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Aquaculture
March 2006; 253(1-4) : 618-627
http://dx.doi.org/10.1016/j.aquaculture.2005.09.011
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New microalgae for the Pacific oyster Crassostrea gigas larvae

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Abstract: The number of microalgae of high nutritional value actually available to mollusc hatcheries is limited to a few species and this represents a constraint for the industry. Fifteen microalgal species belonging to seven different taxonomic classes were selected from the Algobank-Caen microalgal culture collection of the University of Caen. An initial screening of these microalgae, based on their cytomorphological characteristics and growth performances, led to the selection of four species (Imantonia rotunda, Emiliania huxleyi, Pseudoisochrysis paradoxa and Diacronema vlkianum), which were then tested as food for Crassostrea gigas larvae. Two species (I. rotunda and E. huxleyi) were of poor food value for oyster larvae, while P. paradoxa and D. vlkianum resulted in high growth rate and low mortality. These two microalgae were then characterised (dry weight, ash, gross composition, fatty acids) at different stages of growth and their productivity in standard hatchery conditions (2-l glass carboys, 300-l cylinders) was assessed in order to evaluate the potential of these species for use in commercial hatcheries.

Keywords: Microalgae; Mollusc hatchery; Larval nutrition; Biochemical composition

Introduction

Mollusc culture comprises a considerable part of world aquaculture production, representing 14 million tonnes in the year 2000 (from FAO statistics in Helm and Bourne, 2004). Traditionally, mollusc culture depends on juvenile collection from the natural environment. However, due to the high seasonality (spat can only be collected during a short period of the year) and unpredictability (variation in recruitment from year to year) of this activity, spat production in hatcheries is increasingly important. Moreover, the use of genetic tools in mollusc hatcheries allows the production of triploid spat, characterized by increased growth. Published records of mollusc production in hatcheries are scarce. For the Pacific oyster (*Crassostrea gigas*), Muller-Feuga (2000) reported that hatchery production covered 80% of juvenile requirements on the west coast of the USA, but only 10% in French hatcheries. At present, we estimate the latter value to have increased up to 20-25%.

Spat production in hatcheries is strictly dependent on the quality and quantity of the cultured microalgae used as feed. High quality microalgae are particularly important for feeding early stage bivalves (larvae and post-larvae) and for broodstock conditioning. Although about 50 species of microalgae have been tested over the years as food for bivalves (Davis and Guillard, 1958; Enright et al., 1986; Walne, 1970; Brown, 1991, 1997; Wikfors and Onho, 2001), less than 10 species, belonging to the Bacillariophyceae (*i.e.* diatoms), the Haptophta and the Prasinophyceae are routinely cultured in shellfish hatcheries (Robert et al., 2004). Moreover, in the case of nutritionally highly demanding molluscs such as the Pacific oyster, this number is further reduced.

The limited number of microalgae actually available for mollusc rearing represents a major constraint for shellfish hatcheries and in recent years the identification of novel microalgal species of high nutritional value has been the subject of ongoing research in many experimental laboratories worldwide (Brown et al., 1998; Southgate et al, 1998; Renaud et al., 1999, 2002; Thinh et al., 1999; Knuckey et al., 2002;). To be successfully used in hatcheries, microalgae should fulfil four main criteria (Muller-Feuga et al., 2003a). They must be of an appropriate size to be efficiently ingested, they should not have a cell covering that inhibits digestion, they should have a high nutritional value particularly with respect to HUFA and, lastly, they should be amenable to production in the different systems and scales operating in hatcheries. The aim of this work was to assess the potential of novel microalgal strains for use in mollusc hatcheries, taking into account the criteria outlined. Fifteen microalgae belonging to seven different taxonomic classes (Prymnesiophyceae, Pavlovophyceae, Prasinophyceae, Dinophyceae, Dictyochophyceae, Cryptophyceae, Bangiophyceae) were screened on the basis of their cytomorphological characteristics and growth performances. This phase led to the selection of four species (*I. rotunda*, *E. huxleyi*, *P. paradoxa* and *D. vlkianum*), which were then tested as food for *C. gigas* larvae. Finally, biochemical characterisation at different stages of growth, and productivity in large volume cultures (300-l Perspex cylinders), were assessed for *D. vlkianum* and *P. paradoxa*, *i.e.* both species exhibited high nutritional value.

2. Materials and methods

2.1 Microalgal culture

Microalgal strains, all from the Algobank-Caen culture collection of the University of Caen, were initially cultured in 250-ml Erlenmeyer flasks and then in 2-l glass carboys and in 300-l Perspex cylinders. Seawater at ambient salinity (33-34‰) was 1-µm filtered, enriched with sterilised Conway medium (Walne, 1966) and either autoclaved (≤ 2 l) or U.V. treated (300 l). For the culture of the diatom *Chaetoceros calcitrans*, forma *pumilum* sodium metasilicate (40 mg l^{-1}) was added as a silica source and salinity was reduced to 25‰ by addition of distilled water.
Temperature and continuous illumination, provided by white fluorescent tubes, ranged between 19-20 °C and 35-50 µmol photons m^{-2} s (flasks) and 22-23 °C and 180-220 µmol photons m^{-2} s (carboys and cylinders), respectively. Carboys and cylinders were aerated with a 3% CO_2/air mixture to support growth and to maintain the pH within a range of 7.5-8.1.

2.2 Oyster culture

Gametes were obtained from broodstock, previously conditioned as described by Robert and Gérard (1999). Embryonic development was carried out at 24 °C in 150-l tanks containing gently aerated seawater (1-µm filtered, and 33-34‰ salinity) at a density of 50 larvae ml^{-1}. Forty-eight h after hatching, straight-hinged larvae were sieved on a 45-µm nylon mesh and transferred, at a density of 5 larvae ml^{-1}, into 2-l glass beakers, each containing 1.8-l of 1-µm filtered seawater. Two feeding trials, each lasting 14 days, were carried out with diets run in triplicate. Microalgae (*P. paradoxa*, *D. vlkianum*, *E. huxleyi*, *I. rotunda*) were tested in bispecific diets, in association with *C. calcitrans f. pumilum*. The food ration, based on a bispecific assemblage, 20% diatom-80% flagellate, was provided every second day, at a concentration of 50 cells $µl^{-1}$ during the first week and then at 100 cells $µl^{-1}$ (Helm and Bourne, 2004). All microalgae were considered approximately equivalent to the Tahitian clone of *Isochrysis aff. galbana* (T-Iso) in terms of cell volume. A negative control consisting of unfed larvae and a positive control fed with *C. calcitrans f. pumilum* + T-Iso were also included in each trial. For each replicate, at least 200 larvae were sampled at the beginning (day 2 from fertilisation), middle (day 9) and end (day 16) of each feeding trial. Mortality was determined by counting the translucent larval shells under the microscope and growth was assessed by measuring the major shell length by means of image processing (software IMAQ Vision Builder, National Instruments).

2.3 *Analytical procedures*

Growth of microalgal cultures was estimated daily using a Malassez haemocytometer while cell size, expressed in equivalent diameter, was determined with a Coulter Counter (ZM). Growth rate was calculated during the active growth phases, as: $\mu = [(\ln N_1 - \ln N_0)/(t_1 - t_0)]/\ln 2$, where N_1 = measurement at time 1 (t_1), N_0 = measurement at time 0 (t_0). For dry weight, 50 ml of biomass were harvested in triplicate and then centrifuged (3200 g, 10 min); pellets were then rinsed with 20 ml of ammonium formate 0.5 M, re-centrifuged and placed

in pre-weighed tin capsules. Dry weight and ash were measured after heating at 80 °C (overnight) and 450 °C (4 h), respectively. Biochemical analyses were performed on *D. vlkianum* and *P. paradoxa* samples harvested during the exponential and stationary phases of growth. To this end, cultures were grown in duplicate and kept at 22 (±1) °C under continuous illumination (205 µmol photons m^{-2} s). For gross composition 50 ml of culture were harvested and centrifuged (3200 g, 10 min). Pellets were stored at –20°C for a period of up to six months prior to analysis. Proteins, lipids and carbohydrates were measured according to the methods described by Lowry et al (1951), Bligh and Dyer (1959) and Dubois et al. (1956), respectively. For fatty acid and sterol analyses, 10-50 ml of culture (according to cellular concentration) were filtered through 47-mm precombusted (450 °C for 8 h) Whatman GF/F glass-fibre filters and stored at -20 °C in chloroform-methanol (2/1) for a period of up to six months prior to analysis. After sonication (10 min), neutral and polar lipids were separated on a silica (Si100) micro-column and each fraction was eluted with a solution of chloroform-methanol (98/2) and with methanol, respectively. Fatty acids were analysed according to Marty et al. (1992), using a gas-chromatograph (Hewlett-Packard, HP6890) equipped with a J&W 65 DB Wax column (30 m x 0.25 mm; 0.25-µm film thickness) and 23:0 as an internal standard. Neutral sterols were analysed according to the method described by Soudant et al. (2000), using a gas-chromatograph (Chrompack CP 9002) equipped with a Rt X 65 Restek column (15 m x 0.25 mm; 0.25-µm film thickness) and cholestane as an internal standard. Polar sterols were extracted by trans-methylation using a 98/2 sulphuric acid-methanol solution and were analysed using the same method as that described for neutral sterols.

Biochemical analysis results (gross, fatty acids and sterol compositions) were expressed in relative terms (percentage of organic matter or percentage of total fatty acids or total sterols) allowing comparisons independently of microalgae cells weight which increased from exponential to stationary phases. Total fatty acids and total sterols weights were also reported to allow quantitative terms calculations (weight of each component per cell = total weight* relative composition).

Significant differences between experimental conditions were detected by means of ANOVA and Scheffe tests ($P<0.05$) and data expressed in percentage previously transformed (arcsine square root $x_i\ 100^{-1}$) before statistical treatments.

3. Results

3.1 Microalgal screening and larval feeding trials

Among the different species, three prymnesiophytes (*E. huxleyi*, *I. rotunda*, *P. paradoxa*) and two pavlovophytes (*D. vlkianum, P. virescens*) showed high productivity, equal to or greater than *P. lutheri* and T-Iso used here as controls (Table 1). These species were then cultured in 2-l glass carboys and their nutritional value assessed on *C. gigas* larvae. At this stage, *P. virescens* was discarded due to low growth in carboys.

Larvae fed *P. paradoxa* or *D. vlkianum* exhibited high survival (>90% during the first week, >84% during the second week) and growth which was similar to that of larvae fed with the diatom solely (Fig. 1A). Indeed, after the first week of the trial, larvae fed diet

including *P. paradoxa* exhibited growth similar to the positive control (*C. calcitrans f. pumilum* + T-Iso), while no differences were detected between diets composed by *P. paradoxa* or by *D. vlkianum* at the end of the trial (second week). In contrast, *I. rotunda* and *E. huxleyi* led to poor larval development, far lower than the single diet (*C. calcitrans f. pumilum*) and in fact similar to unfed larvae for the diet including *I. rotunda* during the first week of rearing (Fig. 1B). Moreover, larval survival was negatively affected by these diets (<63% for both species after the second week).

Table 1

Size, cell density and growth rate of several microalgae selected from Algobank-Caen

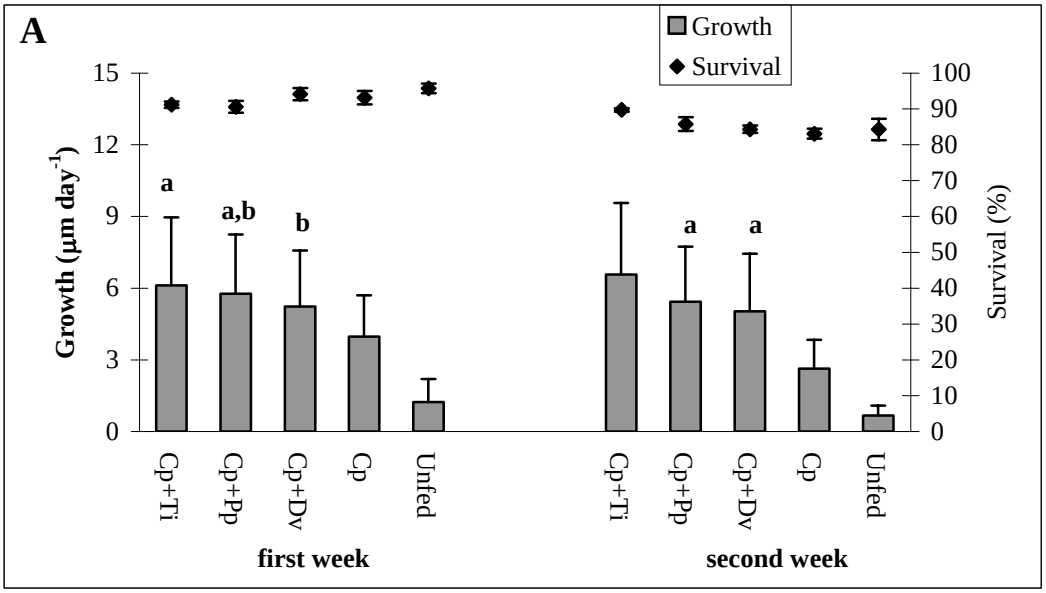
	Code Algobank-Caen	Size (μm)	Cells (ml^{-1} x10^6)	Growth rate (division day^{-1})
Prymnesiophyceae				
Imantonia rotunda	AC23	3.32 ± 0.32	9.7	0.89
Emiliania huxleyi haploid phase	AC472	3.66 ± 0.39	3.5	0.50
Isochrysis sp.	AC66	4.27 ± 0.40	n.d.	n.d.
Pseudoisochrysis paradoxa	AC80	4.29 ± 0.59	30.6	0.25
Isochrysis aff. galbana (T-Iso)*	AC102	6.35 ± 0.72	15.6	0.33
Pavlovophyceae				
Diacronema vlkianum	AC67	5.83 ± 0.41	14.2	0.38
*Pavlova lutheri**	AC44	6.31 ± 0.15	8.9	0.36
Pavlova virescens	AC16	5.45 ± 0.56	5.0	0.43
Pavlova sp.	AC33	4.95 ± 0.25	2.2	0.18
Prasinophyceae				
Nephroselmis astigmata	AC259	9.75 ± 1.22	3.6	0.25
Dinophyceae				
Gymnodium sp.	AC212	13.41 ± 1.08	0.4	0.27
Gymnodium sp. Dinox	AC210	11.92 ± 0.99	0.9	0.23
Dictyochophyceae				
Pseudopedinella pyriforme	AC5	6.89 ± 0.33	0.7	0.11
Cryptophyceae				
Chroomonas sp. CryptoB	AC161	10.92 ± 1.84	1.5	0.19
Rhodomonas sp. Crypto R	AC160	11.90 ± 0.55	2.5	0.20
Bangiophyceae				
Porphyridium purpureum	AC120	5.82 ± 0.45	20	0.20

*Controls; n.d. = none detected

3.2 Characterisation of P. paradoxa and D. vlkianum

The main characteristics of *D. vlkianum* and *P. paradoxa*, as well as their productivity in 2-l glass carboys and 300-l Perspex cylinders, are presented in Table 2. These species attained high cell concentrations in both systems (>25 x 10^6 cells ml^{-1} in carboys, >10 x 10^6 cells ml^{-1} in cylinders) with good daily productivities (0.8 and 0.5 division day^{-1}, respectively).
Cellular size and dry weight were clearly higher in *P. paradoxa* and *D. vlkianum* harvested in the stationary phase of growth while ash content remained constant (insignificant differences: Table 2).

Fig 1 A and B. Length and survival (average ± S.D.) of *C. gigas* larvae fed on different diets, after 1 and 2 weeks. Initial shell length: first trial (A) = 83.0 ± 5.4 µm; second trial (B) = 78.2 ± 4.8 µm. Values within the same column sharing a common superscript letter are not significantly different (P>0.05).
Cp+T-Iso = *C. calcitrans* f. *pumilum* + *Isochrysis aff. galbana* clone T-Iso;
Cp+Pp = *C. calcitrans* f. *pumilum* + *Pseudoisochrysis paradoxa*;
Cp+Dv = *C. calcitrans* f. *pumilum* + *Diacronema vlkianum;*
Cp+Eh = *C. calcitrans* f. *pumilum* + *Emiliania huxleyi*;
Cp+Ir = *C. calcitrans* f. *pumilum* + *Imantonia rotunda;*
Cp20 = *C. calcitrans* f. *pumilum* (20% of ration);
Unfed = starved larvae.



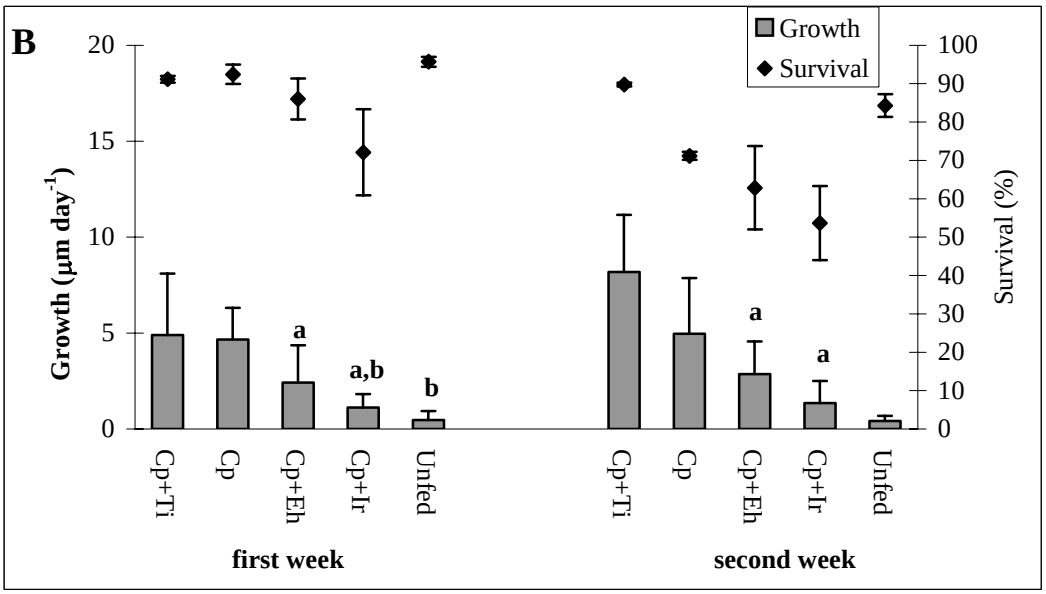


Table 2. Biochemical and productivity characteristics of *D. vlkianum* and *P. paradoxa*

harvested in exponential (Exp.) or stationary (St.) phase of growth (mean ± SD; size n≥15 000; dry weight, ash gross composition and productivity n≥2)

	D. vlkianum			***P. paradoxa***		
	Exp.	St.		Exp.	St.	
Size (µm)	4.37 ± 0.40	4.57 ± 0.58	n.d.	3.98 ± 0.34	4.64 ± 1.23	n.d.
Dry weight (pg cell^{-1})	14.53 ± 0.75	19.27 ± 0.39	*	10.32 ± 1.09	12.68 ±0.11	*
Ash (pg cell^{-1})	0.72 ± 0.30	0.45 ± 0.17		1.21 ± 0.08	1.15 ± 0.13	
Proteins (% organic matter)	24.65 ± 0.06	29.83 ± 1.50	*	48.75 ± 1.40	30.67 ± 0.55	*
Carbohydrates (% OM)	31.49 ± 0.97	23.40 ± 6.64		11.41 ± 0.52	29.16 ± 0.88	*
Lipids (% OM)	33.58 ± 3.43	39.16 ± 3.69		29.97 ± 0.36	35.50 ± 0.03	*
Productivity 2-l carboys (division day^{-1})	0.90 ± 0.01			0.99 ± 0.01		
Productivity 300-l cylinders (division day^{-1})	0.76 ± 0.23			0.52 ± 0.13		

*: -P<0.05; n.d. = none detected

Lipids represented the main component of *D. vlkianum* (33-39% of organic matter, OM), while proteins and carbohydrates accounted for 24-30% and 23-31% of organic matter, respectively. A significant increase in protein content was observed in *D. vlkianum* during the stationary phase of growth.

In the exponential phase of growth, *P. paradoxa* was rich in protein (49% OM), while carbohydrate and lipid represented 11% and 30%, respectively. In the stationary phase of growth, significant increases in carbohydrate (29% OM) and lipid (35% OM) and a decrease in protein (31% OM) were recorded.

Total fatty acid content was higher for both species during the stationary phase of growth (Table 3). In *D. vlkianum* the major fatty acids were the unsaturated 14:0 and 16:0, the monounsaturated 16:1n-7 and the polyunsaturated EPA; these fatty acids accounted for more than 69% of total fatty acids. During the exponential phase of growth, *D. vlkianum* was richer in polyunsaturated fatty acids, particularly in the n-3 fraction, including 18:4n-3, EPA and DHA. The n-9 monounsaturated and n-6 polyunsaturated fatty acid fractions, as well as 16:0, 18:1n-9 and 18:2n-6 content increased significantly in the stationary phase of growth.

Table 3. Fatty acid composition (mean ± SD; n=2) of *D. vlkianum* and *P. paradoxa* in exponential (Exp.) or stationary (St.) phase of growth. Individual data are reported as percentage of total fatty acids while total fatty acids (TO FA) are expressed either total dry weight or percentage of total organic matter content (OM)

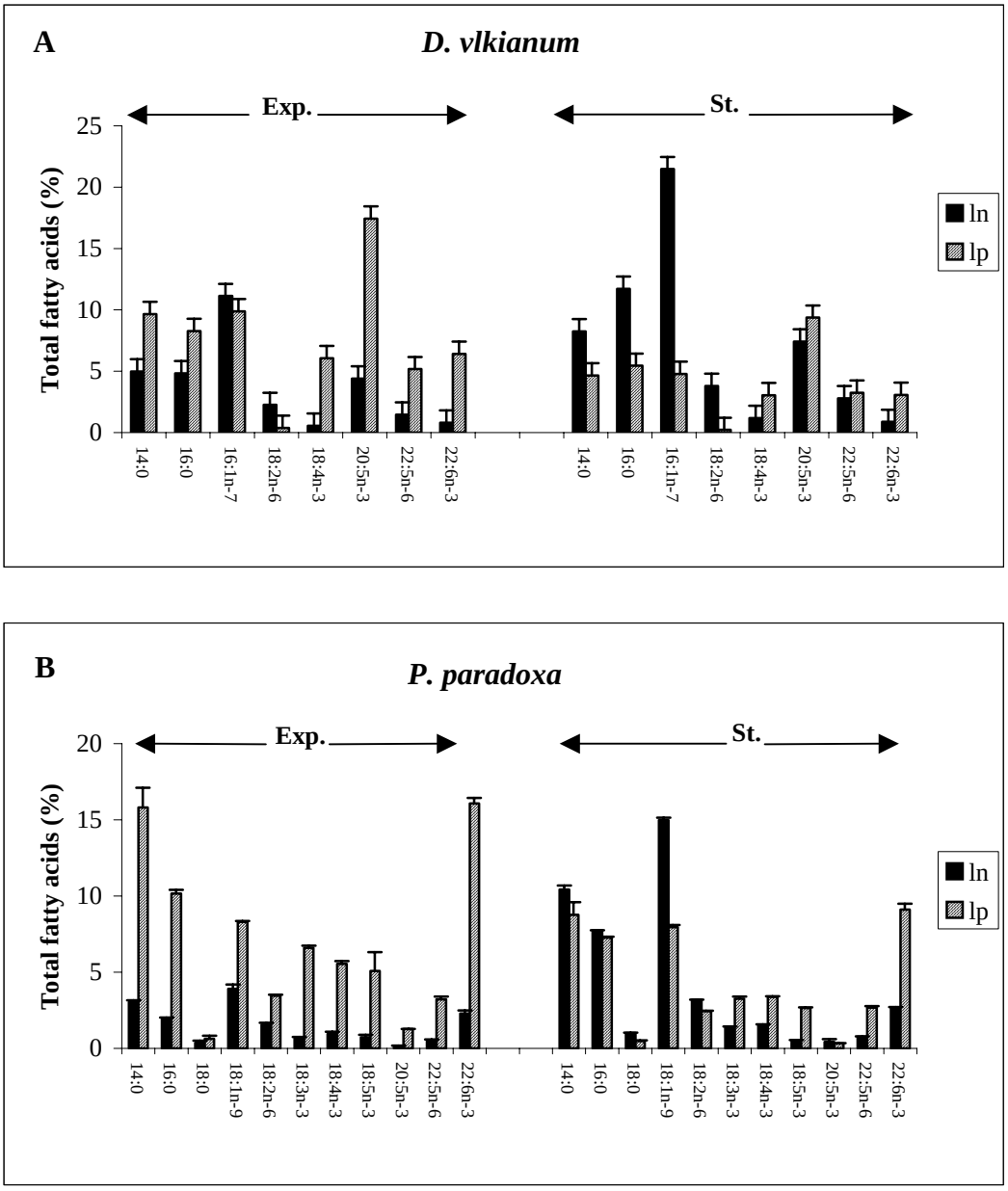
D. vlkianum				***P. paradoxa***			
	Exp.	St.			Exp.	St.	
14:0	14.64 ± 3.16	12.91 ± 0.82		14:0	18.85 ± 1.13	19.19 ± 0.96	
16:0	13.10 ± 1.92	17.17 ± 1.01	*	16:0	12.10 ± 0.30	15.03 ± 0.12	*
16:1n-7	21.03 ± 1.94	26.25 ± 1.07		18:0	1.00 ± 0.38	1.49 ± 0.11	
18:1n-9	1.09 ± 0.13	1.44 ± 0.06	*	16:1n-7	2.10 ± 0.14	1.50 ± 0.25	
18:2n-6	2.63 ± 0.13	4.01 ± 0.07	*	18:1n-9	12.17 ± 0.16	22.94 ± 0.80	*
18:4n-3	6.64 ± 1.26	4.23 ± 0.11	*	18:1n-7	1.02 ± 0.13	1.34 ± 0.39	
20:4n-6	0.75 ± 0.08	2.14 ± 0.10	*	18:2n-6	5.01 ± 0.10	5.65 ± 0.08	*
20:5n-3	21.84 ± 3.68	16.80 ± 0.63	*	18:3n-3	7.28 ± 0.14	4.64 ± 0.31	*
22:5n-6	6.63 ± 1.07	6.03 ± 0.12	*	18:4n-3	6.52 ± 0.06	4.90 ± 0.01	*
22:6n-3	7.24 ± 1.37	3.95 ± 0.01	*	18:5n-3	5.83 ± 1.36	3.19 ± 0.16	
				20:5n-3	1.41 ± 0.05	0.75 ± 0.17	*
				22:5n-6	3.74 ± 0.25	3.45 ± 0.01	
				22:6n-3	18.34 ± 0.68	11.79 ± 0.02	*
TO.SAT	28.32 ± 5.18	30.53 ± 1.86		TO.SAT.	32.17 ± 1.88	36.31 ± 0.54	
TO.MONO	23.06 ± 2.43	28.71 ± 1.20		TO.MONO	15.69 ± 0.34	26.18 ± 0.61	*
TO.(n-9)	1.09 ± 0.13	1.44 ± 0.06	*	TO.(n-9)	12.29 ± 0.08	23.17 ± 0.86	*
TO.(n-7)	21.97 ± 2.29	27.27 ± 1.14		TO.(n-7)	3.35 ± 0.18	3.01 ± 0.25	
TO.POLY	48.32 ± 7.93	40.51 ± 0.32	*	TO.POLY	51.79 ± 1.50	37.26 ± 0.12	*
TO.(n-6)	10.87 ± 1.32	13.65 ± 0.33	*	TO.(n-6)	11.08 ± 1.85	11.05 ± 0.16	
TO.(n-3)	36.40 ± 6.41	25.65 ± 0.78	*	TO.(n-3)	39.71 ± 0.34	25.78 ± 0.30	*
TO FA weight (fg cell^{-1})	2501.74 ± 372.89	4683.90 ± 76.28	*	TO FA weight (fg cell^{-1})	1641.23 ± 57.80	2309.44 ± 42.80	*
TO FA (%OM)	18.12 ± 1.98	24.89 ± 0.41	*	TO FA (%OM)	18.02 ± 0.63	20.03 ± 0.37	

*: $P<0.05$

The fatty acid composition of *P. paradoxa* included 14:0, 16:0, 18:1n-9 and DHA, which together accounted for more than 60% of total fatty acids. Total polyunsaturated fatty acid content, and particularly the n-3 fraction which included 18:4n-3, 18:3n-3, EPA and DHA, was higher during the exponential phase of growth, while monounsaturated fatty acids, the n-9 fraction, as well as 16:0, 18:1n-9 and 18:2n-6 were higher during the stationary phase of growth.

For *D. vlkianum* and *P. paradoxa* fatty acids were principally allocated in the polar fraction (>65% of total) during the exponential phase of growth while in the stationary phase the neutral fraction prevailed in *D. vlkianum* (>65% of total) and both fractions were equally represented in *P. paradoxa* (Fig. 2).

Fig. 2. Fatty acid distribution among neutral (ln) and polar (lp) lipid fractions of *D. vlkianum* (A) and *P. paradoxa* (B) in exponential (Exp.) or stationary (St.) phase of growth. Data are expressed as percentage of total fatty acids.



Nine different sterols were detected in *D. vlkianum* (Table 4); among them β–sitosterol and stigmasterol accounted for more than 70% of total sterol content. Stigmasterol and fucosterol were higher during the exponential phase of growth, while cholesterol, campesterol β-sitosterol, isofucosterol and ethylpavlovol were higher during stationary phase. *D. vlkianum* sterols were mainly allocated in the neutral lipid fraction in both phases of growth, with the exception of the pavlovophyte specific sterols methylpavlovol and ethylpavlovol (Fig. 3).

The sterol composition of *P. paradoxa* comprised brassicasterol, representing ≥97.5% of total sterols, and cholesterol (Table 4). In the exponential phase of growth, cells were rich in cholesterol while total sterol content increased during the stationary phase, due particularly to an increase in brassicasterol. *P. paradoxa* sterols were only detected in the neutral lipid fraction.

Fig. 3. Sterol distribution among neutral (ln) and polar (lp) lipid fractions of *D. vlkianum* harvested in exponential (Exp.) or stationary (St.) phase of growth. Data are expressed as percentage of total sterols.

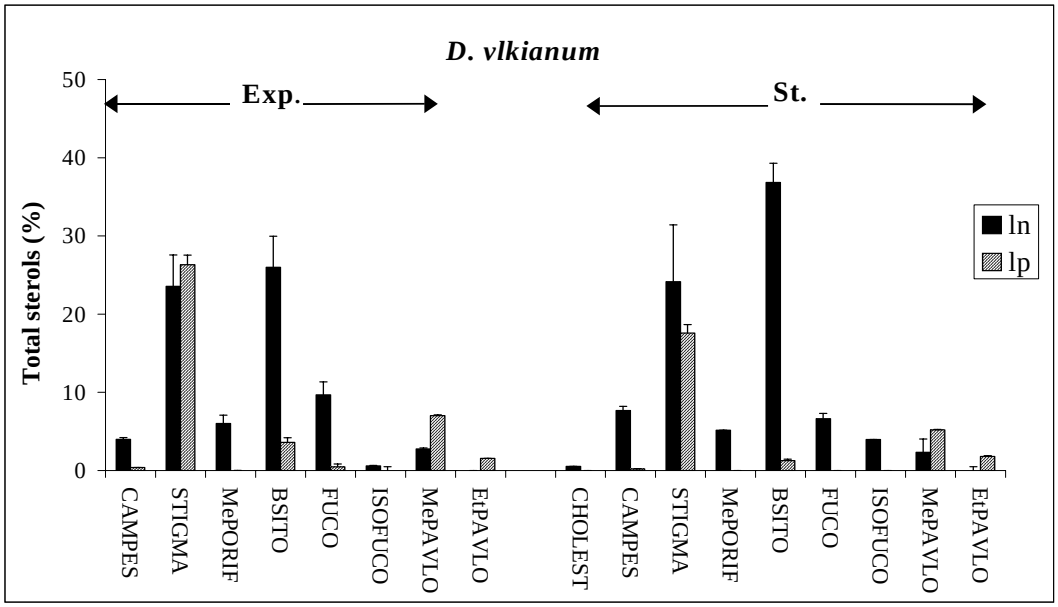


Table 4. Sterol composition of *D. vlkianum* and *P. paradoxa* (mean ± SD; n=2). Individual data are reported as percentage of total sterols while total sterols (TO Sterols) are expressed either total dry weight (fg $cell^{-1}$) or percentage of total organic matter content (% OM).

	D. vlkianum			***P. paradoxa***			
	Exp.	St.			Exp.	St.	
CHOLEST	tr.	0.47 ± 0.07	*	CHOLEST	2.48 ± 0.59	tr.	*
CAMPES	3.89 ± 0.25	7.12 ± 0.01	*	BRASSICA	97.52 ± 0.59	100	*
STIGMA	44.60 ± 1.72	35.95 ± 0.42	*	TO Sterol (fg $cell^{-1}$)	31.86 ± 1.92	17.40 ± 3.51	*
MePORIF	5.37 ± 0.97	4.68 ± 0.02		TO Sterol (% OM)	0.35 ± 0.02	0.19 ± 0.04	*
βSITO	26.41 ± 1.58	34.47 ± 0.56	*				
FUCO	9.04 ± 0.95	6.03 ± 0.29	*				
ISOFUCO	0.53 ± 0.04	3.61 ± 0.46	*				
MePAVLO	8.77 ± 1.12	6.24 ± 0.26					
EtPAVLO	1.39 ± 0.37	1.43 ± 0.01	*				
TO Sterol (fg $cell^{-1}$)	184.48 ± 29.96	248.7 ± 37.13					
TO Sterol (% OM)	1.26 ± 0.11	1.32 ± 0.20					

* $P<0.05$; tr. = traces

CHOLEST = Cholesta-5-en-3β-ol;
CAMPES = 24α-methylcholesta-5-en-3β-ol;
STIGMA = 24β-ethylcholesta-5,22-dien-3β-ol;
MePORIF = 4α-methyl-24α-ethylcholesta-22-en-3β-ol;
βSITO = 24β-ethylcholesta-5-en-3β-ol;
FUCO = 24β-ethylcholesta-5,24(28)-dien-3β-ol;
ISOFUCO = 24β-ethylcholesta-5,24(28)-dien-3β-ol;
MePAVLO = 4α,24β-dimethylcholestan-3β,4β-diol;
EtPAVLO = 4α-methyl-24β-ethylcholestan-3β,4β-diol;
BRASSICA = 24β-methylcholesta-5,22-dien-3β-ol.

4. Discussion

Microalgal culture constitutes a key procedure in shellfish hatcheries, but this activity is far from being optimised and several problems remain to be solved. In particular, the number of good quality microalgae currently available in hatcheries is limited and several species which were previously used in commercial hatcheries have now been discarded due to their poor nutritional value (e.g. *Dunaliella* sp., *Phaeodactylum tricornutum*, *Tetraselmis* sp.). Microalgae are commonly used in plurispecific diets, generally composed of one diatom and one or more flagellates, to assure a better balance in essential nutrients (Coutteau and Sorgeloos, 1992; Robert and Gérard 1999). Several diatoms routinely cultured in mollusc

hatcheries lead to good performances (*Skeletonema costatum*, *Thalassiosira pseudonana*, *Chaetoceros gracilis*, *C. calcitrans*, *C. calcitrans* forma *pumilum*, *C. tenuissimus*-like), while the list of available flagellates is extremely reduced. The present work focused accordingly on flagellates. To address this subject, a step-by-step method was adopted. Firstly, fifteen microalgae were selected on the basis of their cytomorphological characteristics and their productivity was assessed; the species selected on this basis were then tested as food for Pacific oyster larvae and lastly different aspects of those which exhibited good growth were characterised. The fifteen microalgae initially concerned by this work were of suitable size for use as feed in mollusc hatcheries (for larvae, juveniles, and broodstock) but only five species (*P. paradoxa*, the flagellate phase of *E. huxleyi*, *I. rotunda*, *P. virescens*, *D. vlkianum*) gave acceptable productivities in small volume cultures and one of these species (*P. virescens*) was subsequently discarded due to its poor growth in larger volume glass carboys.

All of the microalgae tested in the feeding trials were efficiently ingested by larvae (data not shown), but only *P. paradoxa* and *D. vlkianum* exhibited adequate nutritional value. Moreover, both species showed good growth in various culture systems operating in mollusc hatcheries, reaching high productivities (0.8 and 0.5 division day^{-1}, for *D. vlkianum* and *P. paradoxa*, respectively). Both species can thus be successfully used as feed for oyster larvae as a substitute or in association with T-Iso and a diatom. In contrast, *E. huxleyi* and *I. rotunda* were unsuitable for feeding Pacific oyster larvae.

No data on gross composition of *D. vlkianum* and *P. paradoxa* are available in the literature. Though the relevance of gross composition for the food value of microalgae for molluscs is generally accepted, there are sometimes conflicting reports on the importance of each nutritional component (Brown et al., 1997; Knauer and Southgate, 1999). However, the use of multispecific diets, including microalgae of different classes, allows balancing of such nutrients. In general, larval molluscs require a diet composed of 30-60% protein, 5-30% carbohydrate, while the lipid content does not seem to be directly related to the quality of the diet (Brown et al., 1989). Limited changes in gross composition were observed during *D. vlkianum* growth, while substantial changes occurred in *P. paradoxa*. In the latter species, the change from exponential to stationary phase induced an increment of carbohydrates and lipids, but a decrease in proteins. Such increases in carbohydrate and drastic decreases in protein content have previously been reported for the prymnesiophyte T-Iso from the logarithmic to the stationary phase of growth (Brown et al., 1993).

Both species were characterized by high fatty acid content (≥18% of organic matter content) and by a balanced profile. *D. vlkianum* was rich in 14:0, 16:0, DHA and EPA, while *P. paradoxa* contained 14:0, 16:0 and DHA. The essential role of DHA and EPA for marine animals have been widely demonstrated (Kanazawa et al., 1979), while a positive correlation between 14:0 and 16:0 content and growth of Pacific oyster larvae have been reported by Thompson et al. (1993). Fatty acid composition of *D. vlkianum* was similar to that previously reported by Volkman et al. (1997), even if we found a higher content in 16:1n-7 and a lower content in EPA. Such fatty acid profile also corresponds to that previously reported by Donato et al. (2003) even if we found higher total polyunsaturated and DHA content.

P. paradoxa fatty acid composition partially reflected that reported by Chu and Dupuy (1980); lower quantities of saturated and higher content of polyunsaturated fatty acids were

found by these authors with respect to this work. The change of harvesting regime strongly influenced fatty acid composition. EPA and DHA content of both species were higher during the exponential phase of growth despite total fatty acid content being higher during the stationary phase of growth. The analysis of polar lipid fractions provided information on the metabolic status of microalgae. During the exponential phase of growth, fatty acids are mostly implied in membrane functions, while in the stationary phase they are stored as energy reserves.

Nine different sterols were detected in *D. vlkianum* cells, while Donato et al. (2003) only reported four sterols for the same species. However, in both studies stigmasterol and β-sitosterol represented the main component of the sterol fraction. The sterol profile included methylpavlovol and ethylpavlovol, which are specific to pavlovophytes (Véron et al., 1996). Moreover, an unidentified peak was detected in both chromatograms of polar and neutral sterols of *D. vlkianum*. Further investigation with mass spectrometry is required to determine the nature of this component.

The sterol composition of *P. paradoxa* reflected that described by Lin et al. (1986) and was coherent with that reported for similar species such as *Isochrysis galbana* and T-Iso (Muller-Feuga et al., 2003b).

P. paradoxa is a microalga which has previously been mentioned for its potential in aquaculture (Chu and Dupuy, 1980; Langdon and Robinson, 1996; Wilson et al., 1996). In particular, Langdon and Robinson (1996) detailed the good quality of this species for rearing of the Suminoe and Pacific oyster (*C. ariakensis, C. gigas*); nevertheless this species has not yet been adopted at a commercial scale.

D. vlkianum has been the subject of ultra-structural, taxonomic and biochemical investigations (Green and Hibberd, 1977; Volkman et al., 1997; Donato et al., 2003), but was previously unknown as a food for molluscs.

This study emphasizes the interest of both species for the nutrition of oyster larvae and illustrates the feasibility of their culture in commercial mollusc hatcheries.

Acknowledgments

This study was partially supported by the Groupe Régional d'Action pour la Valorisation Industrielle de la Recherche Basse Normandie, France, through a postdoctoral grant. The authors are indebted to JP Connan for providing assistance in culturing microalgae and to JR Le Coz for his help in biochemical analysis.

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