along the East Coast. Extensive studies focusing on the adverse impacts and ecological consequences of *A. anophagefferens* have been conducted. It has been well confirmed that *A. anophagefferens* can severely impact the feeding activity, growth, survival, and reproduction of bivalve mollusks, such as *Mercenaria mercenaria* (Bricelj et al., 2001; Harke et al., 2011), *Mytilus edulis* (Tracey, 1988; Bricelj et al., 2004), and *Argopecten irradians* (Gallager et al., 1989; Griffith et al., 2019). Beyond that, detrimental effects on microzooplankton were also observed (Lonsdale et al., 1996; Caron et al., 2004; Smith et al., 2008; He et al., 2018). However, the exact reason and mechanism remain ambiguous thus far.

Hypothetical mechanisms have been proposed. One claimed that cellular toxicity contributes to noxious effects. According to Sieburth et al. (1988), organic material forms a diffuse exocellular layer associated with the cell membrane of *A. anophagefferens*. After being hydrolyzed by amylase, the reaction product of the outer polysaccharide layer could reduce the beating frequency of the excised lateral cilia of *M. edulis* (Gainey and Shumway, 1991). The effect was similar to that of dopamine and could also could be blocked by pretreatment with the dopamine antagonist ergometrine. Thus, the reduced grazing rates may be caused by the impact of a dopamine-mimetic compound associated with the cell membrane of *A. anophagefferens* (Gainey and Shumway, 1991). In addition, the ciliary movement of bivalves may also be physically affected by sticky outer polysaccharides (Sieburth et al., 1988; Liu and Buskey, 2000).

Nevertheless, to date, a precise chemical substance related to the noxious effect of brown tide has never been identified. In addition, Caron et al. (2004) found that when serving as a sole food source, *A. anophagefferens* could support the good growth of three protist species (*Oxyrrhis marina*, *Uronema* spp. and *Euplotes* spp.). Slipper limpets (*Crepidula fornicata*) can effectively clear *A. anophagefferens* at biomass-specific rates (Harke et al., 2011). In addition, according to Padilla et al. (2006), *A. anophagefferens* can support a faster development of certain bivalve mollusk species (i.e., *M. mercenaria* larvae) when mixed with *Isochrysis galbana*. Moreover, when moderate-density brown tide cells mixed with *I. galbana* served as the diet, there was no distinct effect compared with the effect of a single species-diet of *I. galbana*, and in certain cases, enhanced growth was generated (Padilla et al., 2006). These findings are all serve as counterevidence to the cellular toxicity of *A. anophagefferens*.

Another hypothesis, which was proposed after Lonsdale et al. (1996) proved *A. anophagefferens* to be a nutritionally inadequate food source for some copepods, declared that the nutritional inadequacy of *A. anophagefferens* might account for its detrimental effects. Smith et al. (2008) found that although the ingestion rates of nauplii of a calanoid copepod, *Acartia tonsa*, were not restrained by *A. anophagefferens* (CCMP 1708), the development of nauplii was severely depressed by *A. anophagefferens* cells. A similar case was discovered when the Chinese strain *A. anophagefferens* served as a monoalgal diet for nauplii of the copepod *Pseudodiaptomus poplesia*. Nauplii exhibited a high ingestion rate for brown tide cells, however, they did not metamorphose and died in the late naupliar phase, in a similar way to those under starvation (He et al., 2018). Evidence for the suspected nutritional inadequacy of *A. anophagefferens* was also found when it served as a food source for bivalves. Conspicuous brown lipid droplets in the hard clam *M. mercenaria* larvae were observed when fed *I. galbana*, while no visible lipid stores were found in larvae when *A. anophagefferens* served as the sole food source (Padilla et al., 2006).

However, *A. anophagefferens* cultured in f/2 medium was affirmed to contain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are highlighted as two vital nutritional constituents for the development of zooplankton and bivalves (Bricelj et al., 1989).

Although the total lipid content and fatty acid composition of *A. anophagefferens* cultured in f/2 culture medium were detected, the biochemical composition of brown tide cells has not been fully elucidated. In addition, no previous study has been conducted regarding the protein, amino acid, and carbohydrate composition of *A. anophagefferens*. These constituents are also important ingredients related to the nutritional quality of food sources

for bivalve mollusks (Hemaiswarya et al., 2011). Additionally, the biochemical composition of microalgae is significantly affected by the source of nitrogen (Levasseur et al., 1993; Lourenco et al., 2002). The brown tides of *A. anophagefferens* often bloom when the ambient dissolved organic nitrogen (DON) level is elevated (Mulholland et al., 2009; Probyn et al., 2010; Gobler and Sunda, 2012), which was also observed along the coast of Qinhuangdao during bloom periods (Zhang et al., 2021). Thus, more information is needed on the biochemical composition of *A. anophagefferens* cultivated in different nitrogen sources.

Nitrate and urea are dominant nitrogen sources among the various forms of nitrogen that are available for phytoplankton communities in nature (Durmaz, 2007), including coastal ecosystems in which *A. anophagefferens* blooms. In this study, an elaborate analysis of the effect of different nitrogen resources (urea, nitrate, and a mixture of both) on the biochemical composition of the Chinese strain *A. anophagefferens* was conducted. The results were also compared with species that are frequently used to feed bivalves (values from the literature), to determine whether *A. anophagefferens* is nutritionally inadequate for filter predators.

2 MATERIAL AND METHOD

2.1 Algal strain and culture

The specie of microalgae used for the experiment was the Chinese strain of *A. anophagefferens* (No. AA-1). The strain was originally isolated from the bloom water column of the Qinhuangdao coast in 2012 (119°37.911′E, 39°54.111′N) and preserved at the Research Center of Harmful Algae and Marine Biology, Jinan University, Guangzhou, China. It was grown in sterilized artificial sea water (ASW; Cavanaugh, 1956) enriched with f/2 culture medium (Guillard and Ryther, 1962) and subcultured routinely. The culture was grown at 20±0.5 °C in an illumination intensity of 100 μmol photons/(m^2·s) along with a 12-h:12-h light:dark cycle.

2.2 Nitrogen source

Three conical flasks, containing 1 000-mL medium and each with nutrient added at a quarter of the f/2 culture medium concentration, were inoculated with approximately 0.6×10^6 cells/mL of *A. anophagefferens*. When the cultures grew to the mid-exponential phase, algal cells were harvested by centrifugation (4 500×*g*, 10 min), re-suspended in 3 000-mL medium containing different nitrogen sources. Nitrogen conditions were designed as follows: Treatment A: 220-μmol/L $NaNO_3$; Treatment B: 110-μmol/L urea; and Treatment C: 110-μmol/L $NaNO_3$ and 55-μmol/L urea. Each treatment was conducted in triplicate.

2.3 Growth phase

Cell number counting was conducted every other day for cell growth rate calculation. After cultivation, algae biomass was harvested by centrifugation, freeze-dried, and grounded before biochemical analysis. Treatment A was harvested on Day 7, Treatments B and C were harvested on Day 5 for total lipid, fatty acid, amino acid, and carbohydrate analysis.

2.4 Ash, moisture, and total lipid content

The total ash and moisture contents of Treatment A were detected according to Van Wychen and Laurens (2016).

The total lipid contents of all the treatments were measured by a modified method based on Bligh and Dyer (1959).

2.5 Amino acid, fatty acid, and carbohydrate analysis

The amino acid content of all treatments was determined by an external, commercial testing organization according to the standard GB 5009.124-2016 acid hydrolysis method.

Fatty acid analysis was adapted from Van Wychen et al. (2016), see Supplementary material for details.

Carbohydrate analysis was conducted using the method modified from Van Wychen and Laurens (2020), see Supplementary material for details.

2.6 Statistical analysis

All results are reported as the mean±standard deviation (SD) of three replicate groups. A comparison between means was analyzed by one-way ANOVA, followed by least-significant difference (LSD) test, and significance was accepted when $P<0.05$. Statistical analysis was conducted by using the SPSS program, version 25 (SPSS Inc., Chicago, IL, USA).

3 RESULT

3.1 Growth rate of *A. anophagefferens*

The growth rates in the exponential phase

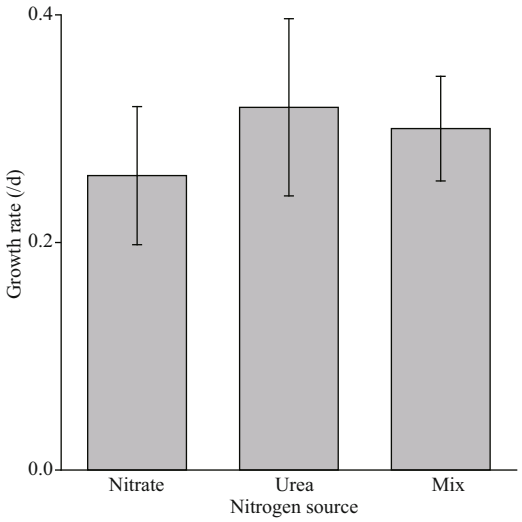

Fig.1 Exponential phase growth rates of *A. anophagefferens* cultivated in nitrate, urea, and nitrate-urea mixture

Error bars represent standard deviation of the means for three replicates.

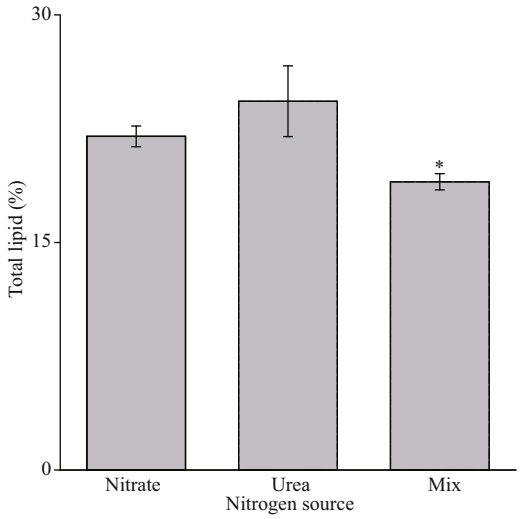

Fig.2 Total extractable lipid contents (% of dry weight) of *A. anophagefferens* cultivated in nitrate, urea, and nitrate-urea mixture

Error bars represent standard deviation of the means for three replicates. Asterisk indicates significantly difference: $P<0.05$.

Table 1 Gross biochemical composition of *Aureococcus anophagefferens* cultivated in different nitrogen sources (% by dry weight, *n*=3)

Gross biochemical composition	Nitrogen source (220 μmol/L)		
	Nitrate	Urea	Mix
Lipid	21.99±0.68^{a}	24.31±2.33^{a}	19.00±0.54^{b}
Protein	39.12±0.48^{b}	40.35±0.79^{ab}	40.88±0.61^{a}
Carbohydrate	10.25±0.27^{a}	7.87±0.13^{b}	7.80±0.05^{b}

Values (mean±SD of three replicates) within the same row with different letters are significantly different ($P<0.05$).

(calculated from Days 1–5) are shown in Fig.1. Growth rates for cultures grown in urea were highest (0.32/d), followed by the nitrate-urea mixture (0.30/d). There were no significant differences among growth rates ($P>0.05$).

3.2 Lipid content and gross biochemical composition

The total extractable lipid content is shown in Fig.2. For *A. anophagefferens* cells grown on urea, the total lipid content was the highest (24.31%), followed by the cells grown on nitrate (21.99%). There were no significant differences between these two groups ($P>0.05$). The total lipid content of the cells grown on the nitrate-urea mixture was the lowest (19.00%), which was significantly lower than that of other two groups ($P<0.05$).

The gross biochemical composition, expressed as a percentage of dry weight, showed the total extractable lipid, protein, and total carbohydrate contents of *A. anophagefferens* under different nitrogen cultivations (Table 1). According to Laurens (2016), the protein content was determined as the summation of amino acids, and the total carbohydrates (including starch) in algal biomass were determined as the summation of monosaccharides. The protein content of the culture on nitrate (39.12%) was not significantly different from that of the culture on urea (40.35%, $P>0.05$) and was significantly lower than that of the culture on the nitrate-urea mixture (40.88%, $P<0.05$). There was no significant difference between the two organic nitrogen addition groups ($P>0.05$). The total carbohydrate contents of *A. anophagefferens* was significantly higher (10.25%) when cultured in nitrate ($P<0.05$), and there was no significant difference between cultures on the urea (7.87%) and the nitrate-urea mixture (7.80%).

3.3 Fatty acid profiles

The fatty acid compositions of *A. anophagefferens* cultivated in different nitrogen conditions are shown in Table 2.

Gas chromatography-mass spectrometry analysis revealed that, as a percent of total fatty acids, *A. anophagefferens* cultured in different nitrogen sources contained 25.96%–28.55% saturated fatty acids (SFAs), 18.05%–19.89% monounsaturated fatty acids (MUFAs), and 51.98%–55.29% PUFAs. The major fatty acids identified in *A. anophagefferens* were C14:0 (myristic acid, 18.81%–22.43%), C16:1n-7 (palmitoleic acid, 11.24%–15.13%),

Table 2 Fatty acids composition of *Aureococcus anophagefferens* cultured in different nitrogen sources shown as content per dry weight (mg/g) and percentage of total fatty acids (%)

Fatty acid	Nitrogen source (220 μmol/L)					
	Nitrate		Urea		Mix	
	(mg/g)	(%)	(mg/g)	(%)	(mg/g)	(%)
C14:0	20.89±1.06 ^{a}	22.43±0.83 ^{x}	13.30±2.11 ^{b}	18.81±0.77 ^{y}	19.45±3.82 ^{a}	20.88±1.60 ^{xy}
C15:0	0.57±0.11 ^{a}	0.62±0.14 ^{xy}	0.60±0.20 ^{a}	0.84±0.23 ^{x}	0.34±0.05 ^{a}	0.36±0.01 ^{y}
C16:0	14.09±0.53 ^{a}	4.83±0.19 ^{y}	7.93±0.99 ^{b}	5.56±0.53 ^{x}	11.94±1.67 ^{a}	4.94±0.22 ^{xy}
C16:1n-7	4.49±0.08 ^{a}	15.13±0.35 ^{x}	3.94±0.72 ^{a}	11.24±0.21 ^{z}	4.58±0.68 ^{a}	12.88±0.26 ^{y}
C18:0	0.63±0.03 ^{a}	0.68±0.02 ^{x}	0.53±0.04 ^{b}	0.75±0.05 ^{x}	0.44±0.01 ^{c}	0.48±0.05 ^{y}
C18:1n-9	4.04±0.22 ^{c}	4.34±0.02 ^{z}	6.07±0.47 ^{a}	8.64±0.46 ^{x}	4.76±0.27 ^{b}	5.17±0.53 ^{y}
C18:2n-6	0.75±0.05 ^{b}	0.80±0.01 ^{z}	1.41±0.12 ^{a}	2.01±0.15 ^{x}	0.85±0.10 ^{b}	0.92±0.03 ^{y}
C18:3n-3	4.64±0.39 ^{a}	4.98±0.19 ^{y}	3.78±0.46 ^{b}	5.37±0.21 ^{x}	3.88±0.42 ^{ab}	4.20±0.05 ^{z}
C18:4n-3	21.42±1.44 ^{ab}	22.98±0.39 ^{y}	19.57±2.33 ^{b}	27.77±0.53 ^{x}	25.05±3.62 ^{a}	26.99±0.68 ^{x}
C18:4n-6	3.59±0.41 ^{b}	3.84±0.32 ^{z}	5.70±0.59 ^{a}	8.11±0.53 ^{x}	5.81±0.30 ^{a}	6.30±0.45 ^{y}
C20:5n-3	4.49±0.31 ^{a}	4.81±0.14 ^{x}	2.26±0.34 ^{b}	3.20±0.08 ^{y}	4.05±0.16 ^{a}	4.40±0.35 ^{x}
C22:6n-3	13.58±0.97 ^{a}	14.56±0.43 ^{x}	5.45±0.97 ^{b}	7.69±0.41 ^{y}	11.48±0.65 ^{a}	12.48±1.27 ^{x}
SFA	36.18±1.57 ^{a}	28.55±1.08 ^{x}	22.36±3.31 ^{b}	25.96±1.44 ^{x}	32.17±5.54 ^{a}	26.66±1.74 ^{x}
MUFA	8.54±0.28 ^{a}	19.47±0.36 ^{x}	10.00±1.16 ^{a}	19.89±0.55 ^{x}	9.34±0.78 ^{a}	18.05±0.27 ^{y}
PUFA	48.46±3.51 ^{a}	51.98±1.43 ^{y}	38.16±4.78 ^{b}	54.15±1.41 ^{xy}	51.11±4.91 ^{a}	55.29±1.47 ^{x}
DHA/EPA	3.03±0.01 ^{a}		2.40±0.07 ^{b}		2.84±0.12 ^{a}	
TFAC	93.18±4.84 ^{a}		70.53±9.03 ^{b}		92.62±11.14 ^{a}	

C14:0: myristic acid; C15:0: pentadecanoic acid; C16:0: hexadecanoic acid; C16:1n-7: palmitoleic acid; C18:0: stearic acid; C18:1n-9: elaidic acid; C18:2n-6: linoleic acid; C18:3n-3: α-linolenic acid; C18:4n-3: stearidonic acid; C18:4n-6: stearidonic acid; C20:5n-3: eicosapentaenoic acid, EPA; C22:6n-3: docosahexaenoic acid, DHA; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFAC: total fatty acids contents. In the same row of each item, the values (mean±SD of three replicates) of quantity levels (mg/g) and percentages (%) with different superscript letters (a, b, c) and (x, y, z) respectively are significantly different (P<0.05).

C18:4n-3 (stearidonic acid, 22.98%–27.77%), and C22:6n-3 (DHA, 7.69%–14.56%). C14:0 and C16:1n-7 comprised 22.43% and 15.13% of the total fatty acids of the culture on nitrate and were significantly higher than those of the culture in urea (18.81% and 11.24% respectively). The culture in the nitrate and the nitrate-urea mixture contained a higher percentage of EPA and DHA than the culture in urea (P<0.05). However, regarding the percentages of all C18 mono- and poly-unsaturated fatty acids, culture in the urea had significantly higher concentrations than both cultures in the nitrate and nitrate-urea mixture, except for C18:4n-3 in which no significant difference between the culture in the urea and nitrate-urea mixture was shown. The culture in urea contained the highest percentage of MUFAs. The culture in the nitrate-urea mixture contained the highest percentage of PUFAs. There was no significant difference among the three cultures in the percentage of SFAs. For all three treatments, no eicosatetraenoic acid (C20:4n-6, ARA) or docosapentaenoic acid (C22:5n-6, DPA) was detected.

3.4 Amino acid profiles

The amino acid content (% by dry weight) of *A. anophagefferens* cultivated in different nitrogen conditions is shown in Table 3.

The percentage composition of nonessential amino acids varied from 23.47% to 24.60%. The percentage composition of essential amino acids varied from 15.65% to 16.28%. The percentage composition of total amino acids varied from 39.12% to 40.88%. The principal fraction of amino acids in the treatment consisted of leucine (3.50%–3.61%), aspartic acid (4.20%–4.39%), and glutamic acid (5.08%–5.43%).

The nonessential amino acid content of the culture in the nitrate was significantly lower than that of the cultures in the urea and the nitrate-urea mixture (P<0.05). Both the essential amino acid and total amino acid contents of the culture in nitrate were not significantly different from those of the contents of the culture in the urea (P>0.05) and were significantly lower than those of the contents of the culture in the nitrate-urea mixture (P<0.05).

Table 3 Amino acids composition of *Aureococcus anophagefferens* cultured in different nitrogen sources (% by dry weight, *n*=3)

Amino acid	Nitrogen source (220 μmol/L)		
	Nitrate	Urea	Mix
Threonine	2.31±0.02 [b]	2.39±0.04 [a]	2.42±0.03 [a]
Valine	2.57±0.05 [a]	2.61±0.10 [a]	2.69±0.03 [a]
Methionine	1.27±0.11 [a]	1.31±0.06 [a]	1.26±0.02 [a]
Isoleucine	1.96±0.05 [a]	2.01±0.09 [a]	2.04±0.02 [a]
Leucine	3.50±0.06 [a]	3.57±0.10 [a]	3.61±0.04 [a]
Phenylalanine	2.11±0.04 [a]	2.18±0.04 [a]	2.21±0.06 [a]
Lysine	1.93±0.01 [ac]	2.05±0.03 [ab]	2.05±0.08 [a]
Aspartic acid	4.20±0.03 [b]	4.34±0.06 [a]	4.39±0.06 [a]
Serine	1.99±0.04 [b]	2.05±0.03 [a]	2.07±0.02 [a]
Glutamic acid	5.08±0.08 [b]	5.18±0.15 [b]	5.43±0.07 [a]
Glycine	2.40±0.02 [b]	2.50±0.04 [a]	2.50±0.03 [a]
Alanine	3.60±0.04 [b]	3.70±0.08 [ab]	3.81±0.06 [a]
Tyrosine	1.40±0.04 [b]	1.49±0.04 [a]	1.46±0.01 [ab]
Histidine	0.67±0.01 [b]	0.69±0.01 [a]	0.71±0.04 [a]
Arginine	2.36±0.04 [a]	2.43±0.05 [a]	2.43±0.04 [a]
Proline	1.78±0.03 [b]	1.86±0.03 [a]	1.81±0.03 [ab]
Total AA	39.12±0.48 [b]	40.35±0.79 [ab]	40.88±0.61 [a]
EAA	15.65±0.25 [b]	16.11±0.36 [ab]	16.28±0.27 [a]
Non-EAA	23.47±0.23 [b]	24.24±0.44 [a]	24.60±0.34 [a]

Values (mean±SD of three replicates) within the same row with different letters are significantly different (P<0.05). AA: amino acid; EAA: essential amino acid; Non-EAA: non-essential amino acid;

3.5 Carbohydrate profiles

The monomeric carbohydrate compositions of *A*. *anophagefferens* cultivated in different nitrogen conditions are shown in Table 4.

Eleven monomeric carbohydrates were detected in *A*. *anophagefferens*, and the major monomeric carbohydrates identified in *A*. *anophagefferens* were galacturonic acid (1.60%–2.90%), glucose (1.30%–1.70%), and galactose (0.96%–1.59%). Except for glucose and galactose, the contents of nine other monomeric carbohydrates were significantly higher in the cultures in nitrate (P<0.05). The glucose content was highest in the culture in urea, and the galactose content was highest in the culture in the nitrate-urea mixture.

4 DISCUSSION

4.1 Growth of *A. anophagefferens* cultured in different nitrogen sources

In this study, *A*. *anophagefferens* grew well when urea served as the sole nitrogen supplement. Cultures with urea and the nitrate-urea mixture were associated with higher growth rates and entered the stationary phase two days earlier than the culture with nitrate. A similar phenomenon occurred during *A*. *anophagefferens* blooms in the coast of Qinhuangdao, China: inorganic nitrogen was negatively correlated with the abundance of *A*. *anophagefferens*, and half of the total dissolved nutrient pools were contributed by DON and DOP (Yao et al., 2019; Zhang et al., 2021). The results also matched previous nutrient enrichment studies in Quantuck Bay and Narragansett Bay, USA, in which, both mesocosms and field measurements showed that dissolved inorganic phosphorus (DIN) concentration was inversely correlated with *A*. *anophagefferens* cell density, and that the initiation of *A*. *anophagefferens* blooms was usually associated with increasing DON levels (Keller and Rice, 1989; Kana et al., 2004; Gobler and Sunda, 2012).

Table 4 Monomeric carbohydrates composition of *Aureococcus anophagefferens* cultured in different nitrogen sources (% by dry weight, *n*=3)

Carbohydrate	Nitrogen source (220 μmol/L)		
	Nitrate	Urea	Mixture
Fucose	1.26±0.02 [a]	0.47±0.01 [b]	0.45±0.01 [b]
Rhamnose	1.10±0.04 [a]	0.40±0.01 [b]	0.44±0.01 [b]
Galactosamine·HCl	0.10±0.01 [a]	0.05±0.00 [b]	0.05±0.01 [b]
Arabinose	0.27±0.02 [a]	0.07±0.01 [b]	0.06±0.01 [b]
Glucosamine·HCl	0.39±0.01 [a]	0.27±0.01 [b]	0.28±0.00 [b]
Galactose	0.96±0.02 [c]	1.59±0.00 [a]	1.47±0.02 [b]
Glucose	1.30±0.14 [b]	1.33±0.11 [b]	1.70±0.05 [a]
Mannose	0.76±0.02 [a]	0.60±0.01 [c]	0.64±0.01 [b]
Xylose	0.69±0.03 [a]	0.64±0.01 [b]	0.64±0.00 [b]
Ribose	0.53±0.02 [a]	0.48±0.01 [b]	0.47±0.01 [b]
Galacturonic acid	2.90±0.19 [a]	1.97±0.18 [b]	1.60±0.07 [c]
Total	10.25±0.33 [a]	7.87±0.13 [b]	7.80±0.05 [b]

Values (mean±SD of three replicates) in the same row with different letters are significantly different (P<0.05).

4.2 Assessment of gross biochemical composition

According to Guedes and Malcata (2012), microalgae grown to the late exponential growth phase usually consist of 5%–15% carbohydrate, 10%–20% lipid, and 30%–40% protein. The *A*. *anophagefferens* cultured in different nitrogen sources contained 7.80%–10.25% carbohydrate, a relatively high amount of protein (39.12%–40.88%,

Table 5 Literature values for gross biochemical composition (% of dry weight) of seven phytoplankton species: *Skeletonema costatum*, *Chaetoceros calcitrans*, *Isochrysis galbana*, *Pavlova lutheri*, *I. galbana* (T-ISO), *Thalassiosira pseudonana*, and *Tetraselmis suecica*

Algae species	Lipid	Protein	Carbohydrate
Skeletonema costatum [1]	10	25	4.6
Chaetoceros calcitrans [1]	16	34	6.0
Pavlova lutheri [1]	12	29	9.0
lsochrysis galbana [1]	23	29	12.9
I. galbana (T-ISO) [1]	20	23	6.0
Thalassiosira pseudonana [1]	19	34	8.8
Tetraselmis suecica [1]	10	31	12.0
Skeletonema costatum [2]	16	23	26.4
I. galbana (T-ISO) [3]	30	48	3.3
A. anophagefferens in nitrate [4]	22	39.1	10.3
A. anophagefferens in urea [4]	24.3	40.4	7.9
A. anophagefferens in mixture [4]	19	40.9	7.8

[1]: Brown, 1991; [2]: Van Houcke et al., 2017; [3]: Martínez-Fernández et al., 2006; [4]: this study.

around the upper limit of the average value), and a high amount of total extractable lipid (up to 24.31% in urea cultivation). The protein and carbohydrate contents were basically within the range, but the total extractable lipid content was higher than that of the average range value. Bricelj et al. (1989) also found that when cultured in f/2 medium, the lipid content of *A. anophagefferens* was quite high (26.4%±6.4% of dry weight). The result of the present study is consistent with Bricelj's work.

When served as food for bivalve mollusks, the nutritional value of the algal diet depends largely on the gross biochemical components (Marshall et al., 2010). Saucedo et al. (2013) discovered that elevated algal diet protein levels were associated with enhanced growth and respiration of juvenile lion-paw scallop, *Nodipecten subnodosus*. Uriarte and Farías (1999) discovered that both the growth and survival of the Chilean scallop, *Argopecten purpuratus*, are positively correlated with dietary diet protein levels. Tang et al. (2006) proved that the feeding behavior of hard clam *Meretrix meretrix* is positively correlated with the total lipid content of algal diet. Wikfors et al. (1992) found that the gross biochemical composition of algae affects the growth of hard clams *M. mercenaria*, one of the most adversely affected species by *A. anophagefferens*. To support rapid growth, both dietary protein and lipids must be present in sufficient quantities. For bivalve mollusks, neutral lipids are the principal energy-providing constituent in algal diets, followed by protein (Fernández-Reiriz et al., 2011; Matias et al., 2011). From this perspective, the relatively high amount of lipids and proteins should make *A. anophagefferens* an appropriate bite.

Skeletonema costatum, *Chaetoceros calcitrans*, *Pavlova lutheri*, *lsochrysis galbana*, *Thalassiosira pseudonana*, and *Tetraselmis suecica* are some of the commonly used microalgae species in bivalve rearing (Kaparapu, 2018). When compared with these species, the gross biochemical composition of *A. anophagefferens* is comparable with all (Table 5). Among them, *Isochrysis* sp. (T-ISO), *P. lutheri*, and *C. calcitrans*, are the most commonly used species when feeding the larva, early juvenile, and broodstock stages of bivalve mollusks (Brown, 2002). Moreover, *S. costatum*, *T. pseudonana*, and *I. galbana* support excellent growth of a great number of bivalve mollusks, including *M. mercenaria* (Wikfors et al., 1992; Greenfield et al., 2004; Bricelj and MacQuarrie, 2007).

4.3 Composition of fatty acids in *A. anophagefferens*

Fatty acids, especially PUFAs, are vital constituents in membrane fluidity and function maintenance and serve as precursors to some bioactive molecules involved in metabolism and reproduction (Da Costa et al., 2015; Cheng et al., 2020). Most animals, including bivalve mollusks, cannot synthesize either n-3 or n-6 family PUFAs de nova (Zhukova, 2019). The ability to produce long-chain PUFAs from shorter chain precursors is also limited (Langdon and Waldock, 1981; Delaunay et al., 1993). The primary source of long-chain PUFAs for bivalves in the field is through the ingestion of algae (Cheng et al., 2020). Therefore, for most bivalves, the content of long-chain PUFAs is emphasized when evaluating the nutritional value of algae.

Bricelj et al. (1989) detected the fatty acid composition of *A. anophagefferens* isolated from the East Coast of the United States. They reported that the amount and composition of fatty acids were remarkably constant between the exponential growth phase and the early stationary phase when cultured in nitrate. The levels of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) in cells in the early stationary phase were 23.2%, 17.5%, and 59.2% respectively. EPA and DHA accounted for 5.1% and 14.0% of the total fatty acids, respectively. In the

Table 6 Literature values for essential fatty acids composition (% of total fatty acids) of six phytoplankton species: *Skeletonema costatum*, *Chaetoceros calcitrans*, *Pavlova lutheri*, *Thalassiosira pseudonana*, *I. galbana* (T-ISO), and *Chaetoceros muelleri*

Algae species	C18:2n-6	C18:3n-3	C18:4n-3	ARA	EPA	DPA	DHA	SFA	MUFA	PUFA
Skeletonema costatum [1]	2.2	0.3	2.2	–	6.0	–	2.0	39.2	32.0	26.1
Chaetoceros calcitrans [1]	0.8	TR	0.5	5.7	11.1	–	0.8	30.2	33.8	33.7
Pavlova lutheri [1]	1.5	1.8	6.0	TR	19.7	2.0	9.4	35.9	20.4	42.0
Thalassiosira pseudonana [1]	0.4	0.1	5.3	0.3	19.3	–	3.9	27.2	19.5	52.6
I. galbana (T-ISO) [2]	3.4	6.2	18.6	0.2	1.9	1.9	18.0	22.5	18.0	59.2
Chaetoceros muelleri [3]	2.9	–	1.8	4.8	7.6	3.8	7.9	33.0	23.7	40.1
A. anophagefferens in nitrate	0.8	5.0	23.0	–	4.8	–	14.6	28.6	19.5	52.0
A. anophagefferens in urea	2.0	5.4	27.8	–	3.2	–	7.7	26.0	19.9	54.2
A. anophagefferens in the mixture	0.9	4.2	27.0	–	4.4	–	12.5	26.7	18.1	55.3

C18:2n-6: linoleic acid; C18:3n-3: α-linolenic acid; C18:4n-3: stearidonic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. [1]: Volkman et al., 1989; [2]: Delaporte et al., 2007; [3]: Milke et al., 2008; [4]: this study. TR: trace; – means no data.

present study, the SFA level was higher (28.55%), and the PUFA level was lower (51.98%). The MUFA, EPA, and DHA levels of the present study were all comparable to the values of Bricelj's results.

When compared with five favorably used microalgae species in bivalve feeding, *A. anophagefferens* is similar to, and, in certain cases higher than, these algal species regarding their content of stearidonic acid (C18:4n-3), α-linolenic acid (C18:3n-3), DHA, and total PUFA (Table 6). Although the EPA content of *A. anophagefferens* is lower than that of most of these species, the content of stearidonic acid (SDA, C18:4n-3), which is an important precursor for EPA (Jónasdóttir, 2019), surpassed those of all the species.

Hard clams (*M. mercenaria*), marine mussels (*M. edulis*) and bay scallops (*A. irradians*) are three bivalve mollusk species that are severely affected by brown tide. Since bivalve nutritional requirements are different among species, a detailed comparison is indispensable. When served as a solo food supplement, both *S. costatum* and *I. galbana* (T-ISO) consistently supported rapid growth of *M. mercenaria* (Helm and Laing, 1987; Wikfors et al., 1992; Bricelj and MacQuarrie, 2007). The C18-PUFA (except C18:2n-6) and DHA contents of *A. anophagefferens* in the nitrate and nitrate-urea mixture were higher than those of *S. costatum*, whereas the EPA level was lower. When compared with *I. galbana* (T-ISO), the C18:4n-3 and EPA levels were higher, while the C18:3n-3 and DHA contents were lower. Portilla et al. (2015) found that an exogenous supplement of EPA and DHA is indispensable for hard calms acclimation to declining temperatures during the overwinter period. The present study proved that these two PUFAs are contained in *A. anophagefferens* cultured in different nitrogen sources.

For *A. irradians*, Milke et al. (2006) reported that, to support fast growth of postlarval and juvenile bay scallops, in addition to EPA and DHA, n-6 PUFAs such as ARA and DPA are also essential. A *Pavlova* sp. (CCMP 459) and *Chaetoceros muelleri* combination diet containing high amounts of ARA and DPA, outperformed all other diets. Similarly, excellent growth of marine mussel *M. edulis* larvae was supported by *C. muelleri*, an algae that contains a high amount of ARA (Leonardos and Lucas, 2000). Although n-6 PUFAs were not detected in the present study or in Bricelj's report (Bricelj et al., 1989), the presence of both ARA (2.35%) and DPA (4.55%) in *A. anophagefferens* was detected by Bigelow et al. (2013), upon culture in L1 medium. Moreover, the fast growth of *M. edulis* larvae was also supported by *S. costatum*, which contained no ARA or DPA (Leonardos and Lucas, 2000).

The fatty acid composition of algae is significantly affected by the culture medium. Bigelow et al. (2013) reported that *A. anophagefferens* contained less C14:0 (10.99%), C16:1n-7 (4.55%), and MUFAs (7.38%), and more C18:2n-6 (3.92%), C18:4n-3 (28.57%), and PUFAs (68.76%) when cultured in L1 medium. The present study found that both EPA and DHA levels were significantly lower when cultured in urea (P<0.05). Moreover, when expressed as per unit of algal dry weight (mg/g), cultures in urea contained significantly lower levels of EPA, DHA, SFA, PUFA, and TFAC than cultures in nitrate and the nitrate-

urea mixture (P<0.05). The nutritional value of *A. anophagefferens* dropped when cultured in urea; nevertheless, both EPA and DHA contents were still comparable with *S. costatum*, which support good growth of *M. mercenaria* and *M. edulis* larvae. The lack of n-6 PUFAs may make *A. anophagefferens* a nutritionally inadequate food source for *A. irradians*. Paradoxically, if this is true, instead of expecting feeding cessation and starvation, a low growth rate and delayed development of the bivalve should be expected.

4.4 Amino acid composition of *A. anophagefferens*

In total, 11 amino acids are essential for bivalve mollusks: threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, arginine, proline and tryptophan (Brown, 1991). Except for tryptophan, which was destroyed by hydrolysis during analysis, the other 10 essential amino acids were detected in *A. anophagefferens*. Moreover, when cultivated in urea, the levels of all 10 amino acids were either not significantly or significantly higher than those of cells cultured in nitrate. Ou et al. (2018a) found that, compared with nitrate-cultivated cells, *A. anophagefferens* cultured in urea contained significantly higher amounts of protein in the stationary phase. Dong et al. (2014) reported that many transcripts encoding enzymes involved in amino acid synthesis increased when *A. anophagefferens* was cultured in urea. The results of the present study are in agreement with Ou et al. (2018a) and Dong et al. (2014)'s observations.

When compared with favorably used species in mariculture, the amino acid composition of *A. anophagefferens* is similar to that of these species. Unlike the amount of protein, the quality of the protein is unlikely to be an element that contributes to differences in nutritional value among microalgae, since it is generally accepted that the amino acid composition is rather similar among species (Guedes and Malcata, 2012). Therefore, when applied to bivalve feeding, the amino acid composition of algae is unlikely to be responsible for differential growth performance.

4.5 Carbohydrate composition of *A. anophagefferens*

Glucose is the principal sugar in most species used in mariculture (Brown, 1991). However, the present study showed that galacturonic acid is the primary monomeric carbohydrate in the *A. anophagefferens* hydrolysate. Although available research does not suggest a close connection between the monomeric carbohydrate composition and the nutritional value of microalgae (Wikfors et al., 1992), the proportion of glucose in the readily hydrolysable carbohydrate may be related to the nutritional quality of microalgae (Knauer and Southgate, 1999). The glucose content is 21%–87% in species that are frequently used in aquaculture, when expressed as a percent of the total carbohydrates (Brown, 1991). Compared with them, *A. anophagefferens* contained relatively lower amounts of glucose (12.65%–21.74%).

The best growth of juvenile oysters and larval scallops was associated with high levels of carbohydrates provided in algal diets, when sufficient protein and essential fatty acids were also supplied (Guedes and Malcata, 2012). Compared with cultures in nitrate, the carbohydrate concentration of *A. anophagefferens* significantly dropped when cultured in urea and nitrate-urea mixture, which may reduce the nutritional value. Nevertheless, it is still comparable with some of the favorably used species in bivalve feeding (Table 5).

4.6 The speculated reason for bivalve starvation caused by *A. anophagefferens*

To serve as an appropriate diet for bivalves, apart from having high nutritional value, microalgae should be easily digested, nontoxic and of adequate size (Cheng et al., 2020). Recruitment failure, reduced filter feeding rate, and cessation of feeding were observed when *A. anophagefferens* reached a certain cell density (Tracey, 1988; Bricelj et al., 2001; Gobler and Sunda, 2012). The retention efficiency of bivalves usually decreases with decreasing particle size (Cranford, 2019). The small size of *A. anophagefferens* may adversely affect bivalve growth. However, it seems not be an adequate explanation that accounts for starvation. In addition, the high absorbance efficiency (up to 90%) (Bricelj and Kuenstner, 1989) of *A. anophagefferens* by *M. edulis* and *A. irradians* apparently rules out indigestibility. Moreover, the relatively low nutritional value of *A. anophagefferens* cultured in urea is still comparable with algae such as *S. costatum* that supports fast bivalve growth. A plausible cause of detrimental effects is the toxicity of cells, which should be seriously evaluated.

5 CONCLUSION

This study found that the gross biochemical compositions of *A. anophagefferens* are comparable with the values found in the literature for species that

are frequently used to feed bivalves. When cultured in nitrate and a nitrate-urea mixture, fatty acid levels, especially PUFAs, including EPA and DHA, are comparable to and in certain cases higher than, the values found in the literature. Although the DHA, EPA, and PUFA contents were significantly decreased when cultivated in urea, they are still comparable to those of *S. costatum*, which supports good growth of *M. mercenaria* and *M. edulis* larvae. In summary, we found that *A. anophagefferens* is not a nutritionally inadequate food source for bivalve mollusks. Cytotoxicity is the more likely cause of detrimental effects.

6 DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Electronic supplementary material

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