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The ascorbic acid content of eleven species of microalgae used in mariculture

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Abstract

The ascorbic acid (vitamin C) concentrations in 11 species of microalgae commonly used in mariculture were determined. The species examined were 4 diatoms (*Chaetoceros calcitrans* (Paulsen) Takano, *Chaetoceros gracilis* Schütt, *Skeletonema costatum* (Greville) Cleve, *Thalassiosira pseudonana* (Hustedt, clone 3H) Hasle and Heimdal); 2 prymnesiophytes (*Isochrysis* sp. (clone T.ISO) Parke, *Pavlova lutheri* (Droop) Green); 1 prasinophyte (*Tetraselmis suecica* (Kylin) Butcher); 2 chlorophytes (*Dunaliella tertiolecta* Butcher, *Nannochloris atomus* Butcher); 1 eustigmatophyte (*Nannochloropsis oculata* (Droop) Green); and 1 cryptophyte (*Chroomonas salina* (Wislouch) Butcher). Duplicate cultures of each species were grown under defined conditions and analysed during both logarithmic and stationary phase of growth.

Average values for ascorbic acid ranged from 9.4 fg cell^{-1} (*N. oculata*, stationary phase) to 700 fg cell^{-1} (*S. costatum*, stationary phase). This value was generally related to cell size. Levels of ascorbic acid cell^{-1} increased during the stationary growth phase for *S. costatum* and *D. tertiolecta* and decreased for *C. gracilis*, *T. pseudonana*, *C. salina* and *N. oculata*. Levels did not change significantly for the remaining species.

Average values for per cent ascorbic acid ranged from 0.11% (*T. pseudonana*, stationary phase) to 1.62% of dry weight (*C. gracilis*, logarithmic phase). The per cent ascorbic acid was not related to algal class. Also, the percentage between logarithmic and stationary phase cultures differed for many of the species, but differences were unrelated to algal class. *Chaetoceros gracilis*, *T. pseudonana*, *N. oculata* and *Isochrysis* sp. (T.ISO) had higher per cent ascorbic acid during the logarithmic phase, whereas *D. tertiolecta* and *N. atomus* contained more per cent ascorbic acid during the stationary phase.

Despite the differences in the composition of the different microalgae (0.11–1.62% ascorbic acid), all species would provide a rich source of ascorbic acid for maricultured animals, which can require 0.003–0.02% of the vitamin in their diet.

Introduction

Microalgae are an important food source in mariculture, as they are eaten by all growth stages of bivalves, and larval stages of some crustacean and fish species. Microalgae are also eaten by zooplankton (rotifers, copepods and brine shrimp) reared as food for late-larval and juvenile

stages of some crustacean and fish species. Not all microalgal species have been successful in supporting the growth of maricultured animals (Epifanio *et al.*, 1981; Enright *et al.*, 1986). The shape and size, toxicity, digestibility and biochemical composition of the microalgae can affect their value as food (Webb & Chu, 1983; Brown *et al.*, 1989).

In an attempt to correlate the composition of microalgae with their nutritional value, researchers have studied the levels of total protein, lipid and carbohydrate (Whyte, 1987; Brown, 1991), as well as the composition of amino acids (Enright *et al.*, 1986; Brown, 1991), fatty acids (Volkman *et al.*, 1989; Thompson *et al.*, 1990), sugars (Whyte, 1987; Brown, 1991) and sterols (Lin *et al.*, 1982) in many species of microalgae. The vitamin content of microalgae used in mariculture has been less well studied, although a detailed analysis of five species has recently been reported (De Roeck-Holtzhauer *et al.*, 1991).

In this study, we report on the ascorbic acid (vitamin C) content of eleven species of microalgae commonly used in mariculture (Table 1). All species studied have been used successfully either as a single-species diet or as part of multi-species diets (see reviews by Webb & Chu, 1983; Brown *et al.*, 1989). The composition of fatty acids, amino acids and polysaccharide sugars of all these species has been determined from studies in our laboratories (Volkman *et al.*, 1989; Brown, 1991; J.K. Volkman *et al.*, unpublished). The ascorbic acid content of many of these microalgae has not been reported previously. The content of

Table 1. Species used for ascorbic acid analysis. All microalgae came originally from the Guillard-Provasolii Culture Collection of Marine Phytoplankton, Bigelow Laboratory for Ocean Science, West Boothbay Harbor, Maine, USA, except *Nannochloropsis oculata*, from the Australian Antarctic Division, Kingston, Tas., Australia. Previously known as [1]*Cyclotella nana*, [2]*Chlorella* sp. (Japanese isolate), [3]*Platymonas suecica*, and [4]*Monochrysis lutheri*.

Algal class/species	CSIRO culture no.	Deposition or origin code	Axenic	Culture medium	Mean cell volume (μm^3)*
Bacillariophyceae					
Chaetoceros calcitrans (Paulsen) Takano	CS-178	C.CAL	Yes	f_2	5
Chaetoceros gracilis Schutt	CS-176	CHGRA	No	f_2	38
Skeletonema costatum (Greville) Cleve	CS-181	SKEL	Yes	f_2	154
Thalassiosira pseudonana[1] (Hustedt, clone 3H) Hasle & Heimdal	CS-173	3H	Yes	G_2	32
Chlorophyceae					
Dunaliella tertiolecta Butcher	CS-175	WHOIIDUN	Yes	f_2	295
Nannochloris atomus Butcher	CS-183	251/4B	Yes	f_2	57
Cryptophyceae					
Chroomonas salina (Wislouch) Butcher	CS-174	3C	Yes	f_E	340
Eustigmatophyceae					
Nannochloropsis oculata (Droop) Green[2]	CS-179	–	No	f_2	14
Prasinophyceae					
Tetraselmis suecica (Kylin) Butcher[3]	CS-187	WTET	Yes	f_2	640
Prymnesiophyceae					
Isochrysis sp. (clone T.ISO) Parke	CS-177	T.ISO	Yes	f_2	51
Pavlova lutheri (Droop) Green[4]	CS-182	MONO	Yes	f_2	91

* From Brown (1991).

Dunaliella tertiolecta has been reported, although the growth conditions and stage of harvest were not given (Happette & Poulet, 1990a). Cultures of *T. suecica*, *P. lutheri*, *S. costatum* and *C. calcitrans* grown under continuous illumination have been analysed (De Roeck-Holtzhauer *et al.*, 1991). However the majority of hatcheries in Australia adopt a 12:12 h light:dark photoperiod for algal culture; therefore this light regime was chosen for the present study.

Materials and methods

Microalgal cultures

Eleven microalgal species were studied (Table 1). Microalgae were cultured in medium f_2 (Guillard & Ryther, 1962), medium f_E containing EDTA (Jeffrey, 1980) or medium G_2 (Loeblich & Smith, 1968). Duplicate aliquots (100 ml) from starter cultures (mid-log phase) were each inoculated in 1.4 l of culture medium in 2 l Erlenmeyer flasks. The duplicate cultures of each species (designated as #1 and #2 hereafter; e.g. *Chaetoceros calcitrans* #1) were grown simultaneously, except for cultures of *Chroomonas salina*, which were grown 2 weeks apart. All cultures were placed on glass shelves and maintained at 20 °C (± 0.5 °C) and at pH 7.4–7.8 by bubbling with a 1% CO_2/air mixture at a total flow rate of 0.4 l min^{-1} $flask^{-1}$.

Cultures of the eleven species were illuminated with 100 μmol photon m^{-2} s^{-1} fluorescent light (Philips daylight tubes) on 12:12 h light:dark cycles. To compare the effects of light photoperiod and intensity on composition, additional duplicate cultures of one species, i.e. *T. pseudonana* were also grown under continuous light, at 50 and 100 μmol photon $m^{-2}s^{-1}$.

An aliquot (300 ml) from each culture was removed at mid-late logarithmic growth phase (5–8 d from inoculation) and stationary phase (10–14 d) for cell counts, dry weight and ascorbic acid analysis. Stationary phase samples were collected 2–4 d after growth of the cultures ceased, as determined by daily cell counts.

Cell counts and dry weight analysis

Cell counts were determined with a Neubauer haemacytometer. The coefficients of variation (minimum of four replicate counts/species) ranged from 3–12% (average 7%) for the different species. The cell volumes for all the species were determined previously (Brown, 1991) by the method of Smayda (1978).

For determination of dry weight $cell^{-1}$, duplicate aliquots (80 ml for logarithmic phase cultures; 40 ml for stationary phase) from each culture were filtered under vacuum (≤ 100 mm Hg) through pre-weighed, pre-combusted (@450 °C; 16 h) glass-fibre filters (Whatman 47 mm; GFF for *C. calcitrans* (the smallest species), GFC for all other species). The filters were then washed with 0.5 M ammonium formate (30 ml) to remove residual salts, and dried at 100 °C for 16 h to volatilise ammonium formate. Filters were re-weighed to determine the dry weight $cell^{-1}$.

Extraction of ascorbic acid from microalgae

An aliquot from each culture (80 ml for logarithmic phase cultures; 40 ml for stationary phase) was filtered under vacuum through a pre-combusted glass-fibre filter (same types as describe above). All subsequent extraction and derivitisation steps were carried out in the dark; at 4 °C, unless otherwise specified.

After filtration, filters were immediately placed in 10 ml plastic centrifuge tubes containing 4.0 ml of 3% metaphosphoric acid + 8% acetic acid (MPA), 1.0 ml of water and 20 μl solution of EGTA-glutathione solution (0.24 M ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid and 0.20 M glutathione; pH 6.5). Culture filtrates were treated identically except that 1.0 ml of filtrate was substituted for 1.0 ml of water; only one of the filtrates of the duplicate cultures was analysed. All samples were vortexed (30 s), sonicated (30 s) and then left for a further 2 min. These mixing steps were repeated twice before leaving the samples for 30 min to complete extraction of ascorbic acid.

Pilot studies showed that all the ascorbic acid was efficiently extracted from the microalgae by this procedure, except with *Nannochloropsis oculata*. Maximum extraction from this microalga required overnight incubation in the extracting medium.

After extraction, all tubes were centrifuged at $2000 \times g$ for 10 min. To ensure that extracted ascorbic acid was in the quantifiable range for the assay, two aliquots of different volumes (1.5 and 0.3 ml) were taken from each supernatant and transferred to separate tubes. The volume of the latter was made up to a total of 1.5 ml with the MPA solution. Sodium acetate buffer (4.5 M pH 6.2; 0.4 ml) was added to each tube, then the solution was equilibrated at 37 °C for 10 min prior to incubation with ascorbic acid oxidase.

The solution of MPA used in the present study was found by Happette and Poulet (1990a) to be one of the most effective for the extraction of ascorbic acid from plankton (including microalgae). In preliminary trials, we tested the relative efficiency of MPA against 0.3 M trichloroacetic acid (Speek *et al.*, 1984) for the extraction of ascorbic acid from *Chaetoceros gracilis*. In otherwise identical extraction protocols, 0.3 M trichloroacetic acid extraction recovered only 60% of the ascorbic acid compared to MPA extraction. On this basis, MPA was chosen as the extraction solution for all microalgal cultures in the present study.

Derivitisation and analysis of ascorbic acid

The extracted ascorbic acid was derivitised and analysed by the method of Speek *et al.* (1984), with minor modification, due to the use of MPA as the extractant instead of 0.3 M trichloroacetic acid. The basis of the assay is the enzymic oxidation of ascorbic acid to dehydroascorbic acid and the condensation of the latter with o-phenylenediamine (OPD) to form 3-(1,2-dihydroxyethyl) furo[3,4-*b*]-quinoxaline-1-one. This fluorescent derivitive is then chromatographed and analysed by high-performance liquid chromatography (HPLC).

An ascorbic acid-oxidase spatula (Boehringer) was added to each tube from above, and tubes were incubated for 5 min at 37 °C in a shaking water bath, mixing the tubes once at 2–3 min. The spatulas were removed and 0.25 ml of a freshly prepared solution of 0.1% (w/v) OPD was added. Tubes were incubated at 37 °C for 30 min, then filtered (0.45 μm syringe filter; Millipore) and stored until analysed (within 24 h).

For the derivitisation of standard, duplicate samples of 0, 0.25, 0.50, 0.75 and 1.0 ml of ascorbic acid solution (7 $\mu g\ ml^{-1}$) were added to 10 ml tubes, and the volume of each tube made up to 1.0 ml with water. To each tube was added 4.0 ml of 3% MPA and 1.2 ml of sodium acetate buffer (4.5 M pH 6.2). Standards were then incubated with the ascorbic acid-oxidase spatula. They were derivitised with OPD solution as for the samples, except that a greater volume (0.5 ml) of the 0.1% OPD solution was used.

HPLC analysis of samples and standards was carried out with a Varian Model 5000 LC by injecting 50 μl of the derivitized extracts onto a C18 Novapak (Waters; 3.9×150 mm) column. The column was eluted isocratically with 80% of 0.08 M potassium dihydrogen phosphate:20% methanol at a flow rate of $0.8\ ml\ min^{-1}$. The ascorbic acid derivative was detected with a Varian Fluorichrom set for an excitation maximum of 355 nm and an emission maximum of 425 nm. The peak area was quantified with a Vista 402 combined integrator and plotter.

Results

Reproducibility of extraction, derivitisation and analysis of samples

The ascorbic acid content for all but one of the cultures presented in Table 2 represents the analysis of a single filter, although two different aliquot volumes were derivitised from each extracted filter, to ensure that at least one assay was within the quantifiable range. For *Chaetoceros calcitrans* (#2 culture), log phase harvest, five individual filters were extracted and derivitised to establish

Table 2. Ascorbic acid concentrations of microalgae harvested at logarithmic and stationary growth phases. *T. pseudonana* (50 $\mu Em^{-2} s^{-1}$ cont.) and *T. pseudonana (100* μmol photon $m^{-2} s^{-1}$ cont.) denote cultures grown under continuous light at 50 and 100 $\mu Em^{-2} s^{-1}$ respectively; all other cultures grown at 100 μmol photon $m^{-2} s^{-1}$ under 12:12 h light:dark photoperiod. Average co-efficient of variation for the analyses of duplicate cultures: cells ml^{-1} 6.1%, dry weight (pg $cell^{-1}$) 11.9%; ascorbic acid (fg $cell^{-1}$) 7.7%; ascorbic acid (% dry weight) 13.9%. All ascorbic acid data represent the analysis of a single culture aliquot, except for *C. calcitrans* (log. phase), where $n = 5$ (mean $\pm$ S.D. given).

Algal species	Culture number	Cells ml^{-1} ($\times 10^{-6}$)		Dry weight (pg $cell^{-1}$)		Ascorbic acid (fg $cell^{-1}$)	Ascorbic acid (% dry wt.)		
		Log.	Stat.	Log.	Stat.	Log.	Stat.	Log.	Stat.
Chaetoceros calcitrans	#1	2.07	15.20	16.5	22.9	44	38	0.27	0.17
	#2	2.61	19.60	15.5	9.17	44 ($\pm$ 3.5)	33	0.28 ($\pm$ 0.022)	0.36
Chaetoceros gracilis	#1	3.10	6.89	29.8	57.9	560	171	1.87	0.29
	#2	3.50	6.89	34.1	43.1	460	144	1.36	0.34
Skeletonema costatum	#1	1.79	3.07	99.0	89.7	380	625	0.39	0.70
	#2	2.01	2.40	56.0	72.1	380	774	0.68	1.04
Thalassiosira pseudonana	#1	5.58	11.0	23.5	18.7	42	19	0.18	0.10
	#2	5.85	9.39	15.3	20.1	50	22	0.32	0.11
T. pseudonana (50 $\mu Em^{-2} s^{-1}$ cont.)	#1	8.49	n.d.	12.3	n.d.	30	n.d.	0.24	n.d.
	#2	7.29	n.d.	12.2	n.d.	34	n.d.	0.28	n.d.
T. pseudonana (100 $\mu Em^{-2} s^{-1}$ cont.)	#1	7.21	n.d.	15.2	n.d.	58	n.d.	0.38	n.d.
	#2	8.37	n.d.	19.4	n.d.	60	n.d.	0.31	n.d.
Dunaliella tertiolecta	#1	0.70	3.31	137.1	113.6	180	320	0.13	0.28
	#2	0.80	3.13	88.8	137.7	150	311	0.17	0.23
Nannochloris atomus	#1	3.19	31.6	16.8	11.5	88	89	0.53	0.77
	#2	2.95	28.9	14.9	12.4	64	92	0.43	0.74
Chroomonas salina	#1	1.49	3.08	117.3	80.5	250	163	0.21	0.20
	#2	0.45	n.d.	169.1	n.d.*	340	n.d.*	0.20	0.18
Nannochloropsis oculata	#1	7.23	69.0	9.1	4.8	55	9.3	0.60	0.19
	#2	7.44	68.0	6.6	4.6	67	9.4	1.01	0.20
Tetraselmis suecica	#1	0.40	1.74	227.2	279.7	580	500	0.26	0.18
	#2	0.35	1.48	281.3	285.8	480	370	0.17	0.13
Isochrysis sp. (T.ISO)	#1	5.40	13.3	16.4	36.1	73	59	0.44	0.16
	#2	5.50	11.6	21.0	36.4	79	78	0.38	0.21
Pavlova lutheri	#1	1.41	8.99	34.1	45.7	39	50	0.11	0.11
	#2	1.96	8.03	28.2	37.7	41	62	0.15	0.16

n.d. = not determined; * for this culture, the clumping of cells prohibited an accurate cell count being made.

the reproducibility of the extraction and the derivitisation procedure for samples. The co-efficient of variation for this analysis was 7.9%. The average co-efficient of variation of standards was 3.7%.

Variation in composition between duplicate cultures

The ascorbic acid levels were determined in the 11 species of microalgae harvested at logarithmic and stationary growth phases (Table 2). Calcula-

tion of cell density and dry weight for the cultures at harvest allowed ascorbic acid to be expressed as fg cell^{-1}, per cent cell dry weight and (for logarithmic phase cultures only) fg μm^{-3}.

For the individual cultures, levels of ascorbic acid (fg cell^{-1}) ranged from 9.3 (*N. oculata* #1; stationary phase) to 770 (*S. costatum* #2; stationary phase). Values for per cent ascorbic acid dry weight ranged from 0.10% (*T. pseudonana* #1; stationary phase) to 1.87% (*C. gracilis* #1; logarithmic phase). There were generally only minor differences in cellular levels of ascorbic acid between duplicate cultures of the same species; the average co-efficient of variation was 7.7%. The differences in levels of ascorbic acid as per cent dry weight between duplicate cultures was greater (average co-efficient of variation 13.9%); this was mainly because some duplicate cultures differed significantly in the total dry weight cell^{-1} (average co-efficient of variation 11.9%). Although great care was taken to ensure identical conditions between duplicate flasks, slight differences in culture growth factors such as light intensity and/or aeration rates could contribute, in part, to some of the differences noted between duplicate cultures.

Ascorbic acid cell^{-1}

The average values of ascorbic acid for the species ranged from 9.4 (*N. oculata*, stationary phase) to 700 fg cell^{-1} (*S. costatum*, stationary phase) (Fig. 1a). The levels of ascorbic acid were generally related to cell volume (Table 1). *S. costatum* and *T. suecica*, two of the largest algae studied, had the highest content of ascorbic acid cell^{-1}. *Chaetoceros calcitrans*, *T. pseudonana* and *N. oculata*, the three smallest algae studied, also had the lowest content of ascorbic acid cell^{-1}.

There was no general relationship between the stage of harvest and ascorbic acid cell^{-1}. *Chaetoceros gracilis*, *T. pseudonana*, *C. salina* and *N. oculata* had significantly more ascorbic acid cell^{-1} in logarithmic phase cultures than the stationary phase cultures, whereas the reverse was true for *S. costatum* and *D. tertiolecta*. *Nannochloropsis oculata* had the greatest difference; its logarithmic phase cultures contained approximately 6.5 times more ascorbic acid than stationary phase cultures. *Chaetoceros calcitrans*, *N. atomus*, *T. suecica*, *Isochrysis* sp. (T.ISO) and *P. lutheri* showed little or no difference in ascorbic acid cell^{-1} between logarithmic and stationary phase cultures.

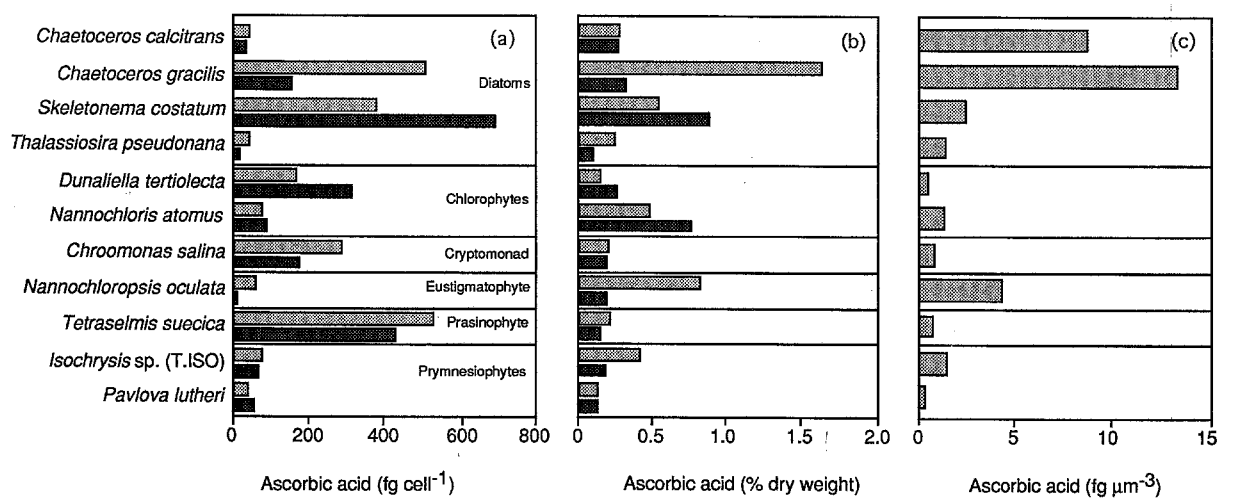

Fig. 1. Ascorbic acid in the microalgae harvested from logarithmic (▨) and stationary phase (■) cultures. (a) average ascorbic acid levels (fg cell^{-1}), (b) average ascorbic acid percentages (% dry weight) and (c) average ascorbic acid concentrations (fg μm^{-3}).

Per cent ascorbic acid

Because of the large differences in the size of the species studied (Table 1), expressing ascorbic acid as per cent dry weight gave a better relative measure of the composition of the species. There were major differences in the per cent ascorbic acid dry weight of many of the species (Fig. 1b). Average values ranged from 0.11% (*T. pseudonana*, stationary phase) to 1.62% (*C. gracilis*, logarithmic phase). This latter alga contained approximately twice the percentage of the next highest (0.87%; *S. costatum*, stationary phase). *Pavlova lutheri* contained the lowest per cent ascorbic acid during logarithmic phase (0.13%), the preferred stage of harvest for mariculture.

The differences noted in per cent ascorbic acid were not class related (Table 1, Fig. 1b). A significant difference was also noted in species from the same genus; for example, logarithmic phase cultures of *C. gracilis* contained approximately 6 times the per cent ascorbic acid as the same cultures of *C. calcitrans*.

The ascorbic acid per cent dry weight for many of the species differed significantly between logarithmic and stationary phase cultures (Fig. 1b). The differences were species-dependent and generally not related to algal class. *Chaetoceros gracilis*, *T. pseudonana*, *N. oculata* and *Isochrysis* sp. (T.ISO) had higher ascorbic acid per cent dry weight in logarithmic phase cultures than in stationary phase cultures. *Chaetoceros gracilis* also showed the greatest change in ascorbic acid per cent dry weight between growth phases; logarithmic phase cultures contained approximately five times more ascorbic acid than stationary phase cultures. *Skeletonema costatum*, and both the chlorophytes, *D. tertiolecta*, and *N. atomus*, contained higher per cent ascorbic acid in stationary phase. Cultures of *C. calcitrans*, *C. salina*, *T. suecica* and *P. lutheri* contained similar amounts in both the logarithmic phase and stationary phase.

Effect of light intensity on the content and per cent of ascorbic acid in Thalassiosira pseudonana

Additional cultures of *T. pseudonana* were grown under continuous light to assess the effect of light intensity and photoperiod on the content and per cent of ascorbic acid. Two light intensities were chosen: 50 and 100 μmol photon $m^{-2}s^{-1}$. Continuous light cultures at 50 μmol photon $m^{-2}s^{-1}$ received the same number of quanta d^{-1} as the standard 12:12 h light:dark cultures grown at 100 μmol photon $m^{-2}s^{-1}$. The content and per cent of ascorbic acid of these cultures harvested at logarithmic phase are compared in Table 2.

The cultures grown under the 12:12 h light:dark regime had less ascorbic acid $cell^{-1}$ (average 22 fg) compared to those grown under continuous light at 50 (average 26 fg) and 100 μmol photon $m^{-2}s^{-1}$ (average 35 fg). Per cent ascorbic acid in the duplicate cultures grown under the 12:12 h light:dark regime differed significantly; culture #1 contained 0.18% (less than all other *T. pseudonana* cultures) whereas culture #2 had 0.32% (more than all other *T. pseudonana* cultures, except 100 μmol photon $m^{-2}s^{-1}$ continuous light culture #1). The continuous light cultures grown at 100 μmol photon $m^{-2}s^{-1}$ contained more ascorbic acid $cell^{-1}$ (average 0.35%) than those grown at 50 μmol photon $m^{-2}s^{-1}$ (average 0.26%).

Concentration of ascorbic acid

To compare species of different sizes, the ascorbic acid contents were also normalised with respect to cell volume. The concentrations of ascorbic acid per cell volume (fg μm^{-3}) were calculated for logarithmic phase cultures (Fig. 1c). Both species of *Chaetoceros* had significantly higher concentrations of ascorbic acid (13.5 and 8.8 fg μm^{-3} for *C. gracilis* and *C. calcitrans*, respectively) than all remaining species (0.44 to 4.4 fg μm^{-3}).

Extracellular ascorbic acid

The extracellular culture media in one of the duplicate cultures from all the species (culture #1,

as indicated in Table 2) were analysed for ascorbic acid. The vitamin was not detected in any filtrates of logarithmic phase cultures, but was in the filtrates of three stationary phase cultures; *D. tertiolecta* (0.77 $\mu g\ ml^{-1}$), *Isochrysis* sp. (T.ISO) (0.11 $\mu g\ ml^{-1}$) and *C. salina* (0.035 $\mu g\ ml^{-1}$). Levels corresponded to 42%, 12% and 6.5%, respectively, of the total ascorbic acid (i.e. intracellular plus extracellular) in the cultures of these species. Microscopic examination of the cells from these cultures (prior to filtration) showed no visible signs of cell lysis or damage that might otherwise have accounted for the ascorbic acid in the medium.

Discussion

Ascorbic acid performs a range of important biochemical and physiological functions in plants and animals (Tolbert, 1979). At the biochemical level, it is an important reductant required by a range a mono- and di-oxygenase enzymes (Wefers & Sies, 1988; Englard & Seifter, 1986; Tolbert, 1979). In animals it promotes collagen formation in connective tissues (Hunter *et al.*, 1979; Sato *et al.*, 1982). It may also stimulate reproduction in fish (Collier *et al.*, 1956; Sandnes & Braekkan, 1981) and moulting in crustaceans (Desjardins *et al.*, 1985). Whether this micronutrient is essential for most marine animals is not known. However, in those animals that have been studied, most appear unable to biosynthesise the molecule, and so require it in their diet.

Fish that require the vitamin include rainbow trout *Salmo gairdneri*, coho salmon *Oncorhynchus kisutch* (Halver *et al.*, 1969), channel catfish *Ictalurus punctatus* (Durve & Lovell, 1982) and tilapia *Tilapia aurea* (Stickney *et al.*, 1984). Crustaceans that require ascorbic acid include *Penaeus japonicus* (Kanazawa, 1985) and *Penaeus stylirostris* (Lightner *et al.*, 1977). At least one crustacean, – juvenile lobster (*Homarus americanus*), – is capable of *de novo* biosynthesis of the vitamin (Desjardins *et al.*, 1985). Copepods, which are commonly used in mariculture as food for fish and crustacean larvae, may also be unable to biosynthesize ascorbic acid (Happette & Poulet, 1990b). Whether ascorbic acid is essential for maricultured mollusc species has not yet been established.

Most microalgal species that have been examined for their ascorbic acid content are not commonly used in mariculture. Bayanova and Trubachev (1981) analysed seven species including *Chlorella vulgaris*, *Platymonas viridis* (chlorophytes), *Synechococcus elongatus*, *Coccopedia* sp., *Spirulina platensis* (cyanophytes), *Cyanidium caldarium* and *Porphyridium cruentum* (rhodophytes) and found levels ranging from 0.054% (*P. viridis*) to 0.24% (*C. vulgaris*). Happette and Poulet (1990a, b) analysed six species from four classes, i.e. *Dunaliella primolecta*, *D. tertiolecta* (chlorophytes), *Thalassiosira weissflogii*, *Chaetoceros* sp. (diatoms), *Cryptomonas maculata* (cryptomonad) and *Phaeocystis pouchetii* (prymnesiophyte). Ascorbic acid values ranged from 35 (*P. pouchetti*; small cell) to 6450 fg $cell^{-1}$ (*C. maculata*; large cell). *Dunaliella tertiolecta* contained 347 fg $cell^{-1}$, a level similar to that found for the strain of same alga in this study during its stationary phase (320 fg $cell^{-1}$).

Several other species studied here have been analysed before. De Roeck *et al.* (1991) analysed the percent ascorbic acid in cultures of *T. suecica*, *P. lutheri*, *S. costatum* and *C. calcitrans* grown under continuous light. However, they did not report the light intensity or the growth phase of the algae when analysed. The values reported were all significantly lower than those reported here for the sames species. They found *S. costatum*, *C. calcitrans*, *T. suecica* and *P. lutheri* to contain 0.006%, O.012%, 0.050% and 0.084% ascorbic acid respectively. The same algae in this study contained ranges of per cent (from the duplicate cultures; both logarithmic and stationary growth phases) from 0.39–1.04%, 0.17–0.36%, 0.13–0.26% and 0.11–0.16% respectively. Of note, the alga De Roeck *et al.* (1991) found to be the richest, i.e. *P. lutheri*, we found to be the least. While differences in culture conditions could account in part for these differences, more likely inappropriate storage or extraction by De Roeck *et al.* (1991) could have led to their underestimat-

ing the vitamin. Unfortunately, De Roeck *et al.* (1991) did not specify whether the alga were freshly harvested, frozen or freeze-dried prior to analysis. We have noted a >90% reduction in ascorbic acid content in filtered samples of *S. costatum* stored at −20 °C for 2 months (Brown & Miller, unpublished). For this reason, only freshly harvested algae were analysed in the present study.

The effects of light intensity and photoperiod on the content and per cent of ascorbic acid in logarithmic phase cultures of *T. pseudonana* were studied. Cultures grown under continuous light were rich in ascorbic acid and both the content and per cent ascorbic acid increased with light intensity. In fact, cultures grown under a 12:12 h light:dark regime contained less ascorbic acid cell^{-1} than continuous light cultures. The relationship between photoperiod and per cent ascorbic acid in the cultures was not clear, due to the variability in composition between the duplicate 12:12 h light dark cultures.

The per cent of ascorbic acid in the logarithmic phase culture of *C. gracilis* #1 (1.87%) represents the highest percentage of the vitamin reported in a microalga. The next highest value reported (1.5%) is for the freshwater chlorophyte *Chlorella vulgaris* (Aaronson *et al.*, 1977). Interestingly, the two species containing the highest % ascorbic acid during logarithmic phase (i.e. *C. gracilis* and *N. oculata*) were not axenic. Possibly, bacteria might be contributing to or stimulating the production of ascorbic acid (by microalgae) in these cultures. Although this hypothesis was not tested here, this aspect could be worthy of future investigation.

The average values of per cent ascorbic acid in the microalgae reported here (0.11–1.62%) are similar to those reported for higher plants. Bayanova and Trubachev (1981) analysed the root crop and leaves of carrot, beet, radish and tomato, as well as cucumber, dill, kale and onion. They found the vitamin level ranged from 0.06% (carrot root crop) to 0.56% (radish root crop).

Some species of microalgae may excrete significant quantities of ascorbic acid into the extracellular culture medium (Aaronson *et al.*, 1977). This is nutritionally important when such algae are added to animal-rearing tanks because animals can absorb dissolved organic molecules such as vitamins (Scott, 1981), amino acids (Manahan & Stephens, 1983) and carbohydrates (DiDomenico & Iverson, 1977) directly from seawater. Aaronson *et al.* (1971) found *Poteriochromonas danica* grown in the light, contained only 0.083% ascorbic acid but excreted approximately 2.5 times this amount into the culture medium. When the same alga was cultured in the dark, using glucose, virtually all the ascorbic acid was excreted into the medium, reaching a concentration of 600–1100 $\mu g\, l^{-1}$ at harvest (Grün and Loewus, 1983). A similar excretion of ascorbic acid occurs in the freshwater chlorophyte, *Euglena gracilis* (Baker *et al.*, 1981). In the present study, ascorbic acid was recovered from stationary phase culture filtrates of only three of the 11 species: i.e. *D. tertiolecta*, *C. salina*, *Isochrysis* sp. (T.ISO). For *D. tertiolecta*, this represented a significant amount (42%) of the total ascorbic acid in cultures, whereas it was less significant for *Isochrysis* sp. (T.ISO) and *C. salina* (11.8 and 6.5%, respectively). Cell damage from the filtering process, with subsequent cell leakage of ascorbic acid, may have contributed to the vitamin detected externally. Although great care was taken during the filtering process (vacuum maintained at ≤100 mm Hg), *D. tertiolecta* and *C. salina* have been shown by Goldman and Dennett (1985) to be susceptible to cell damage under such conditions.

The present work shows that microalgae, which are used as basic feedstock for most mariculture food chains, differ significantly in their individual content of ascorbic acid. Average per cent ascorbic acid ranged from 0.11 to 1.62% dry weight, and cell concentrations from 0.44 to 13.5 fg ascorbic acid μm^{-3}. By comparing microalgae vitamin levels with dietary requirements, it is possible to assess whether microalgae are supplying enough ascorbic acid for the needs of animals feeding on them. Although dietary requirements are unavailable for the larval life stages of animals feeding directly on algae, indications can be drawn

from the known requirements of juveniles and adults of some marine species. Daily requirements of ascorbic acid for fish are 0.003–0.015% of dry diet, dependent on species and life stage (Millikin 1982; Durve & Lovell, 1982; Stickney *et al.*, 1984). The juvenile prawn *Penaeus japonicus* requires 0.02% of Mg-ascorbyl phosphate (a biologically active, synthetic analogue of ascorbic acid which is more stable) in artificial diets for optimal growth (Shigueno & Itoh, 1988). From the data, it would seem likely that all the microalgae presently studied supply enough ascorbic acid to animals feeding directly on them. Although ascorbic acid requirements are not established for molluscs, *P. lutheri* (the alga containing the least ascorbic acid in log phase cultures) has proved to be excellent food for a range of molluscs (Brown *et al.*, 1989).

Ascorbic acid transfer between trophic levels is important for fish larvae and late larval/early juvenile crustaceans that are reared on algal-fed zooplankton. Happette and Poulet (1990b) fed microalgae to previously starved populations of the copepods *Calanus helgolandicus* and *Acartia clausi*. They noted increases in the ascorbic acid levels of 50% and 60%, respectively, for the animals. Their results supported the hypotheses that ascorbic acid from the microalgae can be incorporated with high efficiency through direct feeding. Despite the 15-fold range in the per cent ascorbic acid of the microalgae studied here, all the species, if ingested and digested, should provide an adequate supply of ascorbic acid to cultured animals at the next trophic level of the mariculture food chain.

In conclusion, significant differences were found in the composition of ascorbic acid between some of the microalgae studied. Also, for many of the species, differences in the level of the vitamin were noted between logarithmic and stationary phase cultures. Despite the variations, all the microalgae could provide a rich source of ascorbic acid to mariculture food chains. Feeding trials are now essential for rigorous confirmation.

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References

Aaronson S, DeAngelis B, Frank O, Baker H (1971) Secretions of vitamins and amino acids into the environment by *Ochromonas danica*. J. Phycol. 7: 215–218.

Aaronson S, Dhawale SW, Patni J, DeAngelis B, Frank O, Baker H (1977) The cell content and secretion of water-soluble vitamins in several freshwater algae. Arch. Microbiol. 112: 57–59.

Baker JT (1984) Seaweeds in pharmaceutical studies and applications. Hydrobiolgia 116/117: 29–40.

Bayanova YI, Trubachev IN (1981) Comparative evaluation of the vitamin composition of some unicellular algae and higher plants grown under artificial conditions. Appl. Biochem. Microbiol. 17: 292–298.

Brown MR (1991) The amino-acid and sugar composition of 16 species of microalgae used in mariculture. J. exp. mar. Biol. Ecol. 145: 79–99.

Collier A, Ray S, Wilson WB (1956) Some effects of specific organic compounds on marine organisms. Science 124: 220.

De Roeck-Holtzhauer Y, Quere I, Claire C (1991) Vitamin analysis of five planktonic microalgae and one macroalga. J. appl. Phycol. 3: 259–264.

Desjardins LM, Castell, JD, Kean, JC (1985) Synthesis of dehydroascorbic acid by subadult lobsters (*Homarus americanus*). Can. J. Fish. aquat. Sci. 42: 370–373.

DiDomenico DA, Iverson RL (1977) Uptake of glycollic acid by a marine bivalve. J. exp. mar. Biol. Ecol. 28: 234–254.

Durve VS, Lovell RT (1982) Vitamin C and disease resistence in channel catfish (*Ictalurus punctatus*). Can. J. Fish. aquat. Sci. 39: 948–951.

Englard S, Seifter S (1986) The biochemical functions of ascorbic acid. Ann. Rev. Nutr. 6: 365–406.

Enright CT, Newkirk GF, Craigie JS, Castell JD (1986) Evaluation of phytoplankton as diets for juvenile *Ostrea edulis* L. J. exp. mar. Biol. Ecol. 96: 1–13.

Epifanio CE, Valenti CC, Turk CL (1981) A comparison of *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* as foods for the oyster, *Crassostrea virginica*. Aquaculture 23: 347–353.

Goldman JC, Dennett MR (1985) Susceptibility of some marine phytoplankton species to cell breakage during filtration

and post filtration rinsing. J. exp. mar. Biol. Ecol. 86: 47–58.

Grün M, Loewus FA (1983) Determination of ascorbic acid in algae by high-performance liquid chromatography on strong cation-exchange resin with electrochemical detection. Analyt. Biochem. 130: 191–198.

Guillard RRL, Ryther JH (1962) Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol. 8: 229–239.

Halver JE, Ashley LM, Smith RR (1969) Ascorbic acid requirement of coho salmon and rainbow trout. Trans. am. Fish. Soc. 98: 762–771.

Hapette AM, Poulet SA (1990a) Application of high-performance liquid chromatography to the determination of ascorbic acid in marine plankton. J. Liquid Chromatogr. 13: 357–370.

Hapette AM, Poulet SA (1990b) Variation of vitamin C in some common species of marine plankton. Mar. Ecol. Prog. Ser. 64: 69–79.

Hunter B, Magarelli PC Jr, Lightner DV, Colvin LB (1979) Ascorbic acid-dependent collagen formation in penaeid shrimp. Comp. Biochem. Physiol. 64B: 381–385.

Jeffrey SW (1980) Cultivating uni-cellular marine plants. In CSIRO Division of Fisheries and Oceanography Research Report, 1977–1979, pp. 22–43.

Kanazawa A (1985) Nutrition of penaeid prawns and shrimps. In Taki Y, Primavera JJ, Llobrera JA (eds), Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps, Aquaculture Dept., SEAFDEC, Iloilo City, Philippines, 1984. pp. 123–130.

Langdon CJ, Waldock MJ (1981) The effect of algal and artificial diets on the growth and fatty acid composition of *Crassostrea gigas* spat. J. mar. biol. Ass., U.K. 61: 431–448.

Lightner DV, Colvin LB, Brand C, Danald DA (1977) 'Black Death', a disease syndrome of penaeid shrimp related to a dietary deficiency of ascorbic acid. Proc. World Maricul. Soc. 8: 611–618.

Lin DS, Ilias AM, Connor WE, Caldwell RS, Cory HT, Daves, GD (1982) Composition and biosynthesis of sterols in selected marine phytoplankton. Lipids 17: 818–824.

Loeblich AR, Smith VE (1968) Chloroplast pigments of the marine dinoflagellate *Gyrodinium resplendens*. Lipids 3: 5–13.

Manahan DT, Stephens GC (1983) The use of high performance liquid chromatography to measure dissolved organic compounds in bivalve aquaculture systems. Aquaculture 32: 339–346.

Millikin MR (1982) Qualitative and quantitative nutrient requirements of fishes: a review. Fish. Bull. U.S. 80: 655–686.

Sandnes K, Braekkan OR (1981) Ascorbic acid and the reproductive cycle of ovaries in cod (*Gadus morrhua*). Comp. Biochem. Physiol. (A) 70: 545–546.

Sato M, Kondo T, Yashinaka R, Ikeda S (1982) Effect of dietary ascorbic acid levels on collagen formation in rainbow trout. Bull Jap. Soc. sci. Fish. 48: 553–556.

Scott JM (1981). The vitamin B_{12} requirement of the marine rotifier *Brachionus plicatilis*. J. mar. biol. Ass., U.K. 61: 983–994.

Shigueno K, Itoh S (1988) Use of Mg-L-ascorbyl-2-phosphate as a vitamin C source in shrimp diets. J. World Aquacult. Soc. 19: 168–174.

Smayda TJ (1978) From phytoplankters to biomass. In Sournia A (ed.), Phytoplankton manual, UNESCO monographs on oceanographic methodology UNESCO, 273–279.

Speek AJ, Schrijver J, Schreurs WHP (1984) Fluorometric determination of total vitamin C in whole blood by high-performance liquid chromatography with pre-column derivatization. J. Chromatogr. 305: 53–60.

Stickney RR, McGeachin RB, Lewis DH, Mars J, Riggs A, Sis RF, Robinson EH, Wurts W (1984) Response of *Tilapia aurea* to dietary vitamin C. J. World Maricult. Soc. 15: 179–185.

Thompson PA, Harrison PJ, Whyte JNC (1990) Influence of irradiance on the fatty acid composition of phytoplankton. J. Phycol. 26: 278–288.

Tolbert BM (1979) Ascorbic acid metabolism and physiological functions. Int. J. Vit. Nutr. Res., Suppl. 19: 127–142.

Volkman JK, Jeffrey SW, Nichols PD, Rogers GI, Garland CD (1989) Fatty acids and lipid classes of ten species of microalgae used in mariculture. J. exp. mar. Biol. Ecol. 128: 219–240.

Webb KL, Chu FE (1983) Phytoplankton as a food source for bivalve larvae. In Pruder DG *et al.* (eds), Proc. of the 2nd int. conf. aquaculture nutrition, World Mariculture Society, Spec. Publ. No. 2, Louisiana State University, Louisiana, pp. 272–291.

Wefers H, Sies H (1988) The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependant on vitamin E. Eur. J. Biochem. 174: 353–357.

Whyte JNC (1987) Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. Aquaculture 60: 231–241.

Wikfors GH, Twarog TW, Ukeles R (1984) Influence of chemical composition of algal food sources on growth of juvenile oysters, *Crassostrea virginica*. Biol. Bull. 167: 251–263.