ORIGINAL PAPER

Use of the dinoflagellate *Karlodinium veneficum* as a sustainable source of biodiesel production

**Claudio Fuentes-Grünewald · Esther Garcés ·
Sergio Rossi · Jordi Camp**

Abstract Microalgae are microscopic heterotrophic–autotrophic photosynthesizing organisms with enormous potential as a source of biofuel. Dinoflagellates, a class of microalgae, contain large amounts of high-quality lipids, the principal component of fatty acid methyl esters. The biotic characteristics of the dinoflagellate species *Karlodinium veneficum* include a growth rate of 0.14 day^{-1}, a wet biomass of 16.4 g/L, a growth period of approximately 30 days, and an approximate 97% increase in fatty acid content during the transition from exponential phase to stationary phase. These parameters make *K. veneficum* a suitable choice as a bioresource for biodiesel production. Similarly, two other species were also determined to be appropriate for biodiesel production: the Dinophyceae *Alexandrium andersoni* and the Raphidophyte *Heterosigma akashiwo*.

Keywords Biodiesel · Dinoflagellates · *Karlodinium veneficum* · Lipids · Microalgae

Introduction

Biodiesel is a biofuel that, through transesterification, can be produced from different feedstocks, including grease, vegetable oils, waste oils, animal fats, and microalgae. In this reaction, triglycerides are converted into fatty acid methyl esters (FAMEs) in the presence of an alcohol, such as methanol or ethanol, and either an alkaline or acidic catalyst. The reaction produces two immiscible layers, biodiesel, as the primary product, and glycerol, as a by-product [1].

The unstable price of fossil fuel, worldwide interest in reducing the amount of CO_2 emitted into the atmosphere, and the attempts of petroleum-dependent countries to enlarge their energy matrix have led to increasing interest in biofuel production. Until recently, the synthesis of biodiesel derived mostly from terrestrial plants. This strategy has become controversial due to the lack of sustainability of plant-based biofuel—specifically, the resulting deforestation of extensive land otherwise devoted to the cultivation of soybean, palm, sugarcane, rapeseed, and other food plants [2], the consumption of scarce water resources, the degradation of arable land, and the reduced amount of CO_2 fixation. Moreover, the transformation of primary food resources into biofuels has led to a clash of interests, as plant-derived biodiesel has deprived poor countries of food and increased its cost [3]. All of these factors have stimulated the search for other sources of biodiesel production that are both sustainable and economical [4].

Microalgae are microscopic heterotrophic–autotrophic photosynthesizing organisms that inhabit many different types of environments, including freshwater, brackish water, and seawater. More than 40,000 different species of microalgae have been identified, most of which have a high content of lipids, accounting for between 20 and 50% of their total biomass [4]. Accordingly, microalgae have the potential to synthesize 30-fold more oil per hectare than terrestrial plants [5]. They are currently widely used in industry in the synthesis of pigments and additives, as a source of protein, and in biofuel production.

C. Fuentes-Grünewald (✉) · E. Garcés · J. Camp
Institut de Ciències del Mar/Consejo Superior de Investigaciones
Científicas (ICM/CSIC), Passeig Marítim de la Barceloneta,
37-49, 08003 Barcelona, Spain
e-mail: cfuentes@cmima.csic.es

C. Fuentes-Grünewald · S. Rossi
Instituto de Ciencias y Tecnología Ambiental,
Universidad Autónoma de Barcelona, 08193 Bellaterra, Spain

Marine microalgae have several advantages compared to other sources of biodiesel production. Due to their high growth rate, they have the potential to satisfy the enormous demand for biofuels, but they can be cultured on non-agricultural land or even in coastal areas—and without the need of freshwater. In addition, the tolerance of microalgae to a high CO_2 content in gas streams allows high-efficiency CO_2 mitigation [6, 7]. Biodiesel from microalgae does not contain sulfurs, is highly biodegradable, and is associated with minimal nitrous oxide release. Ultimately, microalgal farming has the potential to more cost-effective than conventional farming [8, 9].

In the 1970s, as a response to the petroleum crisis, the U.S. government made a major effort to identify the optimal type of algae for biodiesel production [10]. As a result, more than 3000 different microalgae species, including those belonging to the Bacillariophyceae, Chlorophyceae, Cyanophyceae, Prymnesiophyceae, Eustigmatophyceae, and Prasinophyceae, with natural habitats in different parts of the country, were examined. A few of these algae were shown to be cultivable on a large scale, such as in a photobioreactor or in ponds.

Nonetheless, the production of biodiesel from microalgae has thus far been restricted to a few species, i.e., those for which the culture conditions in high biomass systems are known: the cyanobacteria *Spirulina platensis* (protein production), the Chlorophyceae *Chlorella prototothecoids* (heterotrophic cultivation in photobioreactors for biomass), *Tetraselmis suecica* (food source in aquaculture hatcheries), and *Haematoccocus pluvialis* (pigment production). Despite their relatively widespread use in these and other applications, these species are controversial sources of biodiesel because their cultivation requires the input of large amounts of freshwater (*Spirulina*, *Chlorella*, *Haematoccocus*) or because their oil content is too low to be of economic interest (*Tetraselmis*).

Microalgae with a high content of fatty acids, neutral lipids, and polar lipids as well as a high growth rate in the natural environment have yet to be exploited for biodiesel,

and the isolation and characterization of microalgae with the potential for more efficient lipid/oil production remain the focus of continuing research [11].

A high content of fatty acids, as neutral lipids or triacylglycerols (TAGs), is found naturally in a group of microalgae, the dinoflagellates. Additionally, these organisms occasionally form explosive and extensive proliferations (blooms) in coastal waters all over the world. These episodic blooms extend for hundreds of kilometers, with cell concentrations reaching millions per liter [12, 13]. Such properties make dinoflagellates a potential candidate as a source of biofuel. In the study reported here, we investigated three genus of dinoflagellates and one raphidophyte species (*Alexandrium*, *Karlodinium*, *Scripsiella*, and *Heterosigma*) for their potential as alternatives for biodiesel production and as sources of biomass in biofuel production. Specifically, the properties of these microalgae were compared with those of microalgae traditionally used in biodiesel production in terms of growth rate, cell yield, and quantity and quality of their oil content.

Materials and methods

Strain cultures

All species were cultivated in multiple and in batch cultures at the Marine Science Institute (ICM/CSIC). The characteristics of the species are presented in Table 1. All strains were grown in L1 medium under the same conditions [14]. The ten strains, belonging to seven genera, were inoculated at an average concentration of 4200 cells/mL seawater (salinity 36, neutral pH) into 2-L Nalgene flasks and incubated at $21 \pm 1°C$ in prefiltered air (Iwaki filter, pore size 0.2 µm; Iwatic, Tokyo, Japan) under a 12/12-h (light/dark) photoperiod, with illumination provided by fluorescence tubes (Gyrolux, Sylvania, Germany) emitting a photon

Table 1 Algal strains analyzed in this study

Class	Species	Strain	Number in this study	History of the cultures	
				Year of isolation	Location
Dinophyceae	*Alexandrium catenella*	ICMB 223	1	2006	Tarragona (Spain)
Dinophyceae	*Alexandrium minutum*	AMP 4	2	1975	Port Palma (Spain)
Dinophyceae	*Alexandrium andersoni*	ICMB 222	3	2007	Alfacs (Spain)
Dinophyceae	*Alexandrium insuetum*	ICMB 218	4	2006	Arenys de Mar (Spain)
Dinophyceae	*Karlodinium veneficum*	ICMB 252	5	2007	Alfacs (Spain)
Dinophyceae	*Scrippsiella trochoidea*	ICMB 213	6	2007	Arenys de Mar (Spain)
Raphidophyceae	*Heterosigma akashiwo*	ICMB 830	7	2007	Vilanova i la Geltru (Spain)
Bacillariophyceae	*Chaetoceros affinis*	ICMB 008	8	1997	Masnou (Spain)
Bacillariophyceae	*Pseudo-nitzschia delicatissima*	ICMBF2B2	9	2007	Arenys de Mar (Spain)
Prasinophyceae	*Tetraselmis suecica*	CCAP 66/4	10	2003	UK

irradiance of 110 μmol photons $m^{-2} s^{-1}$ (measured with a Li-Cor sensor; Li-Cor Biosciences, Lincoln, NB).

Growth rates

Subsamples (10 mL) of each culture were fixed in Lugol's iodine. During the lag phase, the cultures were sampled every day and during stationary phase every 4 days. Samples were counted in a Sedgewick-Rafter chamber under an inverted optical microscope (Leica-Leitz DM-II; Leica Microsystems GMbH, Wetzlar, Germany) at 200–400× magnification. Cell abundance data were used to calculate the exponential growth rate of the cultures. Species-specific net growth rates were estimated from $\mu = \ln(N_0/N_t)/t$, where N_0 and N_t are the initial and final cell densities, respectively, and t is the time interval in days [15].

Wet weight (biomass)

Wet weight (WW) was determined by filtering duplicate subsamples (10 mL) through pre-weighed glass-fiber filters (Whatman GF/F: 25 mm, nominal pore size 0.7 μm; GE Healthcare Life Sciences, Piscataway, NJ) and then weighing the filters on a Sartorius balance (precision of 0.001; Satorius, Germany).

Lipid extraction and fatty acid analyses

Primary lipid analyses were carried out for all strains at the Institute of Science and Environmental Technology (ICTA, Autonomous University of Barcelona, Spain). The lipids of one control strain (*T. suecica*; number 10, see Table 1) and of five different strains (numbers 1, 2, 3, 5, 7, see Table 1) were analyzed at three different times in the growth curve: lag phase (day 6), exponential phase (day 21), and stationary phase (day 35).

Triplicates of a 50-mL subsample were filtered on previously combusted (450°C, 4 h) GF/F Whatman glass-fiber filters, immediately frozen in liquid N_2, freeze-dried for 12 h, and then stored at −20°C until analysis. The filters were placed in a tube with 3:1 dichloromethane–methanol (DCM:MeOH), spiked with an internal standard (2-octyldodecanoic acid and 5β-cholanic acid), and the lipids extracted using a microwave-assisted technique (5 min at 70°C). After centrifugation, the extract was taken to near dryness in a centrifugal vacuum concentrator maintained at constant temperature and then fractionated by solid-phase extraction according to a previously published method [16]. The sample was subsequently re-dissolved in 0.5 mL chloroform and eluted through a 500-mg aminopropyl mini-column (Sep-Pak SPE cartridges; Waters, Milford, MA) previously activated with 4 mL *n*-hexane. The first fraction was eluted with 3 mL chloroform:2-propanol (2:1) and the fatty acids recovered

with 8.5 mL of diethyl ether:acetic acid (98:2). Despite reported concerns on the background levels of free fatty acids (FFAs) in the aminopropyl columns [17], the concentrations of target FFAs in the SPE cartridges used were below the detection limit. The fraction of FFAs was methylated using a 20% solution of MeOH/BF$_3$ heated at 90°C for 1 h, and the reaction was quenched with 4 mL NaCl–saturated water. The FAMEs were recovered by extracting the samples twice with 3 mL of *n*-hexane. The combined extracts were taken to near dryness, re-dissolved with 1.5 mL chloroform, eluted through a glass column filled with Na_2SO_4 to remove residual water and, after removal of the chloroform, subjected to nitrogen evaporation. The extracted sample was stored at −20°C until gas chromatography analysis.

The gas chromatography analysis of extracts re-dissolved in 30 μL iso-octane was carried out in a Thermo Finnigan Trace GC Ultra instrument (Thermo Fisher Scientific, Waltham, MA) equipped with a flame ionization detector and a splitless injector and fitted with a DB-5 Agilent column (length 30 m, internal diameter 0.25 mm, phase thickness 0.25 μm). Helium was used as the carrier gas, delivered at a rate of 33 cm/s. The oven temperature was programmed to increase from 50 to 320°C at 10°C/min. Injector and detector temperatures were 300 and 320°C, respectively. The FAMEs were identified by comparison of their retention times with those of standard fatty acids (37 FAME compounds; Supelco Mix C4-C24; Supelco, Bellefonte, PA) and quantified by integrating the areas under the curves in the gas chromatograph traces (Chromquest 4.1 software), using calibrations derived from internal standards.

### Lipids fluorescence in microalgae

The intracellular neutral lipid distribution in microalgal cells was examined by staining a 3-mL suspension of the algae with 10 μL ($7.8 \times 10^{-4} M$) of Nile Red fluorescent dye (Sigma-Aldrich, St. Louis, MO) dissolved in acetone (final concentration 0.26 μM). The samples were examined by epifluorescent microscopy (Leica-Leitz DM-II; Leica Microsystems GMbH, Wetzlar, Germany) with an excitation wavelength of 486 nm; the emission was measured at 570 nm, following the method of Cooney et al. [18]. Photographs were taken with a SigmaPro software image analyzer, which was also used to calculate the percentage of positively stained cells.

Results

Growth rate of strains

The net growth rate differed among the examined species (Fig. 1) and was highest for *T. suecica* (Prasinophyceae),

Fig. 1 Growth curve of the cultures obtained from the populations of different marine microalgae incubated at 21°C, neutral pH, in medium with a salinity of 36, and in prefiltered air. The flasks were cultured under a 12/12-h (light/dark) photoperiod at a photon irradiance of 110 μmol photons m^{-2} s^{-1}. *Error bars* denote standard deviations among replicates
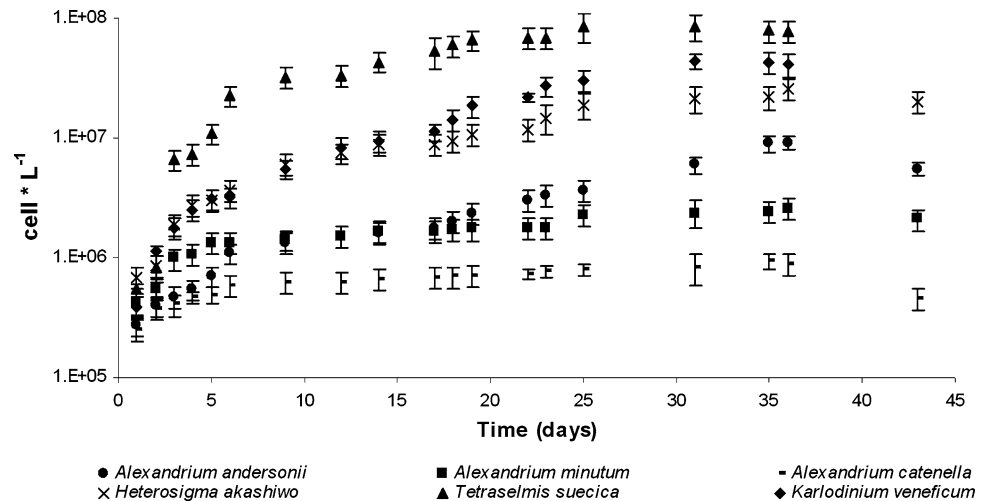
which grew at a rate of 0.23 day^{-1} (one division every 4.4 days) during the exponential phase of growth. Accordingly, at 24 days of culture, the cell abundance was nearly 85×10^6 cells/L, after which the culture entered stationary phase and decayed. Among the three dinoflagellates examined here, the growth rate of *K. veneficum* was the highest, 0.14 day^{-1} in the exponential phase, corresponding to an abundance of 44×10^6 cells/L at day 30 of culture. For *H. akashiwo*, maximum abundance was approximately 26×10^6 cells/L at day 35 of culture, reflecting a growth rate of 0.10 day^{-1} (one division every 10 days). Among the six species examined in the study, this raphidophyte was unique in that cell abundance was maintained for more than 6 months (data not shown). Moreover, the cells remained healthy without the addition of fresh medium. This was in contrast to the other cultures, which showed a gradual decay such that total cell lysis occurred approximately 2 months after inoculation.

The growth rate of dinoflagellates belonging to the genus *Alexandrium* differed depending on the species. The highest growth rate was achieved by *A. andersoni*, at 0.10 day^{-1}; this was similar to that of the raphidophyte *H. akashiwo*, but the cell abundance of the former (maximum 9×10^6 cells/L) was lower than that of the latter. The growth rates of *A. minutum* and *A. catenella* were two orders of magnitude slower (0.04 day and 0.03 day^{-1}, respectively) than those of faster-growing microalgae. In terms of abundance, *A. minutum* reached a maximum of 2.6×10^6 cells/L and *A catenella* a maximum of 9.4×10^5 cells/L at culture day 36 and 35, respectively.

Wet biomass

In the lag phase, the maximum biomass was achieved by *H. akashiwo* (WW 14.3 g/L) and the minimum by *A. andersoni* (9.4 g/L). In comparison, in the exponential phase, *A. catenella* (20.2 g/L) was found to have the maximum

WW and *K. veneficum*, the lowest (average of 16.1 g/L). The increase in WW during the exponential phase was more evident in the genus *Alexandrium* than in the other microalgae (Fig. 2). The different microalgal strains evidenced similar results, with maximum biomass reached in the late-exponential phase. At the stationary phase, the WW of all the cultures diminished, with the exception of *T. suecica*, which retained the weight it had obtained in exponential phase (18.5 g/L).

Total lipid content

The lipid concentration as a percentage of total fatty acid was determined at the different phases of culture in the strains grown under equivalent culture conditions. The lipid content of the control strain, *T. suecica*, was maintained at almost the same level throughout the experiment, with only a slight decrease (7.8%) in the stationary phase (data not shown). The behavior of the genus *Alexandrium* differed depending on the species. The lipid content of *A. catenella* diminished by 7.0% from the lag phase to the exponential phase and then increased by nearly 48% from the exponential phase to stationary phase. In *A. minutum*, the lipid content increased by approximately 97% from the lag phase to the exponential phase, whereas that of *A. andersoni* diminished by about 27% from the exponential phase to the stationary phase. The best performance in terms of lipid accumulation was that of *K. veneficum*. The lipid content of this dinoflagellate increased throughout the different growth phases: by 40% from the lag phase to the exponential phase followed a large and intense accumulation, approximately 97%, from the exponential phase to the stationary phase. The raphidophyte *H. akashiwo* maintained its total lipid content from the lag phase to the exponential phase, but there was an extreme reduction (approx. 43%) from the exponential phase to the stationary phase.

Fig. 2 Total wet weight (*WW*) of six marine microalgae cultures at different phases of the growth cycle (lag, exponential, and stationary phases). *Error bars* denote standard deviations between two replicates. *Asterisk* indicates significant differences at $P < 0.05$
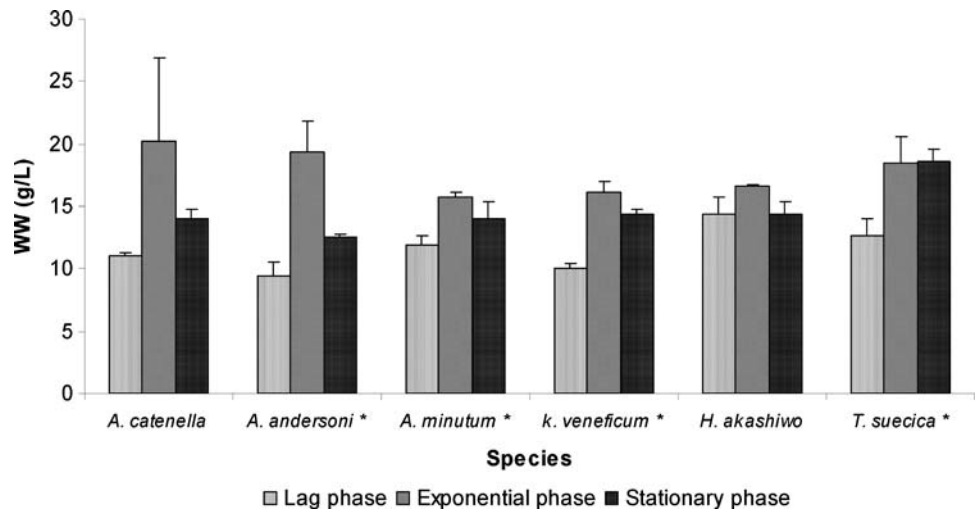
The highest total lipid content (Fig. 3) was achieved at the stationary phase of culture, by the dinoflagellate *K. veneficum*, whereas during this growth phase, among the microalgal strains analyzed, the lipid content of *T. suecica* was the lowest.

Fatty acid composition

The most abundant fatty acids expressed by the different classes of microalgae during the stationary phase were those of the 18:0, 16:0, 20:3, and 17:1 types (Table 2). In general, the highest levels of saturated lipids were found in *A. catenella* (42.3%), *A. minutum* (40.6%), and *K. veneficum* (39.7%). In the diatoms, saturated fatty acid content ranged from 22.3% (the minimum of the six strains tested) in *P. delicatissima* to 32.3% in *C. affinis*. In the dinoflagellate *S. trochoidea*, saturated fatty acids accounted for 29.8% of the lipid content, compared to 39.7% in the control algae *T. suecica*. Monounsaturated fatty acids varied from 5.8 to 9.8% of the total fatty acids. The polyunsaturated fatty acids (PUFAs) identified consisted mainly of

18:5(n3) (0.8–5.0%), 20:3(n3) (2.8–6.4%), and, in lesser amounts, 20:4(n3) (0.4–2.5%).

Change in fatty acid composition at different growth phases

The fatty acid composition in members of the Dinophyceae changed depending on the culture phase (Fig. 4). In most cases, the lipid concentration, especially of PUFAs such as C18:5(n3) and C20:3(n3), increased during the transition from exponential to stationary phase. In *A. minutum* and *A. catenella*, these polyunsaturated acids increased by approximately 5% whereas in *A. andersoni* their amounts did not change. Among the Dinophyceae, *K. veneficum* had the greatest increase in lipid content, 45%, from exponential to stationary phase. This increase was seen not only in the main compounds C16:0 and C18:0, but also in all fatty acids measured. The lipid content of the raphidophyte *H. akashiwo* also increased, by about 20%, between lag phase and stationary phase (data not shown). In the control algae *T. suecica*, a smooth increase of only ∼3%, in the main fatty acids C16:0 and C18:0, was observed (data not shown).

Fig. 3 Total lipid content, as measured by gas chromatography, in terms of oil concentration (g/L) during the stationary phase of culture. *Error bars* denote standard deviations between three replicates
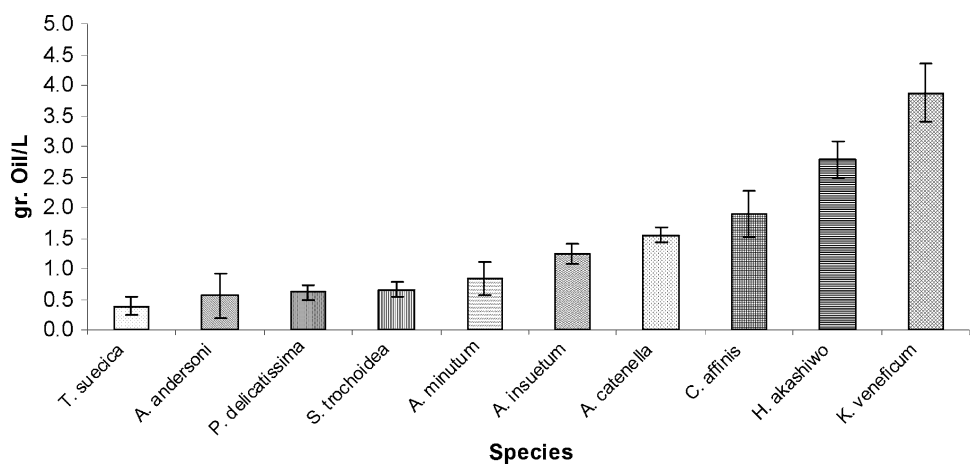
Table 2 Relative abundance (%) of fatty acid composition in different marine microalgae at the stationary phase of culture

Fatty acid	*A. catenella*	SD	*A. minutum*	SD	*A. andersoni*	SD	*A. insueturn*	SD	*K. veneficum*	SD
C12:0	0.6	0.3	0.8	0.4	0.8	0.4	0.3	0.1	0.6	0.1
C13:0	1.6	0.0	1.6	0.0	1.4	0.0	0.0	0.0	0.0	0.0
C14:1	0.6	0.4	0.6	0.3	0.7	0.1	0.2	0.0	0.6	0.1
C14:0	5.2	0.6	3.4	2.3	2.6	1.6	2.1	1.5	6.5	0.2
C15:1	1.2	0.1	1.4	0.2	1.2	0.1	1.0	0.0	1.2	0.2
C15:0	0.5	0.1	0.5	0.1	0.5	0.2	0.5	0.1	0.5	0.1
C16:1	0.7	0.6	0.9	0.2	1.0	0.7	0.7	0.4	0.0	0.0
C16:0	11.5	1.3	11.7	1.2	10.7	2.0	9.5	1.6	11.3	1.8
C17:1	7.7	0.8	7.5	0.5	7.5	2.1	5.8	1.3	8.5	1.1
C17:0	0.9	0.3	0.9	0.3	0.8	0.3	0.6	0.5	0.9	0.3
C18:3	1.4	0.9	2.1	0.0	0.9	0.0	5.0	0.0	1.9	0.0
C18:2	0.1	0.0	0.4	0.2	0.4	0.1	0.6	0.0	0.2	0.0
C18:1	1.4	0.8	1.4	0.8	1.7	0.7	1.8	0.3	1.0	0.3
C18:0	19.8	2.2	19.0	1.7	18.2	4.2	15.2	3.5	19.3	4.0
C20:5	0.5	0.2	0.3	0.0	0.5	0.2	0.5	0.1	0.4	0.1
C20:4	1.8	1.2	0.4	0.2	1.6	1.1	2.5	0.9	1.3	0.6
C20:3	6.0	0.9	2.8	2.3	4.8	0.7	4.8	1.0	4.4	1.1
C20:2	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
C20:1	1.1	0.0	1.1	0.0	0.7	0.5	0.8	0.4	0.8	0.0
C20:0	0.6	0.0	0.3	0.1	0.4	0.1	0.4	0.1	0.3	0.2
C21:0	0.5	0.0	2.0	1.7	0.2	0.0	0.2	0.0	2.2	2.0
C22:6	0.0	0.0	0.1	0.0	0.0	0.0	0.3	0.1	0.0	0.0
C22:2	0.7	0.0	0.5	0.1	0.0	0.0	0.4	0.1	0.0	0.0
C22:1	0.0	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.0	0.0
C22:0	0.0	0.0	0.5	0.0	0.0	0.0	0.1	0.0	0.5	0.0
C23:0	1.3	1.0	1.8	0.0	1.3	1.2	0.2	0.0	0.0	0.0
C24:1	0.3	0.2	0.0	0.0	0.3	0.0	0.3	0.0	0.0	0.0
C24:0	0.4	0.0	0.0	0.0	0.0	0.0	0.8	0.3	0.0	0.0
Total SFA	42.3		40.6		36.8		29.9		39.7	
Total MUFA	13.0		12.9		13.1		10.7		12.0	
Total PUFA	10.5		6.7		8.1		14.3		8.2	
Trace <0.1%	34.2		39.8		42.0		45.1		40.1	

Fatty acid	*S. trochoidea*	SD	*H. akashiwo*	SD	*C. affinis*	SD	*P. delicatissima*	SD	*T. suecica*	SD
C12:0	0.2	0.1	0.7	0.0	0.1	0.0	0.3	0.2	1.2	0.0
C13:0	0.0	0.0	2.1	0.0	0.0	0.0	0.1	0.0	1.3	0.0
C14:1	0.2	0.0	0.5	0.1	0.3	0.0	0.2	0.0	0.6	0.2
C14:0	3.2	2.4	7.1	0.6	8.6	0.0	4.5	0.0	7.7	1.4
C15:1	1.1	0.3	1.3	0.2	1.2	0.5	1.2	0.7	1.2	0.2
C15:0	0.5	0.3	0.8	0.4	0.5	0.1	0.4	0.1	0.9	0.6
C16:1	0.8	0.6	0.5	0.0	0.3	0.0	0.6	0.1	1.2	0.9
C16:0	9.5	2.5	10.4	1.5	7.8	3.3	6.0	2.1	10.4	0.6
C17:1	7.1	0.3	8.7	1.0	8.5	1.3	4.0	1.3	9.8	2.1
C17:0	0.7	0.4	0.7	0.1	0.8	0.3	0.3	0.2	0.8	0.2
C18:3	2.0	0.0	2.1	0.0	1.6	0.0	0.8	0.2	2.2	0.0
C18:2	0.5	0.0	0.4	0.1	0.5	0.2	0.5	0.2	1.6	0.9
C18:1	1.1	0.8	1.2	0.7	1.3	0.7	0.7	0.2	2.0	0.9

Table 2 continued

Fatty acid	S. trochoidea	SD	H. akashiwo	SD	C. affinis	SD	P. delicatissima	SD	T. suecica	SD
C18:0	15.1	4.3	15.5	2.0	13.5	6.2	9.8	2.4	16.9	1.2
C20:5	0.4	0.1	0.5	0.2	0.9	0.3	0.3	0.1	0.5	0.2
C20:4	2.2	0.2	1.5	0.8	2.5	1.9	2.2	0.5	1.7	1.0
C20:3	5.6	0.5	5.0	0.6	6.4	0.4	3.2	0.7	5.8	0.2
C20:2	0.0	0.0	0.2	0.0	0.2	0.0	0.0	0.0	0.0	0.0
C20:1	0.9	0.0	0.9	0.0	0.6	0.3	0.6	0.0	0.6	0.5
C20:0	0.3	0.2	0.5	0.0	0.4	0.1	0.3	0.0	0.2	0.0
C21:0	0.2	0.0	2.2	1.9	0.6	0.0	0.2	0.0	2.9	2.6
C22:6	0.0	0.0	0.1	0.0	0.1	0.0	0.2	0.0	0.0	0.0
C22:2	0.5	0.0	0.5	0.0	0.3	0.1	0.8	0.0	0.0	0.0
C22:1	0.1	0.0	0.0	0.0	0.4	0.0	0.1	0.0	0.0	0.0
C22:0	0.0	0.0	0.4	0.0	0.1	0.0	0.2	0.0	0.0	0.0
C23:0	0.0	0.0	0.2	0.1	0.2	0.1	0.1	0.0	0.2	0.0
C24:1	0.0	0.0	0.0	0.0	0.4	0.0	0.2	0.0	0.0	0.0
C24:0	0.2	0.0	0.2	0.1	0.2	0.0	0.3	0.0	0.0	0.0
Total SFA	29.8		38.7		32.3		22.3		39.7	
Total MUFA	11.4		13.0		13.0		7.6		15.3	
Total PUFA	11.2		10.4		12.4		8.0		11.7	
Trace <0.1%	47.6		37.8		42.3		62.1		33.3	

SFA Saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid

Standard deviation, $n = 3$

Fluorescence of neutral lipids

The liposoluble fluorescence probe Nile Red was used to visualize neutral lipids in the cells. This method has several advantages over in situ screening. The dye is relatively photostable, intensely fluorescent when dissolved in organic solvent and in a hydrophobic environment, and it is sensitive to non-polar lipids in living cells. In this study, microphotographs (Fig. 5) were obtained from stationary-phase cultures.

The highest lipid content, as determined by Nile Red staining, was observed in *K. veneficum*, in which 81% of the cells were lipid-positive. In this species, small drops of neutral lipids were seen dispersed throughout the cytoplasm. While some *A. minutum* cells in the sample stained highly positive for lipid, others did not such that, overall, the percentage of stained cells was low (21.3%). *Tetraselmis suecica* was also comparatively poor in terms of neutral lipid content during the stationary phase. In contrast, many of the cells of *A. andersoni* showed massive concentrations of neutral lipids, usually located in the hypotheca of the cell.

Discussion

The growth curves obtained for the six microalgal species that we investigated are characteristic of marine microalgae in showing that for most of them, the lag phase occurred from day 0 to day 6; the exception was *T. suecica*, which is consistent with previously reported data for this species [19, 20]. The biomass of *T. suecica* doubles every 24 h during the exponential growth phase, thus reaching high densities. For this reason, it has become one of the most frequently used microalgal strain in industrial aquaculture.

Among the Dinophyceae, *K. veneficum* showed the best performance in terms of growth, although the rate measured in this study was much lower than that of wild populations [21]. Nonetheless, it was high enough to yield a large biomass in culture within a reasonable period of time. Further studies will be needed to determine whether the growth rate in culture can be improved, such as by isolating new strains and test different abiotic parameters prior the establishment of the maximum growth rate.

In the genus *Alexandrium*, different strategies of growth and abundance were observed. The best growth results were obtained with *A. andersoni*, with a rate similar to that of wild populations in the Mediterranean Sea. For this species, maximum cell abundance occurred at day 35, and the growth curve was longer than that of either the control algae or the best Dinophyceae. In contrast, both *A. minutum* and *A. catenella* had low growth rates. Moreover, the cell abundance of *A. catenella* was the lowest of the strains studied, specifically, almost two orders of magnitude less than the best microalgae in this study (*T. suecica*). The

Fig. 4 Different types of oil [29] compared with the fatty acid profile obtained from dino-flagellates and raphidophyte in this study (color figure online)
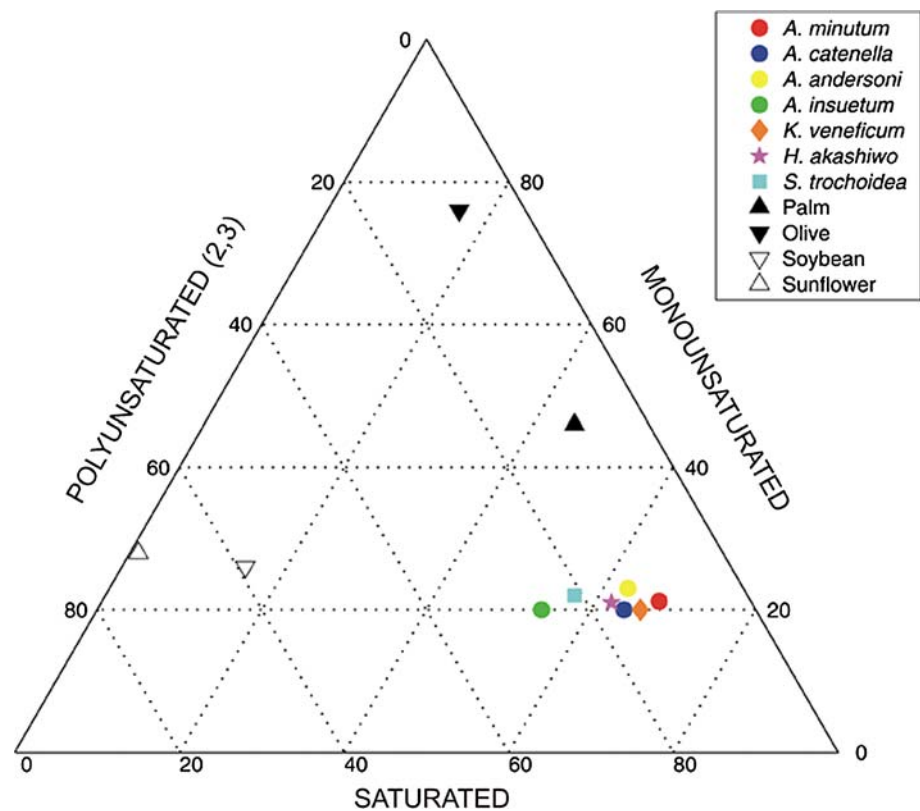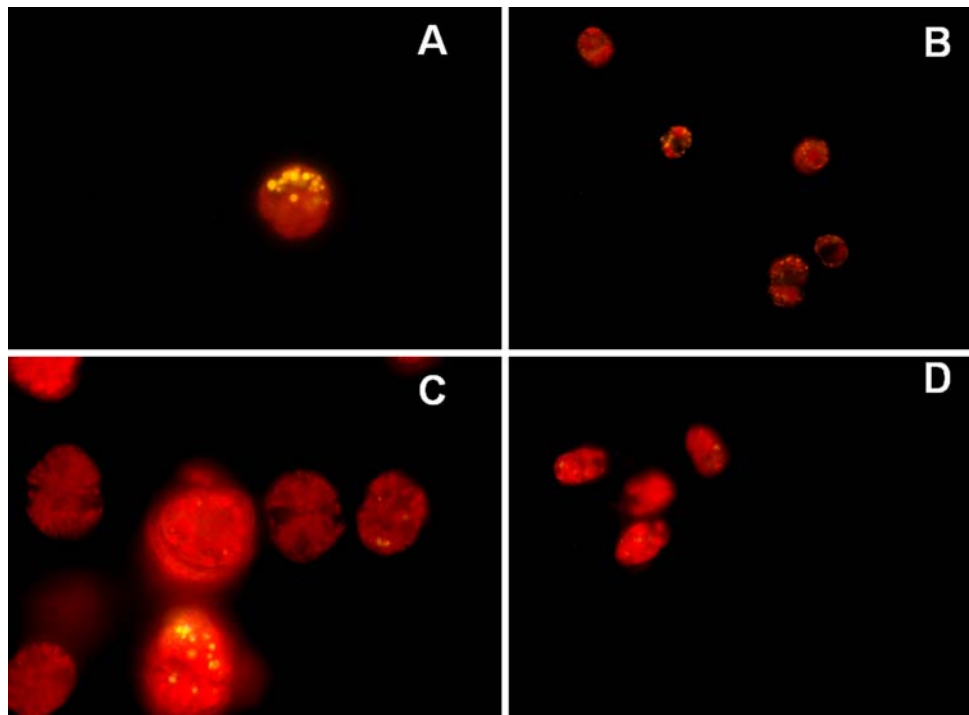

Fig. 5 Epifluorescent micro-photographs (magnification ×60) of microalgae stained with the fluorochrome Nile Red. Neutral lipids are seen as *lighter colored drops* in cells. *A Alexandrium andersoni*, *B Karlodinium veneficum*, *C Alexandrium minutum*, *D Tetraselmis suecica*

growth rate of the raphidophyte *H. akashiwo* was similar to that of *A. andersoni*, with a maximum density at day 36. Furthermore, this latter species was able to remain in stationary phase for more than 6 months. This feature could be taken advantage of to maintain this organism as a constant inoculum in a high-biomass culture strategy. The different growth rates and cell abundances suggest that the biovolume of the cells greatly influences the carrying capacity of the population when the microalgae are cultured in flasks or tanks such as those used in this study.

The biomasses of the different strains were similar during the different culture phase. For example, the biomass of all strains was lower during the lag phase than during the exponential phase, with the maximum biomass being that of *H. akashiwo* and the minimum being that of *A. andersoni*. In the transition from lag phase to exponential phase, the biomass of all strains increased, most importantly in *A. catenella* and *A. andersoni*, as both strains almost doubled their biomass weight within 10 days. In contrast, the biomass of *H. akashiwo* in these two growth phases differed by only a few grams. In the stationary phase, culture biomass decreased in all cases but one: *T. suecica* maintained its biomass between exponential phase and lag phase.

In terms of commercial or industrial applications, it is important that the optimal phase to harvest the microalgae is known. Our results suggest that, in terms of growth phase and biomass in this type of culture (batch cultures), harvest during the late-exponential phase results in the highest yields. For example, the average wet weight of *K. veneficum*, *A. andersoni*, and *H. akashiwo* during the late-exponential phase was approximately 15 g/L. This value is low compared to the 50 g/L reported for *Spirulina platensis*, a typical cyanobacteria used in protein production and cultured in open ponds, but it is similar to the weights obtained following heterotrophic cultivation of *Chlorella protothecoides*, which is used for biodiesel production. The WW of this green algae when cultured in a bioreactor was reported to be about 15.5 g/L in 5-L vessels, 12.8 g/L in 750-L vessels, and 14.2 g/L in 11,000-L vessels [22].

The total lipid content in our strains, especially those belonging to the Dinophyceae and the Raphidophyte, increased from the lag and exponential phases to the stationary phase. These results are consistent with those of previous studies on other dinoflagellate species. Mansour et al. [23] found that in *Gymnodinium* sp. the proportion of triacylglycerols increased almost fourfold during the stationary phase compared to the level measured during the exponential phase. Triacyglycerols function as storage lipids and, therefore, in most microalgae they are usually at their lowest levels during exponential growth, increasing during stationary phase as nitrogen or phosphorus is depleted [24, 25]. This observation can be practically applied in strategies aimed at exploiting the high biomass reached by cultures of dinoflagellates, such as *K. veneficum*. Accordingly, it is important to evaluate growth phase, biomass, and storage lipids with respect to achieving the highest production under different limiting conditions. Based on our results on biomass and lipid content, the late-stationary phase is the optimal time to harvest microalgae with the aim of obtaining the highest concentrations of oil.

The characterization of marine microalgae lipid composition has been suggested as a chemotaxonomic tool to distinguish between orders and classes of these organisms [25, 26]. However, the lipid composition within the same species can vary in response to growth conditions and other related factors. In the microalgae studied here, the characteristic fatty acid composition is C16:0 and C18:0 [27], and these lipids were present in high concentrations in our strains. Additionally, the PUFAs octadecapentaenoic acid (OPA 18:5n3), and 20:5n3 were present in all dinoflagellate species, albeit in varying amounts. A requirement of the raw material used for biodiesel production is that it contain high amounts of saturated fatty acids. Our results show that the oil extracted from dinoflagellates is highly similar to palm oil (Fig. 4). With respect to biodiesel production, the fatty acid profile of the dinoflagellates suggests that the obtained product offers several advantages in terms of quality because it results in a high-cetane fuel and thus a high quality of ignition.

Staining cells with Nile Red confirmed the presence of oils drops distributed throughout the cytoplasm. Within the same culture of *A. minutum*, some cells had large amounts of neutral lipids, whereas others had none. There were indeed differences in the per-cell oil content in this species. In *Karlodinium*, small oil drops were heterogeneously distributed in the cell body, while in almost all cells of *A. andersoni* they were concentrated in the hypotheca. Oil drops were difficult to observe in *T. suecica*, most likely due to the low lipid content of the cells. Taken together, our staining results demonstrate that Nile Red can be used prior to the rapid quantification of neutral lipids by spectrophotometry, as described by other groups [18, 28].

Pilot studies using large-scale cultures are needed to validate our findings that dinoflagellates offer a sustainable approach to biodiesel production. In these studies, a natural source of light should be used and culture strategies, such as photobioreactor and open-pond cultures, should be developed with the aim of enhancing the quantity and the quality of the microalgal lipids.

Conclusion

Dinoflagellates are widely distributed and readily isolated in many different countries. As shown here, they comprise several strategic species that can be used as a source of raw material for biofuels. An analysis of the biotic characteristics (growth rate, biomass, cell yield, lipid content) of several species of microalgae supports their use as feedstocks for biodiesel production. Two species of Dinophyceae, *K. veneficum* and *A. andersoni*, and one Raphidophyte species, *H. akashiwo*, were found to be of particular interest as a bioresource for biodiesel production, based on: (1) their high lipid content; (2) their moderate net growth rate; (3) their high average wet biomass; (4) their short period of growth (28–35 days) compared with terrestrial plants;

Future work will need to focus on improving the biotic features of microalgal cultures relevant to biodiesel production, mainly by changing environmental parameters such as salinity, nutrient, temperature, and light conditions.

References

1. Palligarnai T, Vasudevan PT, Briggs M (2008) Biodiesel production-current state of the art and challenges. J Ind Microbiol Biotechnol 35:421–430. doi:10.1007/s10295-008-0312-2

2. Lian PH (2007) Potential habitat and biodiversity losses from intensified biodiesel feedstock production. Ecol Conserv Biol 21:1373–1375

3. Puppán D (2002) Environmental evaluation of biofuels. Periodica polytechnica. Ser Soc Man Sci 10(1):95–116

4. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306. doi:10.1016/j.biotechadv.2007.02.001

5. U.S. Department of Energy, Energy Information Administration (2006) International Energy Outlook (Dept. of Energy, Washington, DC), DOE Publ. No. EIA-0484

6. Chang EH, Yang SS (2003) Some characteristics of microalgae isolated in Taiwan for biofixation of carbon dioxide. Bot Bull Acad Sin 44(1):43–52

7. Hsueh HT, Chu H, Yu ST (2007) A batch study on the bio-fixation of carbon dioxide in the absorbed solution from a chemical wet scrubber by a hot spring and marine algae. Chemosphere 66(5):878–886. doi:10.1016/j.chemosphere.2006.06.022

8. Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends Biotechnol 26(3):126–131. doi:10.1016/j.tibtech.2007.12.002

9. Yanqun L, Horsman M, Nan W, Christopher QL, Dubois-Calero N (2008) Biofuels from microalgae. Am Chem Soc 24:815–820

10. Sheehan J, Dunahay T, Benemann J, Roesler P (1998) A look back at the US Department of energy's aquatic species program: biodiesel from algae. U.S. Department of Energy National Renewable Energy Laboratory, Golden

11. Qiang H, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54:621–639. doi:10.1111/j.1365-313X.2008.03492.x

12. Clement A, Aguilera A, Fuentes-Grünewald C (2002) Análisis de marea roja en Archipiélago de Chiloe, contingencia verano 2002. In: 22nd Congreso de Ciencias del Mar. Valdivia, Chile

13. Basterretxea G, Garcés E, Jordi A, Masó M, Tintoré J (2005) Breeze conditions as a favoring mechanism of *Alexandrium taylori* blooms at a Mediterranean beach. Estuar Coast Shelf Sci 32:1–12

14. Guillard RRL, Hargraves PE (1993) *Stichocrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32:234–236

15. Guillard RRL (1973) Division rates. In: Stein JR (ed) Handbook of phycological methods. Cambridge University Press, Cambridge, pp 289–312

16. Ruiz J, Antequera T, Andres AI, Petron MJ, Muriel E (2004) Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. Anal Chim Acta 520:201–205

17. Russell JM, Werne JP (2007) The use of solid phase extraction columns in fatty acid purification. Org Geochem 38:48–51

18. Cooney MJ, Elsey D, Jameson D, Raleigh B (2007) Fluorescent measurement of microalgal neutral lipids. J Microbiol Methods 68:639–642

19. Fábregas J, Otero A, Dominguez A, Patiño M (2001) Growth rate of the microalgae *Tetraselmis suecica* changes the biochemical composition of Artemia species. Mar Biotechnol 3:256–263

20. Fábregas J, Abalde J, Herrero C, Cid A (1991) Yields in biomass and chemical constituents of four commercial important marine microalgae with different culture media. Aquac Eng 10:99–110

21. Stolte W, Garcés E (2006) Ecological aspects of harmful algal in situ population growth rates. Ecolological Studies, vol 189. Springer, Berlin Heidelberg

22. Li X, Xu H, Wu Q (2007) Large-scale biodiesel production from microalgae *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnol Bioeng 98(4):764–771

23. Mansour P, Volkman J, Blackburn S (2003) The effect of growth phase on the lipid class, fatty acid and sterol composition in the marine dinoflagellate, *Gymnodinium* sp. in batch culture. Phytochemistry 63:145–153

24. Brown MR, Dunstan GA, Norwood SJ, Miller KA (1996) Effects of harvest stage and light in biochemical composition of the diatom *Thalassiosira pseudonana*. J Phycol 34:712–721

25. Hallegraeff GM, Nichols PD, Volkman JK, Blackburn S, Everit D (1999) Pigments, fatty acids, and sterols of the toxic dinoflagellate *Gymnodinium catenatum*. J Phycol 27:591–599

26. Mooney DB, Nichols PD, De Salas MF, Hallegraeff GM (2007) Lipid, fatty acid, and sterols composition of eight species of kareniaceae (Dinophyta): chemotaxonomy and putative lipid phycotoxins. J Phycol 43:101–111

27. Mathews CK, Van Holde KE (2000) Bioquimica, 2nd edn. MacGraw-Hill/Interamericana, Madrid

28. Gao C, Xiong W, Zhang Y, Yuang W, Wu Q (2008) Rapid quantification of lipids in microalgae by time domain nuclear resonance. J Microbiol Methods 75:437–440

29. Ramos MJ, Fernández, CM, Casas Abraham, et al (2008) Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour Technol 100:261–268