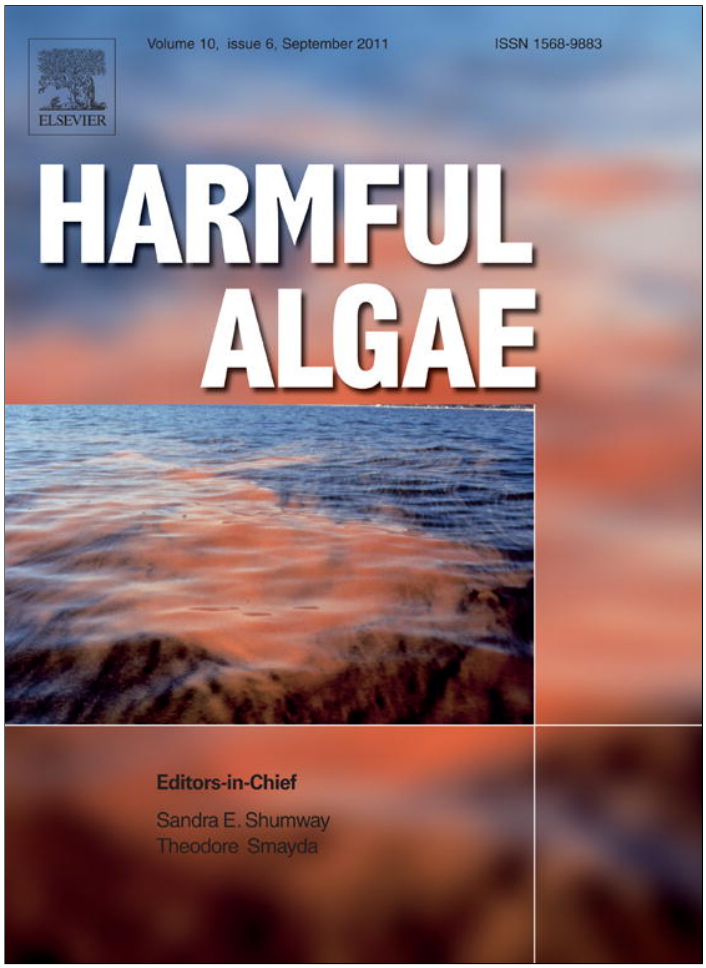

Harmful Algae 10 (2011) 654–667

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Intraspecific variability in *Karlodinium veneficum*: Growth rates, mixotrophy, and lipid composition

Albert Calbet [a,*], Mireia Bertos [a], Claudio Fuentes-Grünewald [a,b], Elisabet Alacid [a], Rosa Figueroa [c,1], Berta Renom [a], Esther Garcés [a]

[a] Department of Marine Biology and Oceanography, Institut de Ciències del Mar (CSIC), P. Marítim de la Barceloneta 37-49. 08003, Barcelona, Spain
[b] Institut de Ciència i Tecnologia Ambientals, Universitat Autònoma de Barcelona, (ICTA-UAB), Edifici Cn Campus UAB - 08193 Cerdanyola del Vallés, Barcelona, Spain
[c] Instituto Español de Oceanografia (IEO), C.O. Vigo, 36280, Vigo, Spain

ARTICLE INFO

Article history:
Received 8 February 2011
Received in revised form 6 May 2011
Accepted 6 May 2011
Available online 13 May 2011

Keywords:
Dinoflagellate
Fatty acids
Growth
Grazing
Harmful algal blooms
Karlodinium veneficum

ABSTRACT

We isolated eleven strains of the harmful algal bloom (HAB)-forming dinoflagellate *Karlodinium veneficum* during a bloom event in the NW Mediterranean coastal waters and we studied the inter-strain variability in several of their physiological and biochemical traits. These included autotrophic growth parameters, feeding capabilities (mixotrophy), lipid composition, and, in some cases, their responses to biotic and abiotic factors. The strains were found to differ in their growth rates (0.27–0.53 d^{-1}) and in the maximum cell concentrations achieved during stationary phase (6.1×10^4–8.6×10^4 cells mL^{-1}). Their ingestion performance, when offered *Rhodomonas salina* as prey, was also diverse (0.22–1.3 cells per *K. veneficum* per day; 8–52% of their daily ration). At least two strains survived for several months under strict heterotrophic conditions (no light, low inorganic nutrients availability, and *R. salina* as food source). These strains also showed very distinct fatty acid compositions, with very low contents of monounsaturated and polyunsaturated fatty acids. According to a Bray Curtis similarity analysis, three or four strain groups able to perform different roles in bloom development were identified. We further analyzed one strain from each of the two most distinct groups with respect to prey concentration, light intensity, nutrient availability, and we determined the functional responses (growth and feeding rates) to food concentration. Taken together, the results served to highlight the role of mixotrophy and clone variability in the formation of HABs.

1. Introduction

A microalgal bloom is caused by a rapid increase in the growth of a group or species of algae, resulting in its preferential and abundant accumulation. When the species involved in such blooms are potentially harmful, either for the environment or socio-economically, they are referred to as harmful algal blooms (HABs). Traditionally, HABs have been seen to derive from a clone of cells (i.e., genetically identical) that interact with other organisms and with the environment in an identical manner. Similarly, blooms formed by the same species are presumed to exhibit the same traits and behaviors. However, recent evidence points to differences in the toxin content of strains comprising many bloom-forming species, such as cyanobacteria (Carrillo et al., 2003) prymnesiophytes (Ichimi et al., 2002; Nejstgaard et al., 2007), and dinoflagellates (Loret et al., 2002; Nascimento et al., 2005; Wang et al., 2006; Adolf et al., 2008; Bachvaroff et al., 2009; Paz et al., 2007), as well as in the feeding behavior of some dinoflagellates (Adolf et al., 2008) and the growth rates of diatoms (Rynearson and Armbrust, 2000) and dinoflagellates (Loret et al., 2002). The conclusions of most such studies, especially those concerning dinoflagellates, were based on cultures of different strains obtained from different locations. In some reports, the strains were considered to be genetically identical, based on similarities in various molecular markers (Loret et al., 2002), while in others differences were determined (Adolf et al., 2008). The present work extends the research of Adolf et al. (2008) and Bachvaroff et al. (2009), who examined different strains of *Karlodinium veneficum* from different locations, by characterizing differences in the growth and feeding activities of 11 strains of *K. veneficum* originating from the same sample, obtained from a bloom in Alfacs Bay (Catalonia, Spain) in June 2007. The question asked in this work was first posed by Bachvaroff et al. (2009): whether there is functional variability (in terms of growth and mixotrophy) within a bloom population, with some individuals more adapted to certain conditions than others. Specifically, we

* Corresponding author. Tel.: +34 932309519; fax: +34 932309555.
E-mail address: acalbet@icm.csic.es (A. Calbet).
[1] Present address: Limnology Division, Department of Ecology, Lund University, S-22362 Lund, Sweden.

1568-9883/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2011.05.001

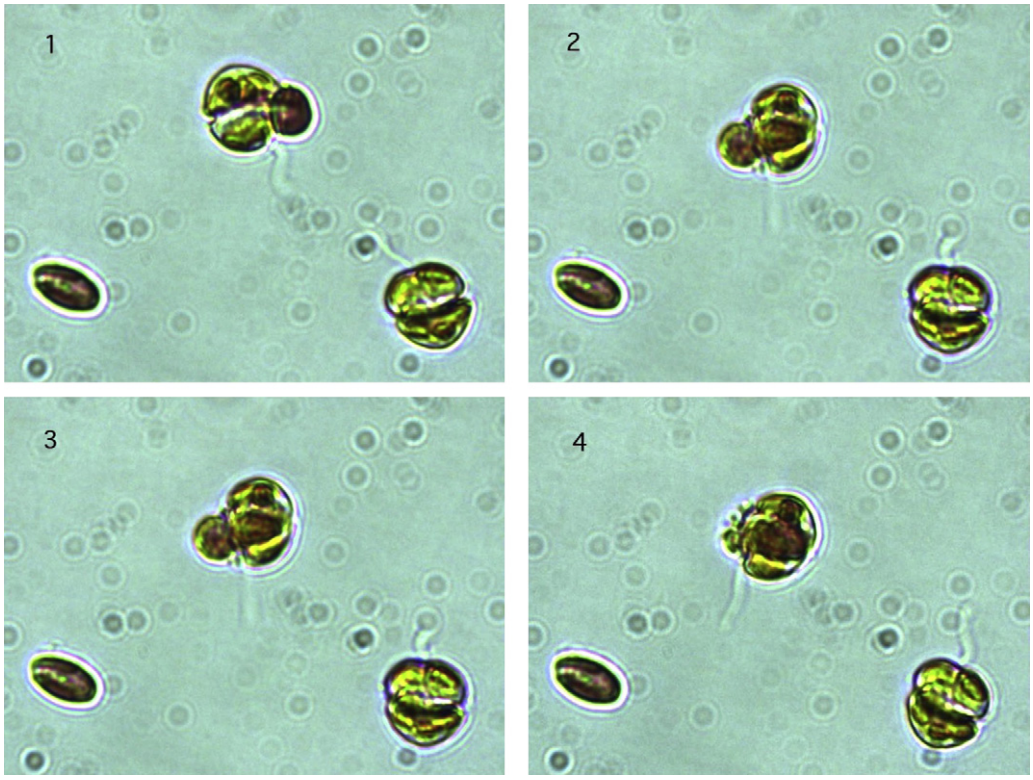

Fig. 1. Representative micrographs of *Karlodinium veneficum* feeding on *Rhodomonas salina* (ca.10 and 6.5 μm equivalent spherical diameter, respectively).

hypothesized that strains with relatively lower autotrophic growth rates would be those adapted to a more mixotrophic (i.e., ingestion of prey) energetic path and, accordingly, should manifest a characteristic lipid profile, i.e., low in the essential lipids usually obtained in a heterotrophic diet, including several polyunsaturated fatty acids (PUFAs) (Klein Breteler et al., 1999; Adolf et al., 2007). In addition, we examined the ecology of *K. veneficum* in further detail by investigating the effects of several biotic and abiotic variables (prey availability, light intensity, nutrient concentration, etc.) on its growth, feeding rates, and biochemical lipid composition.

The microalga chosen, *K. veneficum* (previously, *Woloszynskia micra*, *Gyrodinium esturiale*, *Gyrodinium galatheanum*, *Gymnodinium veneficum*, *K. micrum*), is a widespread and cosmopolitan HAB-forming dinoflagellate. Blooms of *K. veneficum* were first described in South Africa by Braarud (1957) and later in Europe (Bjornland and Tangen, 1979), North America (Li et al., 2000a,b; Terlizzi, 2000), and Australia (Ajani et al., 2001; Cosgrove et al., 2000). In addition to the proven ability of this species to capture and ingest prey (Li et al., 1999, 2000a,b, 2001; Adolf et al., 2008; Sheng et al., 2010; this work, Fig. 1), mixotrophy may be a relevant survival strategy in this species (Li et al., 1999, 2000a), with the presence of suitable prey acting as a trigger for toxic *K. veneficum* blooms (Adolf et al., 2008).

2. Methods

2.1. Strain variability

2.1.1. Sample collection, cell isolation, and culture

We isolated eleven strains of the dinoflagellate *K. veneficum* from single cells of one live sample collected in Alfacs Bay (NW Mediterranean; Table 1) in June 2007, during a bloom ($>1.6 \times 10^6$ cells L^{-1}) that coincided with high mortalities of the mollusk *Ensis ensis* (Fernández-Tejedor et al., 2009). Based on morphological characteristics, determined by optical and epifluorescence microscopy and by the number of chloroplasts per cell, all of the studied strains were identified as members of *K. veneficum*. Analyses of the 5.8S rDNA-ITS regions confirmed the microscopic identification. The ITS sequence codes for GenBank are presented in Table 1.

We transferred the isolated cells into IWAKI tissue-culture multi-wall plates filled with L1 medium without silicate (Guillard, 1975) adjusted to 34 psu (after few months transferred to 38 psu). All established cultures were maintained at 20 °C under a 12:12 light:dark cycle. Illumination was provided by fluorescence tubes (Gro-lux, Sylvania, Germany), generating a photon irradiance of 100 μE m^{-2} s^{-1}.

2.1.2. Growth kinetics in L1 medium

Growth rate parameters were compared for the eleven *K. veneficum* strains. To ensure consistency with future work, we retained the original labeling of the strains, even if not correlative.

Table 1
Summary of the experiments conducted and the strains of *Karlodinium veneficum* used in each experiment. The corresponding ICM (Institut de Ciències del Mar-CSIC) code and GenBank (BankIt1450636 Seq.) sequence code is also indicated.

Experiment	Strains
Growth kinetics in L1 medium, variability in feeding behavior, and variability in fatty acid contents	K1, K3, K4, K6, K12, K16, K17, K21, K22, K24, K30
Functional feeding and growth responses	K4, K24
Effects of light on feeding behavior	K4, K24
Effect of low nutrient availability on feeding behavior	K4, K24
Effects of long-term heterotrophic conditions	K3, K4, K21, K22, K24, K30
Effects of feeding on lipid composition	K24
Sequence codes:	
K1: ICMB-256, not sequenced	K17: ICMB-271, JF906087
K3: ICMB-258, JF906074	K21: ICMB-274, JF906089
K4: ICMB-259, JF906075	K22: ICMB-275, JF906090
K6: ICMB-261, JF906077	K24: ICMB-277, JF906092
K12: ICMB-266, JF906082	K30: ICMB-282, JF906096
K16: ICMB-270, JF906086	

For the experiments presented here, we kept the strains in 75-mL polystyrene culture flasks, in exponential growth phase, for several days prior to the experiment. Duplicate 1.3-L Pyrex bottles filled with L1 medium (38 psu) were then inoculated with aliquots of the different strains to reach an initial concentration of ca. 3000 cells mL^{-1}. Almost daily, the bottles were gently mixed and a 10-mL aliquot was removed to monitor the growth rates. Cells of the microalgae were counted in duplicate samples of 0.5 mL in a Multisizer III particle counter. The experiments lasted 14 days and were carried out at 18 °C under a 12:12 h light:dark cycle (120 μE m^{-2} s^{-1}). The growth rates of *K. veneficum* were calculated as the slope of the linear phase using Ln-transformed data (exponential growth phase).

2.1.3. Strain variability in feeding behavior

We verified the mixotrophic capacities of all eleven strains of *K. veneficum* by providing them with *Rhodomonas salina* as prey, based on the hypothesis that any given difference in autotrophic growth rates would be reflected by differences in feeding rates. We filled culture flasks with 75 mL of L1 medium at 38 psu and added *R. salina* to a final concentration of 5000 cells mL^{-1}. We set three (plus one initial) bottles as controls for *R. salina* growth, and we inoculated batches of three (plus one initial) bottles with the different strains of *K. veneficum* to reach a final concentration of 1500 cells mL^{-1}. To compensate for any dilution effect on the *R. salina* suspension resulting from addition of the grazers, a similar volume of L1 medium was added to all initial and control bottles. One bottle per treatment was sacrificed as the initial sample and the remaining bottles were incubated on a slowly rotating plankton wheel (0.2 rpm) for 48 h at 18 °C under a 12:12 h light:dark cycle (50–100 μE m^{-2} s^{-1}).

Due to the similarity in size between *R. salina* and *K. veneficum* (6.5–7 and 9.5–11 μm equivalent spherical diameter, respectively), they could not be accurately distinguished in an electronic particle counter. Therefore, in the grazing experiments, we preserved duplicate (sometimes triplicate) samples of the cells with Lugol and we counted them microscopically using a Sedgwick Rafter counting chamber. Additionally, evidence of feeding was obtained microscopically for live samples. An example of *R. salina* engulfment by *K. veneficum* is shown in Fig. 1.

Feeding rates were calculated only when significant differences ($p < 0.05$) were found between the growth rates of prey in the control and experimental bottles (*t*-test), based on the equations of Frost (1972) and using the average concentration of grazers throughout the incubation.

2.1.4. Strain variability in fatty acid contents

To characterize the fatty acid profile (FA) of autotrophically growing *K. veneficum*, we sampled the cultures in triplicate (10 mL/sample) during stationary growth phase. The samples were filtered through Whatman GF/F (25 mm) pre-combusted glass-fiber filters, immediately frozen in liquid nitrogen, freeze-dried for 12 h, and stored at −20 °C until analysis. Cell extracts were prepared using 3:1 DCM:MeOH (dichloromethane–methanol) according to the method of (Ruiz et al., 2004). The samples were subsequently redissolved in 0.5 mL of chloroform and eluted through a 500-mg aminopropyl mini-column (Waters Sep-Pak® Cartridges) previously activated with 4 mL of *n*-hexane, according to (Fuentes-Grünewald et al., 2009). The first fraction (neutral lipids) was eluted with 3 mL of chloroform:2-propanol (2:1) and the fatty acids recovered with 8.5 mL of diethyl ether:acetic acid (98:2). The free fatty acid (FFA) fraction was methylated using a 20% solution of $MeOH/BF_3$ followed by heating at 90 °C for 1 h, yielding fatty acid methyl esters (FAMEs). The reaction was quenched with 4 mL of NaCl-saturated water and the FAMEs 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 of chloroform, eluted through a glass column filled with Na_2SO_4 (to remove residual water), and, after chloroform removal, subjected to nitrogen evaporation. The extracts were stored at −20 °C until analyzed by gas chromatography, using a Thermo Finnigan Trace GC ultra instrument equipped with a flame ionization detector and splitless injector and fitted with a DB-5 Agilent column (30-m length, 0.25-mm internal diameter and 0.25-μm phase thickness). Helium was used as the carrier gas at a flow rate of 33 cm s^{-1}. The oven temperature was programmed to increase from 50 °C to 320 °C at 10 °C min^{-1}. The injector and detector temperatures were 300 °C and 320 °C, respectively. Methyl esters of the fatty acids were identified by comparing their retention times with those of the standards (37 FAME compounds, Supelco® Mix C4-C24) Finally, the fatty acids were quantified by integrating the areas under the peaks in the gas chromatograph traces (Chromquest 4.1 software) with calibrations derived from internal standards (2-octyldodecanoic acid and 5β-cholanic acid), and considering losses through the process.

Additionally, we examined whether the ingestion of prey (*R. salina*) modified the lipid composition of *K. veneficum*. Accordingly, the fatty acid profiles of mixotrophic cultures of *K. veneficum* (K24) and *R. salina* were characterized using the above-described methodology. Samples were withdrawn from triplicates cultures initially consisting of 5000 *R. salina* mL^{-1} and 1500 *K. veneficum* mL^{-1} in 150-mL bottles at time 0, 1, 2 days, and 4 days of incubation. The experiment was terminated when neither free cells of *R. salina* nor cryptophyte pigments inside *K. veneficum* cells were observed by epifluorescence microscopy, interpreted as complete digestion of the prey. This occurred after 4 days of incubation at 18 °C and a 12:12 h light:dark regime. The control consisted of autotrophic cultures of *K. veneficum* K24 and *R. salina* exposed to the same culture conditions.

2.1.5. Statistical similarity analysis

To explore similarities in the data obtained from the different *K. veneficum* strains, we used PRIMER software to obtain a multi-dimensional scaling of growth rate, biovolume, ingestion rates, and fatty acids concentration. The resemblance was based on the Bray-Curtis similarity, and similarity boundaries (70, 80, 85, and 90) were estimated.

2.2. Response of K. veneficum to environmental factors

2.2.1. Functional feeding and growth responses

We examined the functional responses of feeding and growth rates with respect to food concentration in the two strains of *K. veneficum* with the highest and lowest autotrophic growth rates (K4 and K24, respectively), based on the results shown in Table 2. The experimental design consisted of a range of prey concentrations (*R. salina*) prepared in three initial and three experimental 75-mL culture flasks filled with L1 medium (Table 2). An additional series of triplicate *R. salina* controls was prepared for each concentration to obtain prey growth rates. The initial concentrations of predators and prey are shown in Table 2. The bottles were incubated for 48 h under the same conditions as in the previous experiment. An incubation >24 h guaranteed that the dinoflagellates had adapted to the presence of the prey (Skovgaard, 1996) and provided a better growth response to prey availability. In parallel with this experiment, K4 and K24 strains in L1 (1500 cells mL^{-1}) were incubated with 5000 *R. salina* mL^{-1} and without prey, to estimate any growth enhancement due to feeding.

2.2.2. Effects of light on feeding behavior

We examined the response of *K. veneficum* to a short-term deprivation of light in order to evaluate whether its feeding behavior was modified under darkness. Therefore, simultaneous

with the functional response experiment, we added a treatment in which 1500 *K. veneficum* (K4 and K24 strains) mL^{-1} were incubated with 5000 *R. salina* mL^{-1} in L1 medium at 18 °C in triplicate bottles and complete darkness (the bottles were covered with aluminum foil). An additional three samples, consisting of *R. salina* (5000 cells mL^{-1}) controls, were included to estimate the growth of these algae in the dark.

2.2.3. Effect of low nutrient availability on feeding behavior

To test whether the feeding behavior of *K. veneficum* was nutrient-dependent, K24 and K4 strains were grown in sterilized filtered seawater (FSW) for 3 days and then used in an experiment in which their feeding rates on *R. salina* were assessed following the same protocol as described above. Accordingly, we incubated 1500 *K. veneficum* (K4 and K24) mL^{-1} in FSW with 5000 *R. salina* mL^{-1} in triplicate for 48 h at 18 °C. In addition, we carried out the same experiment but using L1 medium to compare the ingestion and growth rates of the two strains with and without nutrients. Triplicate controls consisted of *R. salina* alone in FSW and in L1-medium-enriched water (883 μM nitrate, 36 μM phosphate). After 3 days of acclimation to FSW, inorganic nutrient concentrations in the cultures were 7.6 μM nitrate and 0.6 μM phosphate. By the end of the experiment, the FSW cultures contained 2 μM nitrate and 0.2 μM phosphate. Nutrients were analyzed with an AA3 Bran + Luebbe Technicon.

2.2.4. Effects of long-term acclimation to heterotrophic conditions

We kept parallel cultures of *K. veneficum* (K3, K4, K21, K22, K24, and K30) in pasteurized FSW with *R. salina* as prey for 6 months in the dark to determine whether the selected strains could thrive under strict heterotrophic conditions. At the end of the 6-month period, a feeding experiment was set up as described above, in which the ingestion rates of the surviving strains were compared with those of parallel cultures grown for the same period in L1 medium containing an excess of nutrients. As in the previous experiments, the prey concentration was 5000 *R. salina* mL^{-1} and the grazer concentration 1500 *K. veneficum* mL^{-1}. Incubations for the feeding experiment lasted 24 h instead of 48-h to avoid any change in feeding behavior that the presence of nutrients may have had on the heterotrophic strains.

3. Results

3.1. Growth kinetics in L1 medium

The eleven strains of *K. veneficum* showed distinct growth responses, differing in their maximum concentrations at stationary phase and in their growth rates (Fig. 2, Table 3). By the end of the experiment (day 14), all strains, except K1 and K3, had entered stationary phase. The estimated growth rates ranged from 0.27 to 0.53 d^{-1} (Table 3). Note that K17 was not sampled at the same intensity as the other strains due to the unexplained presence of aggregates during the exponential phase of growth, which precluded accurate measurement of the cell concentration.

Table 2
Initial predator (*Karlodinium veneficum*) and prey (*Rhodomonas salina*) concentrations used for the feeding and growth functional-response experiments of strains K4 and K24.

Strain	*R. salina* × mL^{-1}	SD	*K. veneficum* × mL^{-1}	SD
K4	15476	1683.6	4404	89.3
K4	4289	691.4	1857	30.7
K4	2220	349.0	839	131.4
K4	1206	25.5	716	211.5
K4	721	20.4	514	20.5
K4	431	23.3	274	6.4
K24	15642	1831.6	5548	146.9
K24	4522	63.3	2141	236.2
K24	2400	147.5	1139	50.4
K24	1302	117.9	914	94.3
K24	661	33.4	555	28.8
K24	407	32.9	327	46.0

SD: standard deviation.

The maximum *K. veneficum* concentration reached was not significantly correlated with the growth rate; however, growth rates were significantly linearly related ($p < 0.01$) with cell volume, i.e., the larger the cells, the faster the growth rate (Fig. 3). Interestingly, upon reaching stationary phase many of the strains significantly reduced their cell volume, except K16, K17, and K22, whose volumes increased (Table 3).

3.2. Strain variability in feeding behavior

Table 4 shows the *R. salina* ingestion rates by the different strains of *K. veneficum*. The differences between the strains were obvious, ranging from 0.22 to 1.34 cells per *K. veneficum* per day, corresponding to a daily ration (specific ingestion rates × 100) between 8% and 52% of cell carbon (Table 4).

K. veneficum ingestion rates were linearly related to their autotrophic growth rates and cell volume (Fig. 4). Assuming that the relationship between *K. veneficum* growth rate and cell volume (Fig. 3) drove the relationship between ingestion rates and growth rates, we asked whether the significance was retained when the variability associated with cell volume was excluded. Fig. 4 shows a plot of the relationship between *K. veneficum* growth rates and

Table 3
Summary of the results of the autotrophic growth experiment. Growth rates of *K. veneficum* were calculated on Ln-transformed values in the exponential section of the growth curve. Max. cells are the maximum cell concentration attained in the stationary phase. Cell volume (obtained with a Coulter counter) is also indicated for the exponential and stationary phases of the growth curve. Asterisks indicate the cases in which the cell volume was different between exponential and stationary phases at $p < 0.05$ (*t*-test).

Strain	Growth rate (d^{-1})	SE slope	r^2	Max. cells (cells mL^{-1})	Cell volume (μm^3) exponential phase	SE	Cell volume (μm^3) stationary phase	SE
K1	0.32	0.018	0.98	79585	470.6	1.5	450.1	11.3
K3	0.36	0.013	0.99	86600	449.9	7.0	401.8*	4.8
K4	0.27	0.011	0.99	69815	433.8	5.9	389.8*	7.6
K6	0.37	0.009	1.00	71665	416.6	8.1	421.8	1.3
K12	0.43	0.056	0.97	81895	597.1	21.9	531.8*	10.6
K16	0.36	0.028	0.98	72510	509.0	9.9	544.6*	11.0
K17	0.40	0.000	1.00	69365	463.5	4.9	501.6*	1.0
K21	0.40	0.014	0.99	60960	581.4	22.1	564.1	2.9
K22	0.47	0.030	0.99	70535	521.9	2.6	610.3*	7.2
K24	0.53	0.042	0.99	76060	646.8	11.6	516.4*	3.6
K30	0.48	0.031	0.99	75755	585.0	5.3	496.5*	17.9

SE: standard error.

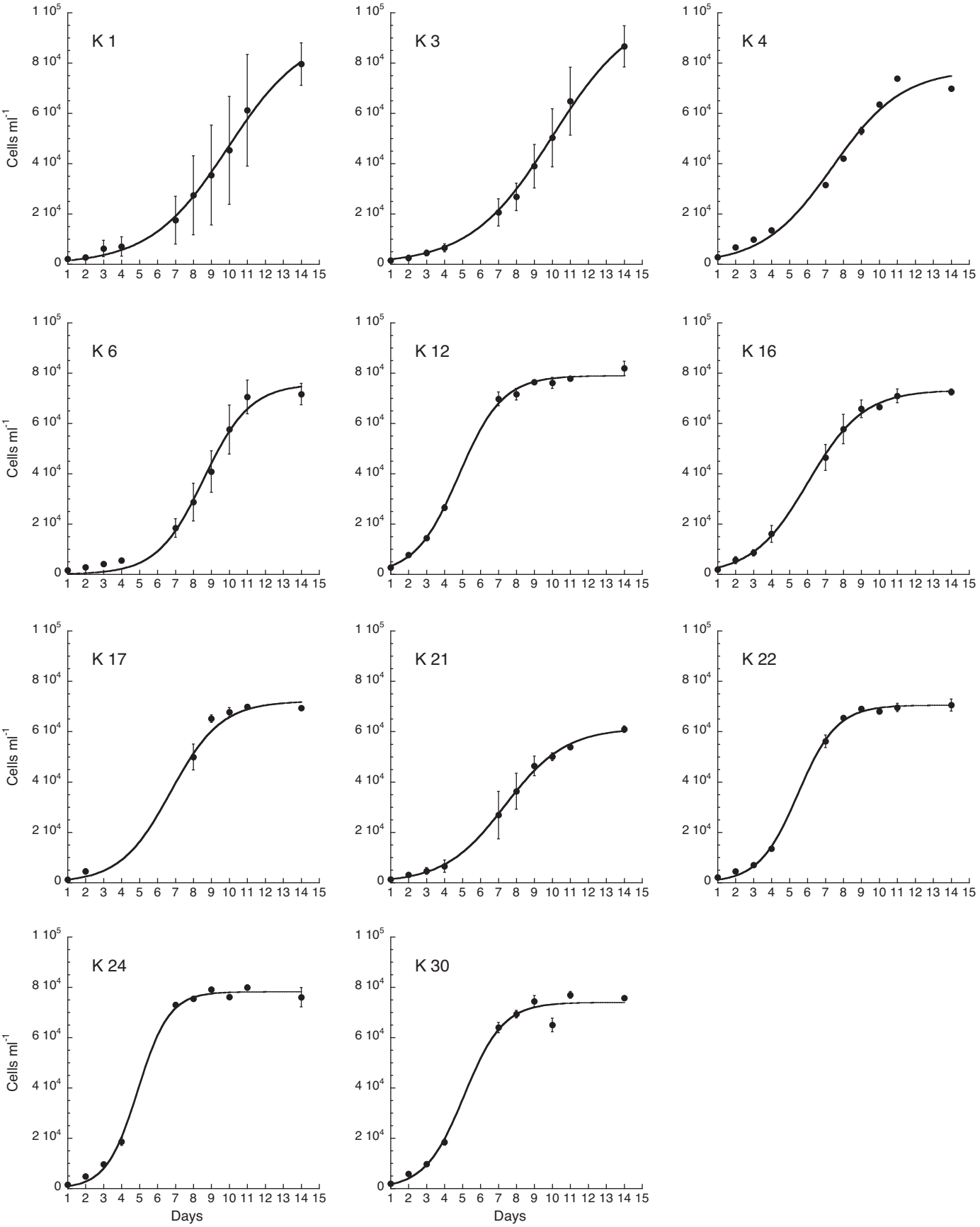

Fig. 2. *Karlodinium veneficum* cell abundance over a period of 14 days in L1 rich medium. The data were fitted to a logistic curve for illustrative purposes. The autotrophic growth rates corresponding to the exponential phase are given in Table 3.

Table 4
Ingestion rates (cells *K. veneficum*$^{-1}$ day^{-1}) and carbon-specific ingestion rates ($\mu gC_{prey}\ \mu gC_{predator}^{-1}\ d^{-1}$) of the different strains of *K. veneficum* feeding on *R. salina*.

Strain	Ingestion rate	SD	Specific ingestion rate	SD
K1	0.69	0.092	0.24	0.033
K3	0.36	0.068	0.13	0.023
K4	0.22	0.146	0.08	0.055
K6	1.34	0.081	0.52	0.031
K12	1.26	0.116	0.37	0.035
K16	0.98	0.119	0.32	0.043
K17	0.79	0.084	0.28	0.029
K21	1.12	0.041	0.33	0.021
K22	1.22	0.070	0.40	0.021
K24	1.03	0.144	0.28	0.040
K30	1.09	0.099	0.32	0.029

SD: standard deviation.

weight-specific (carbon-based) ingestion rate. While the tendency was the same, the relationship was no longer significant ($p = 0.16$). In addition to describing the relationships between variables, the information in Fig. 4 is also useful to characterize associations between plots. For instance, plot 1 (cell volume *vs.* ingestion rates) provides clear evidence for three major groups of strains: those of high volume and high ingestion rates (K12, K16, K21, K22, K24, K30), those with intermediate values (K1, K17), and those of small volume and low ingestion rates (K3, K4). This association is only slightly modified in plot 2 (ingestion rate *vs.* growth rate), with the exception that K6 has moved closer to the main group. Finally, the plot of growth rate *vs.* specific ingestion rate also differs moderately from the other plots, with K4 and K6 defining the upper and lower limits of the specific ingestion rates, respectively. K24 is revealed as a strain with a high growth rate under autotrophic conditions and a medium biomass-specific feeding performance, whereas K22 seems to exhibit considerable growth and ingestion rates.

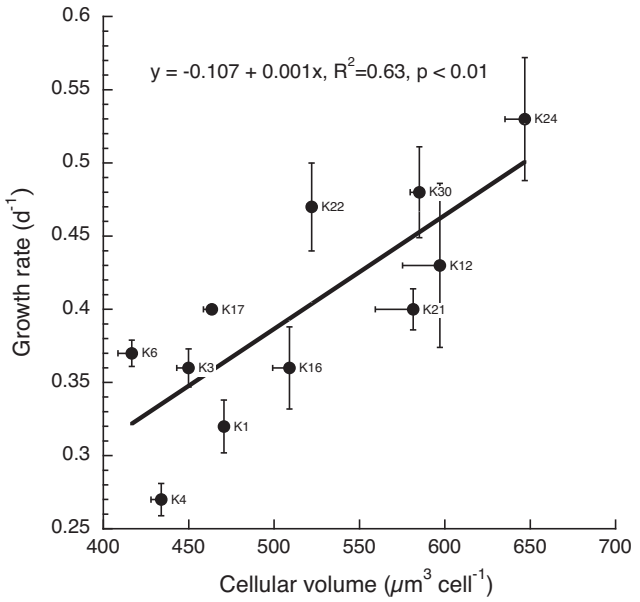

Fig. 3. Relationship between *K. veneficum* cellular volume in the exponential phase and the growth rate under autotrophic conditions (values from Table 3). Deming (Model II) equations were fitted for different variances. The determination coefficient and significance level are shown. Error bars are SE.

Table 5
Relative abundance (%) of fatty acid (FA) composition of different clones of *Karlodinium veneficum* (K1–K30: autotrophic growth; $K24_{mixotr.}$: mixotrophic growth). SAFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acids; SD: standard deviation; –: below detection limits. SD: standard deviation.

FA	K1	SD	K3	SD	K4	SD	K6	SD	K12	SD	K16	SD	K17	SD	K21	SD	K22	SD	K24	SD	K30	SD	$K24_{mixotr.}$	SD	Rhod	SD
C8:0	–		–		1.3	0.5	0.6	0.0	0.7	0.2	0.5	0.7	0.9	0.2	1.0	0.1	6.6	0.0	3.4	4.9	3.6	4.5	–		–	
C11:0	2.5	2.0	1.5	0.4	2.0	1.4	1.2	0.8	0.9	0.1	2.6	2.8	1.2	0.9	0.6	0.4	1.3	0.0	1.0	1.5	2.6	1.4	–		2.8	2.4
C12:0	5.8	4.6	1.1	1.1	0.3	0.0	0.2	0.1	0.1	0.1	0.2	0.3	0.2	0.1	–		–		–		0.1	0.1	–		–	
C14:0	5.1	3.7	1.7	0.7	1.3	0.2	1.6	0.2	1.4	0.0	0.5	0.8	1.6	0.2	1.2	0.1	–		–		0.3	0.4	1.2	0.0	1.2	0.4
C14:1	–		1.4	1.1	0.4	0.6	0.3	0.2	0.4	0.2	0.2	0.3	0.6	0.1	0.2	0.3	–		–		0.1	0.1	–		2.4	0.0
C15:0	6.2	4.0	1.6	1.7	0.1	0.2	–		0.2	0.0	–		–		–		–		–		0.1	0.1	–		–	
C15:1	7.1	0.0	–		–		–		–		–		–		–		–		–		–		–		–	
C16:0	9.9	3.6	11.5	2.4	12.5	1.8	16.4	1.1	17.5	0.9	17.6	0.5	14.8	0.8	18.9	1.9	8.8	0.0	16.2	1.6	16.7	2.0	15.5	0.3	16.4	0.3
C16:1	–		0.6	0.9	1.2	0.3	1.1	0.1	0.8	0.5	1.0	1.4	1.3	0.3	2.6	1.9	–		–		0.4	0.6	2.8	0.6	2.3	1.2
C17:0	–		–		0.2	0.3	0.6	0.1	0.6	0.0	–		–		0.7	0.1	–		–		0.2	0.3	–		–	
C17:1	–		–		–		0.8	1.1	–		–		–		–		–		–		–		–		–	
C18:0	4.6	3.8	0.4	0.6	0.9	0.3	0.4	0.1	0.4	0.0	0.3	0.4	1.6	1.7	2.1	2.0	–		–		0.3	0.5	1.9	0.3	1.8	0.7
C18:1	–		1.1	1.5	2.2	0.7	4.0	0.0	1.9	2.2	3.9	0.1	1.8	2.6	0.6	0.8	–		–		0.9	1.3	–		2.5	0.0
C18:2	–		1.4	1.9	3.2	0.4	–		1.9	2.7	–		–		2.3	3.2	–		–		–		–		–	
C18:3	–		12.5	13.6	17.2	2.7	15.4	0.5	16.6	1.4	8.5	12.0	15.2	2.6	20.1	2.2	21.6	0.0	8.5	12.0	14.2	1.6	15.6	1.0	17.7	0.6
C20:5	–		–		–		–		–		–		–		–		–		–		0.2	0.3	–		–	
C22:6	–		0.6	0.9	1.5	0.3	1.0	0.2	1.2	0.2	1.7	1.2	1.3	0.3	1.4	0.1	1.4	0.0	5.9	0.2	4.1	4.0	5.7	1.1	4.6	2.2
C24:0	–		–		–		–		0.3	0.5	–		–	1.9	–		–		–		0.3	0.4	–		–	
C24:1	–		–		0.5	0.8	–		0.3	0.4	–		1.4		–		–		–		–		0.7	1.0	–	
SAFA	29.3		17.3		17.8		20.6		21.6		21.4		18.7		22.3		16.7		20.6		23.9		16.6		20.4	
MUFA	7.1		3.1		4.3		6.2		3.8		5.1		5.1		3.4		0.0		0.0		1.4		3.5		7.3	
PUFA	0.0		14.5		22.0		16.4		19.7		10.2		16.5		23.8		23.0		22.8		18.5		21.3		22.3	

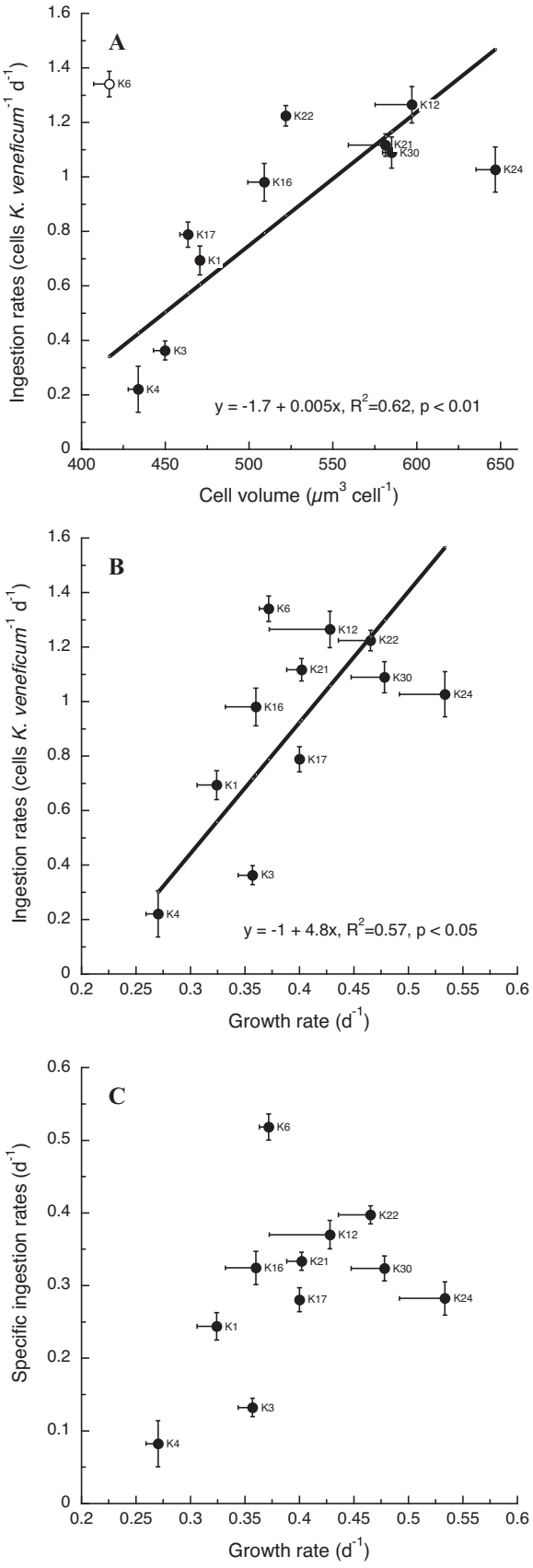

Fig. 4. (A) Relationship between rates of *K. veneficum* ingestion of *R. salina* and cell volume at exponential growth. (B) Relationship between rates of *K. veneficum* ingestion of *R. salina* and the autotrophic growth rates. (C) Relationship between carbon-specific rates of *R. salina ingestion* and *K. veneficum* autotrophic growth rates. One outlier (open circle; K6) in the ingestion rates *vs.* cell volume relationship was not considered for the equation. Deming (Model II) equations were fitted for different variances. The determination coefficient and significance level are also shown. Error bars are SE.

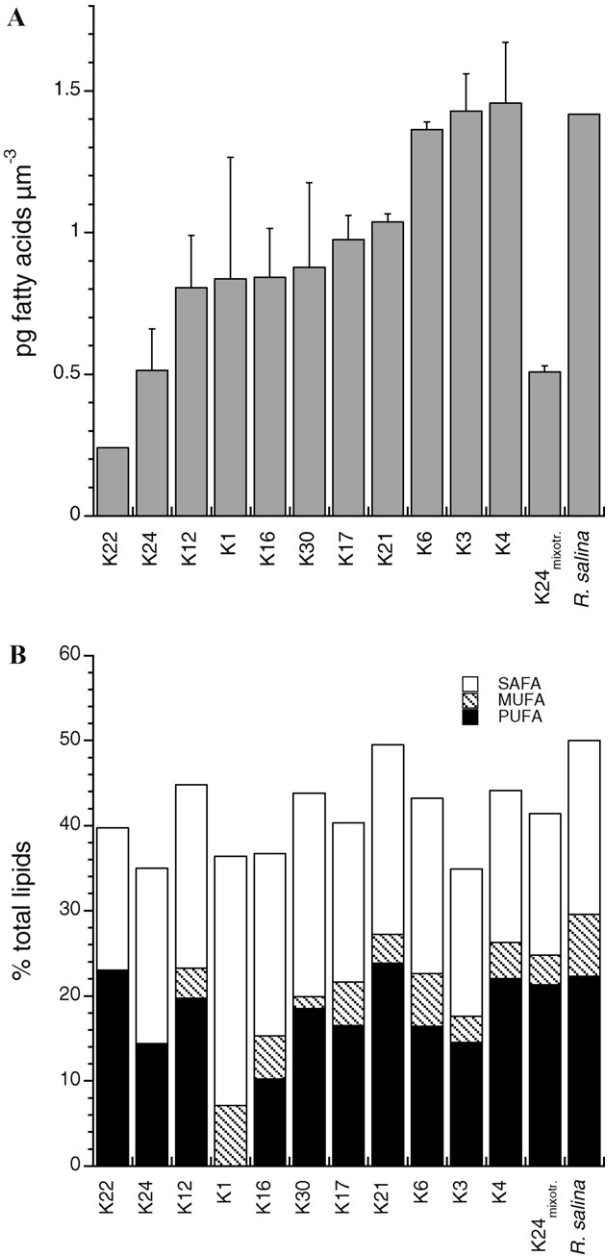

Fig. 5. (A) Fatty acids concentration per μm^3 of cells of the *K. veneficum* strains. (B) Relative abundance (%) of fatty acid composition in *K. veneficum* strains at the stationary phase of culture. K24$_{mixotr.}$ refers to the strain K24 fed with *R. salina* (see text). Error bars are SD.

3.3. Strain variability in fatty acid contents

Under autotrophic conditions, the strains varied considerably in terms of FA content, which ranged from 0.24 to 1.4 pg μm^{-3} (Fig. 5). In autotrophic *K. veneficum* strains, C16:0 accounted for 8.8–18.9%, C18:3n3 for 8.5–21.6%, and C22:6n3 for 0.6–5.9% of total FA. The corresponding values for mixotrophic *K. veneficum* were 15.5%, 15.6%, 5.7%, respectively (Table 5). The highest fatty acid contents were measured in strains K3, K4, and K6, and the lowest in strains K22 and K24. The latter result reflected the non-significant amount of monounsaturated FAs (MUFAs) in both

Table 6
Effects of nutrient depletion on *Karlodinium veneficum* (strains K4 and K24) ingestion (cells *K. veneficum* d^{-1}) and growth rates (d^{-1}). L1: cells grown in nutrient-rich medium, FSW: cells grown for 3 days in filtered seawater.

Treatment	Ingestion rate	SD	Growth rate	SD
K4 L1	0.00	–	0.056	0.070
K4 FSW	0.00	–	0.059	0.055
K24 L1	0.67	0.043	0.27	0.043
K24 FSW	0.48	0.012	0.39	0.012

SD: standard deviation.

strains (Fig. 5; Table 5). Saturated (SAFA) and polyunsaturated (PUFA) FAs accounted for most of the bulk fatty acids in the cells, and MUFAs for the minor proportion in the lipid profiles of the different strains. The phagocytosis by *K. veneficum* of its prey resulted in the incorporation of lipids into the cell, as evidenced by the presence of MUFAs in strain $K24_{mixotr.}$ after feeding on *R. salina* (Fig. 5; Table 5).

3.4. Statistical similarity analysis

The multi-dimensional scaling test based on similitudes among the strains with respect to growth rate, biovolume, ingestion rates, and fatty acid concentrations is presented in Fig. 6. The graph shows the relationship between similarities in the item–item matrix and the location of each strain in low-dimensional space. Some strains were highly similar and appear as a closed group, such as K3–K4 (90% similarity), K22–K24 (85% similarity), and K1–K12–K16–K17–K21–K30 (90% similarity). K6 was similar to the latter group at a similarity boundary of 85%, but differed at 90%.

3.5. Functional feeding and growth responses

The ingestion rate of strain K24 showed a typical functional curve (Holling II), with feeding saturation at ca. 3300 cells mL^{-1} and maximum ingestion rates of ca. 1 cell *R. salinas* per *K. veneficum* per day (Fig. 7). The *K. veneficum* growth rates also corresponded with the feeding response, although growth seemed to become saturated faster than ingestion (at ca. 800 cells mL^{-1}; Fig. 7). Conversely, K4 showed only significant feeding on *R. salina* at two concentrations of prey. The growth rates for K4 were actually quite low, with unexplained negative values at lower prey concentrations (Fig. 7). However, the presence of *R. salina* at high concentrations seemed to stimulate growth of the dinoflagellate, even if no feeding was detected. This pattern was corroborated when the growth rates obtained during *K. veneficum* feeding on *R. salina* were compared with those achieved by autotrophic metabolism only (L1 medium; Fig. 8). For K4, the ingestion rate was not significant (data not shown), although the presence of prey largely and unexplainably enhanced growth rates. However, K24 ingested 0.66 ± 0.029 SE *R. salina* cells per *K. veneficum* per day, which resulted in a slight (4.15%) but not significant enhancement of growth (Fig. 8).

3.6. Effects of light and nutrients on feeding behavior

The effects of short-term light deprivation on the ingestion and growth rates of strains K4 and K24 are shown in Fig. 7. Incubations in the dark did not cause differences in the ingestion rates whereas the growth rates were adversely affected. Indeed, the negative growth (cells were actually dying) of these two strains of *K. veneficum* indicated detrimental effects due to the short-term absence of light. This contrasts with the fact that strain K24 (hereafter K24h) was able to thrive in the dark for at least 6 months, feeding only on *R. salina*. Strain K22 (hereafter K22h) also survived for the entire duration of the experiment, with a higher *R. salina* ingestion rate than controls grown in L1 ($p < 0.01$, *t*-test; Fig. 9). Strain K24h was, unfortunately, accidentally lost before the feeding experiment was completed, and K3, K4, K21, and K30 perished after a few days/weeks of light and nutrient deprivation. It is worth mentioning that both K22h and K24h retained their chloroplasts during light deprivation but these appeared less bright on epifluorescence microscopy.

When grown under conditions of nutrient depletion for 3 days, strains K4 and K24 showed no enhancement of feeding (Table 6). K4 did not exhibit detectable grazing on *R. salina* under either nutrient-rich or nutrient-depleted conditions (data not shown), while there was a slight (although significant, $p < 0.05$ *t*-test) decrease in the ingestion rates of K24 in nutrient depleted *vs.* nutrient-rich conditions (0.48 ± 0.007 *vs.* 0.67 ± 0.025 SE cells *R. salina* per *K. veneficum* per day, respectively).

4. Discussion

This study had two main, interconnected objectives: (1) to assess the physiological and biochemical variability among different strains of *K. veneficum* isolated from one sample of the same bloom, and (2) in a further step, to determine whether these

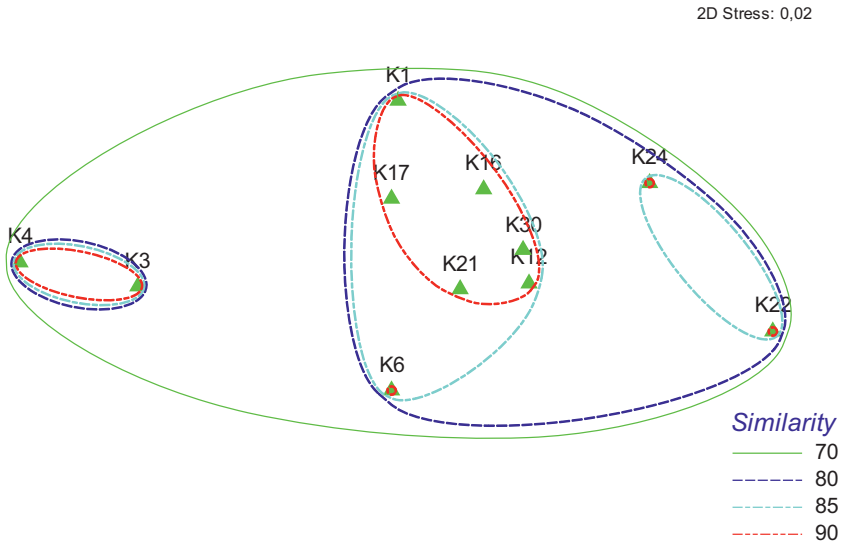

Fig. 6. Multi-dimensional scaling of the *K. veneficum* strains. Similarity boundaries (70, 80, 85, 90) are drawn.

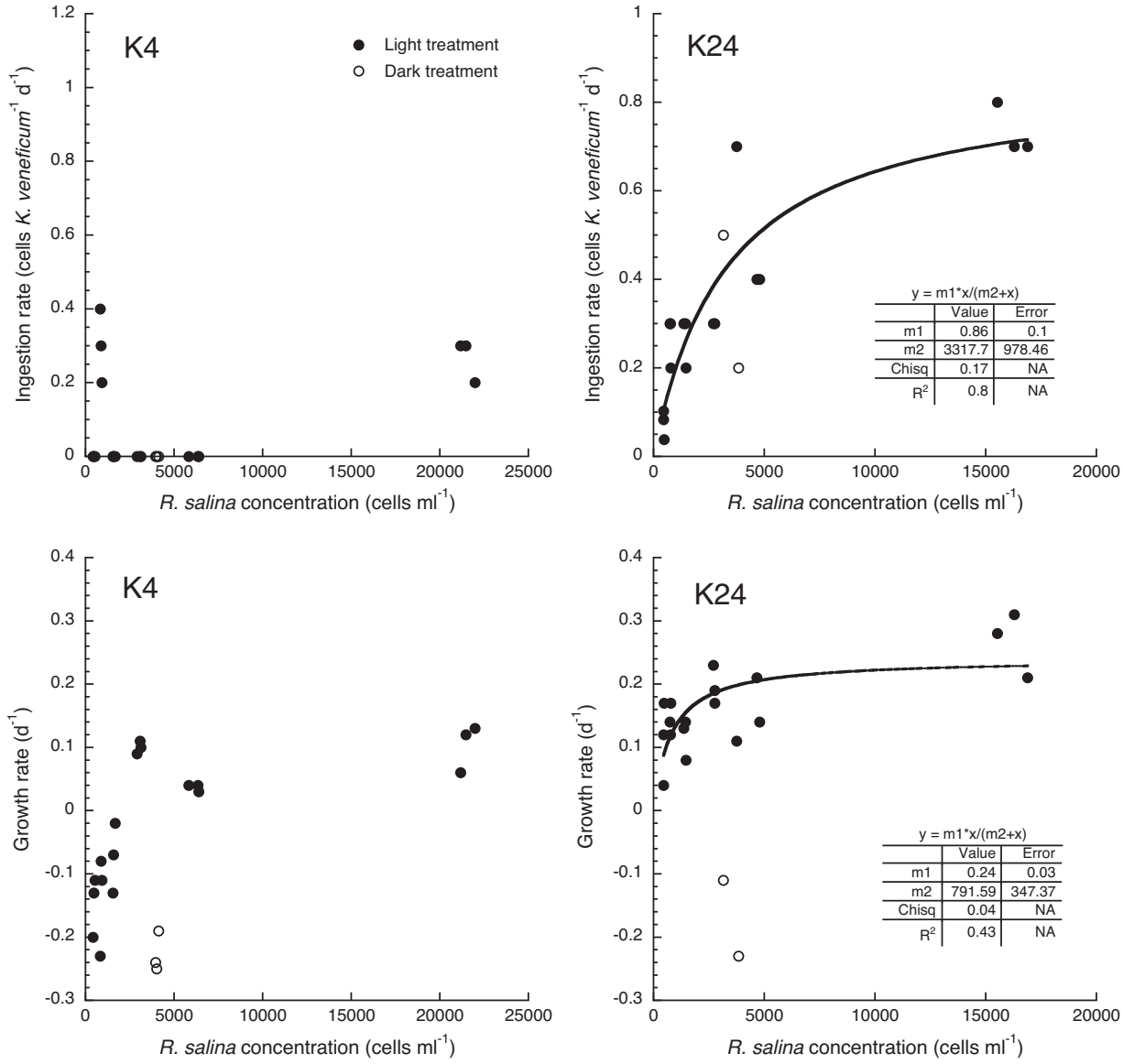

Fig. 7. Functional responses in ingestion and growth rates of strains K4 and K24 of *K. veneficum* feeding on *R. salina*. Open symbols correspond to incubations conducted in the dark. Fittings correspond to a Holling type II functional response.

strains, based on their autotrophic growth parameters, differed in their responses to certain biotic and abiotic factors, such as the presence of prey, light intensity, and nutrient availability. These objectives will be discussed in this order, followed by two brief, more general sections comparing the feeding capacities of *K. veneficum* with those of other protists and examining the role of mixotrophy in *K. veneficum* and other HAB-forming dinoflagellates. Our discussion concludes with remarks about the relevance of this study.

4.1. Strain variability

A significant result of our experiments was that *K. veneficum* strains originating from the same bloom varied remarkably in their growth rates, size, feeding behavior, and lipid composition. Nucleotide sequences analysis of the strains' internal transcribed space regions (ITS; Andree, K. unpublished data) failed to detect any differences among them, indicating genetic homogeneity in this region. It has been reported, however, that dinoflagellates (*Karenia brevis*) with homogeneous ribosomal DNA sequences can differ significantly in growth rates and toxin content (Loret et al., 2002). As pointed out by these authors, ITS sequences, a common marker for species and/or subspecies designation, may not be the best indicator of strain variability; instead, other, faster evolving DNA regions may be more informative. A recent study of the genetic markers of the *K. veneficum* strains analyzed herein found significant genetic differences between some of the strains, as determined by amplified fragment-length polymorphisms (AFLP) (Walworth pers. comm.). This result supports the conclusion that the different strains of *K. veneficum*, or at least certain groups, are distinct clones and is in agreement with our data on metabolic performance and lipid composition.

In this regard, according to our original hypothesis, cells with low autotrophic growth rates should exhibit an enhanced heterotrophic/mixotrophic metabolism, which should be evidenced by a negative relationship between autotrophic growth and ingestion rates. This could be understood as a specialization within a bloom; some cells being able to take advantage of other algae as a resource, thereby also reducing competition, while other cells being fast-growing when nutrients are available in high concentrations (Adolf et al., 2008). However, we found that cells with higher autotrophic growth also had a higher capability to

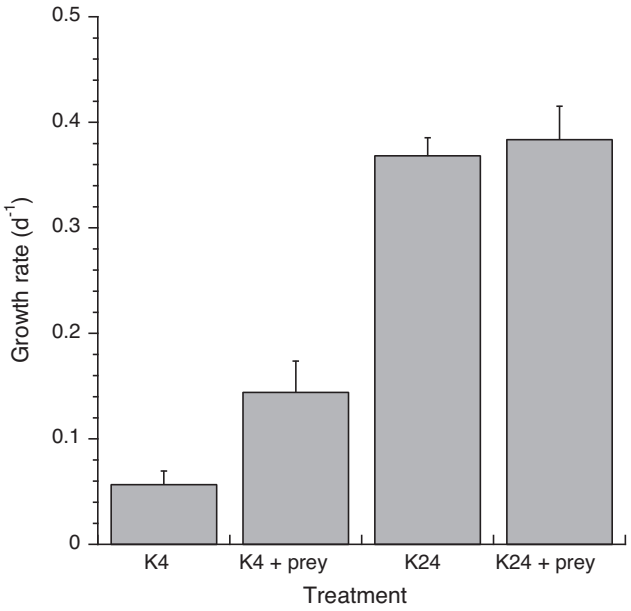

Fig. 8. Growth rates of strains K4 and K24 of *K. veneficum* incubated only with L1 medium or with L1 and prey (*Rhodomonas salina*). Error bars are SE.

phagocytize prey, contradicting this hypothesis. In fact, our results suggest that in a bloom, some strains are "winners" (with enhanced abilities under both autotrophic and heterotrophic conditions), whereas others have limited growth capacities. This idea was previously discussed for different species comprising HABs (Burkholder et al., 2008), although detailed information for different strains within the same bloom was not available.

The variability in growth and feeding rates of the *K. veneficum* strains was also evident in their lipid composition. Lipids are important in the marine environment, constituting a significant part of the total carbon flux through trophic levels. They have been used in the past as biomarkers (Lee et al., 1971; Reuss and Poulsen, 2002), facilitated by the fact that they can be analyzed directly from the environment and, ideally, can be interpreted both quantitatively and qualitatively in terms of *in situ* biomass.

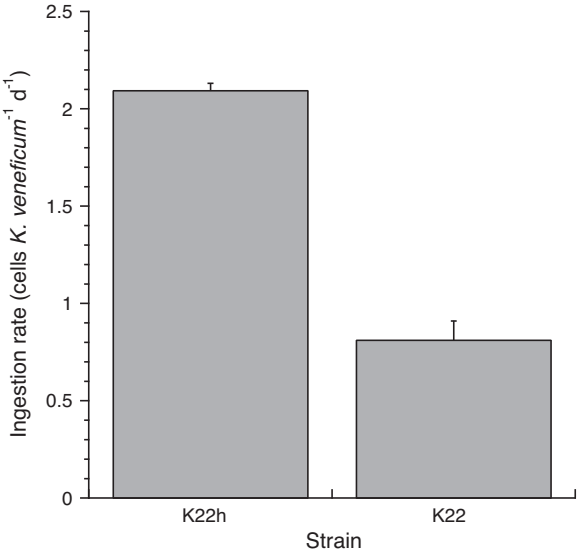

Fig. 9. Comparison of the ingestion rates of *K. veneficum* strain K22 grown under autotrophic conditions (light and excess inorganic nutrients; K22) and under heterotrophic conditions (dark, FSW, and feeding on *R. salina*; K22h). Error bars are SE.

However, lipid profiles in eukaryotic algae may vary over the course of the growth curve or in the presence of stress conditions, e.g., nitrogen deprivation (Hallegraeff et al., 1999; Mansour et al., 2003). Our study points out two other sources of variability in lipid profiling, i.e., between strains and between modes of nutrition (this aspect also evidenced in the work by Adolf et al., 2007). This is certainly a handicap in terms of using lipids as chemotaxonomic tools.

Overall, the fatty acid (FA) contents of our strains were higher than those previously reported for dinoflagellates (Jónasdóttir, 1994; Broglio et al., 2003), most likely because the method used to quantify total FAs and because in our study we analyzed the cultures at stationary phase. Stationary phase is characterized by an accumulation of FAs, mostly SAFAs and MUFAs (see review by, Dalsgaard et al., 2003). Although this abundance of FAs may have partially masked strain-related differences in FA composition, some variability was still noted. Of particular interest was (besides the peculiar FA profile of K1) the observation that some of the strains with enhanced growth and feeding capabilities had distinct lipid profiles, as reported by Adolf et al. (2007), also with *K. veneficum*. For instance, strains K22 and K24 both had low FA contents that did not include significant amounts of MUFAs. However, at least K24 (the only strain tested) incorporated these FAs after feeding on *R. salina*. In this regard, we mostly agree with Adolf et al. (2007) conclusions concerning the particular fatty acid profile of mixotrophic *K. veneficum*. However, we observed remarkable differences on the likely source of 22:6n3. In our analysis both K22 autotrophic and mixotrophic showed similar relatively high contents of this fatty acid. This seems to advocate for a synthesis by the polyketide synthase pathway, rather than by consumption of 18 C precursors, as suggested in Adolf et al. (2007) work. In any case, we do not know for how long a particular FA profile persists in the cell after feeding has ended. Nonetheless, based on the results presented here, under natural conditions *K. veneficum* can be expected to take advantage of the available prey, including the incorporation of FAs.

An intriguing and related question arises from the fact that among the four strains tested, only K22 and K24 were able to survive for several months under strict heterotrophic conditions. In a previous study, *Euglena gracilis* grown in the dark had reduced contents of MUFAs and PUFAs while its chloroplasts regressed to their plastidial condition (Barsanti et al., 2000), suggesting that these FAs are associated with chloroplast formation. Analyses of the lipid composition of our strains was conducted under the same light conditions (PUFA increases under non-limiting light conditions; Dalsgaard et al., 2003) as used in the above-mentioned study; therefore, any difference should be genetically driven and not the result of environmental conditions. It seems reasonable to conclude that the overall heterotrophic/mixotrophic capabilities of strains K22 and K24 were enhanced, reinforcing the theory of relevant genotypic and phenotypic variability within a bloom. We suspect that clones such as these are very important, first, at the initial phases of the bloom, to reduce the competition for nutrients by feeding on the competing algae, and second, as survival specialists under low light/low nutrient conditions. It may well be that these strains also have higher karlotoxin contents, which could be used to immobilize prey, thereby enhancing feeding rates (Sheng et al., 2010), or are able to provide resistance to grazing (Vaqué et al., 2006; Waggett et al., 2008). Unfortunately, we did not analyze the toxin contents of our strains.

The similarity analysis identified two strains (K3 and K4) that also differed from the rest, due to their high fatty acid contents but also to their poor performance under autotrophic and heterotrophic conditions. This finding is another example of the performance variability of cells comprising a bloom.

In summary, the substantial differences among the eleven strains examined herein allowed them to be functionally grouped in terms of growth and mixotrophic feeding capacities. However, it is not clear whether these groups were constituted by the same or by different clones. Nevertheless, based on the physiological data, it is highly likely that our strains of *K. veneficum* are derived from at least three or four different clones.

4.2. Effects of light on K. veneficum mixotrophy

According to Stickney et al. (2000), in an ideal mixotroph the rate of feeding is inversely related to irradiance, whereas in phagocytic alga, when dissolved inorganic nutrients are limiting, the feeding rate directly depends on the irradiance. However, when nutrients are provided in excess, low light levels (or darkness) should trigger an enhanced feeding response in either mixotrophy mode, as previously observed for dinoflagellates (Bird and Kalff, 1989; Jones et al., 1993; Skovgaard, 1996; Legrand et al., 1998). In our experiments, and similar to those of Skovgaard et al. (2000) with *Fragilidium subglobosum*, no significant differences in ingestion rates after 2 days of light deprivation were noted, although the growth rates of the dinoflagellates were certainly adversely affected. By contrast, Li et al. (1999) observed a negative effect of low light intensities on *K. veneficum* feeding activity. This contrasting response to light intensity may reflect differences in the strains used or they may merely be the result of different experimental set-ups. For example, the study of Li et al. (1999) included longer acclimation periods. At very low light intensities, this may have negatively affected the organisms, which, as noted by the authors, are unable to survive in prolonged darkness. The explanation is consistent with our own findings, as four out of six strains did not survive prolonged darkness. By contrast, strains K22h and K24h were able to survive, feed, and grow for months under complete darkness.

There is also a second aspect, not considered thus far, that distinguishes our experiments from those of Li et al. (1999): the use of a plankton wheel. By keeping the culture bottles in movement, we ensured a homogeneously distributed suspension of prey, precluding the formation of patches. In a standing set-up, such that light is more intense on one side of the bottle, both grazers and prey may aggregate, enhancing the encounter rates and artificially increasing ingestion rates. The effect should be more evident at higher light intensities, becoming irrelevant under darkness, although it is likely that cells aggregate according to their geotaxis or vertical migration patterns, if any; however, this does not necessarily produce the same outcome as in a feeding experiment.

4.3. Effects of nutrient limitation on feeding rates

Nutrient limitation is considered a trigger for mixotrophy (Raven, 1997; Jones, 1997, 2000; see also review by Burkholder et al., 2008). For instance, *Heterocapsa triquetra* ingestion of fluorescently labeled algae only occurred in nutrient-depleted medium, not when the dinoflagellate was cultured under high nutrient conditions (Legrand et al., 1998). Similar findings were obtained for other microalgae (Stoecker et al., 1997; Estep et al., 1986; Sanders, 1991; Arenovski et al., 1995). In *K. veneficum*, inorganic nutrient limitation was shown to stimulate feeding on *Storeatula major*, with P deficiency enhancing feeding more than N deficiency (Li et al., 2000a). We did not observe this response in our short nutrient limitation experiment, as even feeding by K24 was slightly adversely affected during nutrient deprivation. However, it should be noted that, by the end of the incubations, nutrients were not fully depleted, and the remaining nutrients could have been enough to sustain autotrophic growth, masking any effect. However, strain K22h, cultivated under complete darkness and in nutrient-poor FSW, significantly increased its feeding rates over those of the control grown in nutrient-rich medium. The ability of this strain (and K24h) to endure fully heterotrophic conditions, whereas other strains could not, and its particular lipid composition indicate extreme variability and thus calls into question most of the research on mixotrophy based on a single strain (see review by Stoecker, 1999).

4.4. Performance of K. veneficum in relation to other dinoflagellates

K. veneficum has been previously described as a mixotrophic species, exhibiting inter-strain variability with respect to ingestion rates (Li et al., 1999, 2001; Adolf et al., 2008). The data presented here corroborate this finding, extending this variability to isolates from the same bloom. Our estimated ingestion rates are in the medium-low range of those reported by Adolf et al. (2008): 0.22–1.34 (2.1 for K22h) *vs.* 0–4 cells *K. veneficum*$^{-1}$ d^{-1}, respectively. This indicates that the variability observed within our isolates from the same bloom is lower than that between strains from different locations. However, in terms of absolute numbers, our experiments are not easily comparable with those of Adolf et al. (2008), as we estimated ingestion rates as cell (*R. salina*) disappearance in 48-h incubations, whereas Adolf et al. (2008) measured orange (*S. major*) fluorescent inclusions after 6.5-h incubations. In addition, not only the prey but also the methods to estimate grazing differed between the two studies. Incubations of 6.5 h may mask any possible feeding rhythm and result in an overestimate of grazing if the digestion time is >6.5 h, which seems to be the case (Li et al., 2001). Other studies, more comparable to ours, showed *in situ* ingestion rates of this species in Chesapeake Bay of 0–0.26 cryptophytes dinoflagellate^{-1} d^{-1} (Li et al., 2001), and from 0 to ~7 cryptophytes dinoflagellate^{-1} d^{-1}, depending on the light conditions (Li et al., 1999).

The ingestion rates of our *K. veneficum* strains corresponded to daily rations of 8–50% of body carbon consumed per day. Compared to other mixotrophic dinoflagellates, reviewed by Yoo et al. (2010), these values are in the high range, trailing only those of *Paragymnodinium shiwhaense*, *Karlodinium armiger*, and *Fragilidinium mexicanum* (Fig. 10). However, heterotrophic dinoflagellates, as expected, have much higher specific ingestion rates because feeding is the only means of obtaining nourishment. This surplus of directly ingested carbon is cheaper to metabolically incorporate than carbon fixed from photosynthesis (e.g., the respiration rate of mixotrophic *F. subglobosum* is less than that of autotrophic *F. subglobosum*; Hansen et al., 2000), and is less preferably respired (Putt, 1990). In the case of *K. veneficum*, particulate carbon assimilation reportedly contributes 31–72% of gross carbon uptake during mixotrophic growth (Adolf et al., 2006). Our lower values likely reflected the fact that the cells were not fully adapted to growth under mixotrophic conditions (48-h experiments). However, in the case of K4, there was an unexplainable enhancement of growth that did not correspond to any significant rate of ingestion. This result does not seem to have been an experimental artifact, because it was obtained in two independent experiments (Figs. 4 and 5); thus, it may be related to the capacity of dinoflagellates to absorb not only particulate but also dissolved carbon released by prey (Glibert and Legrand, 2006).

It has also been suggested that mixotrophs require certain essential elements or growth factors from their prey, even if consumed at very low rates (Caron et al., 1993; Raven, 1997; Jones, 1997). If this is indeed the case for K4, then this dinoflagellate would belong to group C of the mixotrophs classification proposed by Jones (1997): organisms whose primary mode of nutrition is phototrophy, but which must supplement their diet by ingesting prey.

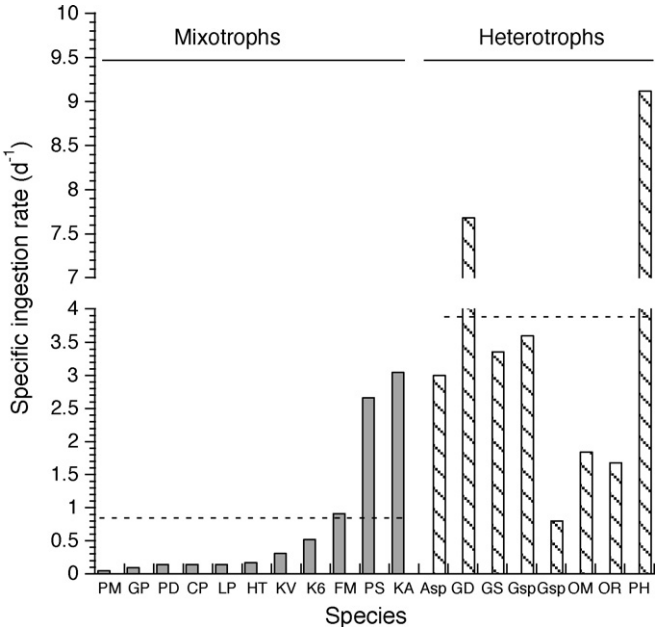

Fig. 10. Comparison of the maximum carbon-specific ingestion rates (d^{-1}) of different mixotrophic and heterotrophic dinoflagellates. The discontinuous line indicates the average for mixotrophs and heterotrophs. Data for mixotrophs other than K6 (this study) are from the review by Yoo et al. (2010), Table 5. PM: *Prorocentrum micans*, GP: *Gonyaulax polygramma*, PD: *P. donghaiense*, CP: *Cochlodinium polykrikoides*, LP: *Lingulodinium polyedrum*, HT: *Heterocapsa triquetra*, KV: *Karlodinium veneficum*, K6: Strain K6 of *K. veneficum*, FM: *Fragilidium mexicanum*, PS: *Paragymnodinium shiwhaense*, KA: *Karlodinium armiger*, Asp: *Amphidinium* sp. (Strom and Morello, 1998), GD: *Gyrodinium dominans* (Nakamura et al., 1995), GS: *Gyrodinium spirale* (Hansen, 1992), Gsp_1: *Gymnodinium* sp. (Bjørnsen and Kuparinen, 1991), Gsp_2: *Gymnodinium* sp. (Strom and Morello, 1998), OM: *Oxyrrhis marina* (Hansen et al., 1996), OR: *Oblea rotunda* (Strom and Buskey, 1993), PH: *Protoperidinium hirobis* (Jacobson and Anderson, 1986).

4.5. Ecological role of mixotrophy in K. veneficum

The benefits of mixotrophy for a bloom-forming organism have been extensively discussed in the literature. For instance, according to Glibert et al. (2009), mixotrophy in *K. brevis* may play a substantial role in sustaining natural populations in inorganic nutrient-poor waters. It has been also suggested that toxic dinoflagellates enhance their growth by using other phytoplankton as prey, a behavior that contributes to bloom initiation (Stoecker et al., 1997; Adolf et al., 2008). The benefits for the mixotrophs would be twofold: they would obtain extra nutrients through feeding while simultaneously reducing the number of competitors for inorganic nutrients. But is this extra energy really relevant for the metabolism of a bloom-forming mixotroph? Assuming the ingested food is converted into growth with an efficiency of 25% (Straile, 1997), the specific maximum ingestion rates shown in Table 6 render a growth rate of $0.17 \pm 0.075\ d^{-1}$, which may be a relevant amount during bloom initiation, when cells are leaving lag phase but have not yet entered exponential phase; however, it is not substantial by the time the algae are exponentially growing.

The literature contains contrasting results regarding the impact of a harmful species feeding on other algae. Li et al. (2001) estimated that the daily grazing impact of *K. veneficum* (*G. galatheanum*) on naturally occurring populations of cryptophytes in Chesapeake Bay was <4% of the standing stock. However, Jeong et al. (2005) derived daily removals of >100% of cryptophyte populations by *Prorocentrum donghaiense*, *H. triquetra*, and *Prorocentrum micans* populations in Masan Bay, Korea, and for *Gonyaulax polygramma* in the coastal waters off Saemankeum, Korea. These later results suggest that, during blooms, algal grazers have a considerable grazing impact on populations of co-occurring autotrophic prey species. On the whole, it is clear from the present study and others discussed herein that the role of mixotrophy in the formation of HABs cannot be easily explained; rather, more information is needed before the dynamics of these complex and important organisms are fully understood.

4.6. Final remarks

Our data and those from other studies leave little room for doubt that HABs are characterized by and may indeed rely on strain variability. It is particularly remarkable that the eleven strains retained their metabolic differences long after being cultivated under homogeneous conditions. It has been suggested that under non-limiting conditions fast growth would be selected during exponential phase (Lakeman et al., 2009), which over a sufficient amount of time could homogenize differences between strains. Similarly, in-culture selection for increased performance of one trait should reduce the presence of a negatively correlated trait (Lakeman et al., 2009). Therefore, for strains of the same species, the ability to grow in rich medium would be negatively correlated with the alternative, trophic pathway (mixotrophy). However, according to our data this is not the case; rather, the opposite may occur. Moreover, the strains compared in the present study were isolated at the same time, from the same fully developed bloom, which, according to the conceptual model of Adolf et al. (2008) could have reduced the variability among them. Similar strain variability was also reported regarding the toxin profiles of 18 strains of *K. veneficum* isolated from different locations (Bachvaroff et al., 2009), and for the specific growth rates of *Alexandrium tamarense* strains simultaneously isolated from the east coast of Scotland (Tillmann et al., 2009). Thus, it is likely that strain variability is a more resilient trait than previously thought. But what about the ability of those cells with lower performance to compete and survive? That question might be answered by our observation of very few poorly performing strains; although it may be that many of such strains were unable to survive the selection process. Nevertheless, it can be assumed that most cells in a bloom are fast growing, as it guarantees that the community prospers. However, genetic variability is crucial for any species/population to evolve and survive; thus, strains/cells not well adapted to fast growth may be better at surviving under very different conditions, due, for example, to a higher resistance to grazing or to a higher capacity to form cysts. These hypotheses need to be tested in future works.

Acknowledgements

This work was funded through projects CTM2004-02575/MAR and CTM2009-08783 from the Ministry of Science and Innovation to A.C. E.G. was supported by a Ramon y Cajal contract of the Spanish Ministry of Science and Innovation (MICINN), and C.F-G. by Comisión Nacional de Investigación Ciencia y Tecnología (CONICYT) Chile. We thank IRTA Institute from the *Generalitat de Catalunya* for the continuous monitoring of HABs and for providing the natural samples. We also thank K. Andree for submitting the ITS sequences. Sequencing work was funded through the project INIA and project RTA2005-00109-00-0. [SS]

References

Adolf, J.E., Bachvaroff, T., Place, A.R., 2008. Can cryptophyte abundance trigger *toxic Karlodinium veneficum* blooms in eutrophic estuaries? Harmful Algae 8 (1), 119–128.

Adolf, J.E., Place, A.R., Stoecker, D.K., Harding, L.W., 2007. Modulation of polyunsaturated fatty acids in mixotrophic *Karlodinium veneficum* (Dinophyceae) and its prey, *Storeatula major* (Cryptophyceae). Journal of Phycology 43, 1259–1270.

Adolf, J.E., Stoecker, D.K., Harding, L.W., 2006. The balance of autotrophy and heterotrophy during mixotrophic growth of *Karlodinium micrum* (Dinophyceae). Journal of Plankton Research 28 (8), 737–751.

Arenovski, A.L., Lim, E.L., Caron, D.A., 1995. Mixotrophic nanoplankton in oligotrophic surface waters of the Sargasso Sea may employ phagotrophy to obtain major nutrients. Journal of Plankton Research 17, 801–820.

Ajani, P., Hallegraeff, G., Pritchard, T., 2001. Historic overview of algal blooms in marine and estuarine waters of New South Wales, Australia. In: Proceedings of the Linnean Society of New South Wales 123, Australia, pp. 1–22.

Bachvaroff, T.R., Adolf, J.E., Place, A.R., 2009. Strain variation in *Karlodinium veneficum* (Dinophyceae): toxin profiles, pigments, and growth characteristics. Journal of Phycology 45 (1), 137–153.

Barsanti, L., Bastianini, A., Passarelli, V., Tredici, M.R., Gualtieri, P., 2000. Fatty acid content in wild type and WZSL mutant of *Euglena gracilis*. Effects of carbon source and growth conditions. Journal of Applied Phycology 12 (3), 515–520.

Bird, D.F., Kalff, J., 1989. Phagotrophic sustenance of a metalimnetic phytoplankton peak. Limnology and Oceanography 34, 155–162.

Bjornland, T., Tangen, K., 1979. Pigmentation and morphology of a marine *Gyrodinium* (Dinophyceae) with a major carotenoid different from peridinin and fucoxanthin. Journal of Phycology 15, 457–463.

Bjørnsen, P.K., Kuparinen, J., 1991. Growth and herbivory by heterotrophic dinoflagellates in the Southern Ocean, studied by microcosm experiments. Marine Biology 109, 397–405.

Braarud, T., 1957. A red water organism from Walvis Bay. Galathea Reports 1, 137–138.

Broglio, E., Jónasdóttir, S.H., Calbet, A., Jakobsen, H.H., Saiz, E., 2003. Effect of heterotrophic versus autotrophic food on feeding and reproduction of the calanoid copepod *Acartia tonsa*: relationship with prey fatty acid composition. Aquatic Microbial Ecology 31 (3), 267–278.

Burkholder, J., Glibert, P., Skelton, H., 2008. Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters. Harmful Algae 8 (1), 77–93.

Caron, D.A., Sandres, R.W., Lim, E.L., Marrasé, C., Amaral, L.A., Whitney, S., Aoki, R.B., Porter, K.G., 1993. Light-dependent phagotrophy in the freshwater mixotrophic Chrysophyte *Dinobryon cylindricum*. Microbial Ecology 25, 93–111.

Carrillo, E., Ferrero, L.M., Alonso Andicoberry, C., Basanta, A., Martin, A., López Rodas, V., Costas, E., 2003. Interstrain variability in toxin production in populations of the cyanobacterium *Microcystis aeruginosa* from water-supply reservoirs of Andalusia and lagoons of Donana National Park (Southern Spain). Phycologia 42 (3), 269–274.

Cosgrove, J., Grigo, S., Hosja, W., Hallegraeff, G., 2000. The investigation of a dinoflagellate associated with a fish kill event in the Murray River/estuary, Western Australia. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J., Lewis, R.J. (Eds.), Abstract in 9th International Conference on Harmful Algal Bloom. UNESCO, Hobart, Tasmania (Australia), p. 518.

Dalsgaard, J., St John, M., Kattner, G., Müller-Navarra, D., Hagen, W., 2003. Fatty acid trophic markers in the pelagic marine environment. Advances in Marine Biology 46, 227–318.

Estep, K.W., Davis, P.G., Keller, M.D., Sieburth, J.M., 1986. How important are oceanic algal flagellates in bacterivory? Limnology and Oceanography 31, 646–650.

Fernández-Tejedor, M., Giménez, G., de la Iglesia, P., Diogène, J., 2009. Resultados del programa de seguimiento de fitoplancton tóxico y biotoxinas en las zonas de producción de bivalvos de Cataluña: años 2007–2008. In: Costa, P.R., Botelho, M.J., Rodrigues, S.M., Palma, A.S., Moita, M.T. (Eds.), Proceedings de la X Reunión Ibérica sobre Fitoplancton Tóxico y Biotoxinas 2009. IPIMAR, Lisboa, Portugal, pp. 15–29.

Frost, B.W., 1972. Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod *Calanus pacificus*. Limnology and Oceanography 17, 805–815.

Fuentes-Grünewald, C., Garcés, E., Rossi, S., Camp, J., 2009. Use of the dinoflagellate *Karlodinium veneficum* as a sustainable source of biodiesel production. Journal of Industrial Microbiology and Biotechnology 36 (9), 1215–1224.

Glibert, P.M., Burkholder, J.M., Kana, T.M., Alexander, J., Skelton, H., Shilling, C., 2009. Grazing by *Karenia brevis* on *Synechococcus* enhances its growth rate and may help to sustain blooms. Aquatic Microbial Ecology 55, 17–30.

Glibert, P.M., Legrand, C., 2006. The diverse nutrient strategies of harmful algae: focus on osmotrophy. In: Granéli, E., Turner, J.T. (Eds.), Ecology of Harmful Algae. Springer-Verlag, Berlin Heidelberg (Ecological Studies, vol. 189).

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York, pp. 29–60.

Hallegraeff, G.M., Nichols, P.D., Volkman, J.K., Blackburn, S.I., Everit, D., 1999. Pigments, fatty acids, and sterols of the toxic dinoflagellate *Gymnodinium catenatum*. Journal of Phycology 27, 591–599.

Hansen, P.J., 1992. Particle size selection, feeding rates and growth dynamics of marine heterotrophic dinoflagellates, with special emphasis on *Gyrodinium spirale*. Marine Biology 114, 327–334.

Hansen, F.C., Witte, H.J., Passarge, J., 1996. Grazing in the heterotrophic dinoflagellate *Oxyrrhis marina*: size selectivity and preference for calcified Emiliana huxleyi cells. Aquatic Microbial Ecology 10, 307–313.

Hansen, G., Daugbjerg, N., Henriksen, P., 2000. Comparative study of *Gymnodinium mikimotoi* and *Gymnodinium aureolum*, comb. nov. (=*Gyrodinium aureolum*) based on morphology, pigment composition, and molecular data. Journal of Phycology 36 (2), 394–410.

Ichimi, K., Suzuki, T., Ito, A., 2002. Variety of PSP toxin profiles in various culture strains of *Alexandrium tamarense* and change of toxin profile in natural *A. tamarense* population. Journal of Experimental Marine Biology and Ecology 273 (1), 51–60.

Jacobson, D.M., Anderson, D.M., 1986. Thecate heterotrophic dinoflagellates feeding behavior and mechanisms. Journal of Phycology 22, 249–258.

Jeong, H.J., Yoo, Y.D., Park, J.Y., Song, J.Y., Kim, S.T., Lee, S.H., Kim, K.Y., Yih, W.H., 2005. Feeding by phototrophic red-tide dinoflagellates: five species newly revealed and six species previously known to be mixotrophic. Aquatic Microbial Ecology 40, 133–150.

Jónasdóttir, S.H., 1994. Effects of food quality on the reproductive success of *Acartia tonsa* and *Acartia hudsonica*: laboratory observations. Marine Biology 121, 67–81.

Jones, H.L.J., 1997. A classification of mixotrophic protists based on their behaviour. Freshwater Biology 37, 35–43.

Jones, H.L.J., Leadbeater, B.S.C., Green, J.C., 1993. Mixotrophy in marine species of *Chrysochromulina* (Prymnesiophyceae): ingestion and digestion of a small green flagellate. Journal of Marine Biology Association UK 73, 283–296.

Jones, R.I., 2000. Mixotrophy in planktonic protists: an overview. Freshwater Biology 45 (2), 219–226.

Klein Breteler, W.C.M., Schogt, N., Baas, M., Schouten, S., Kraay, G.W., 1999. Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids. Marine Biology 135 (1), 191–198.

Lakeman, M.B., von Dassow, P., Cattolico, R.A., 2009. The strain concept in phytoplankton ecology. Harmful Algae 8 (5), 746–758.

Lee, R.F., Nevenzel, J.C., Paffenhöfer, G.A., 1971. Importance of wax ester and other lipids in the marine food chain: phytoplankton and copepods. Marine Biology 9, 99–108.

Legrand, C., Granéli, E., Carlsson, P., 1998. Induced phagotrophy in the photosynthetic dinoflagellate *Heterocapsa triquetra*. Aquatic Microbial Ecology 15, 65–75.

Li, A.S., Stoecker, D.K., Adolf, J.E., 1999. Feeding, pigmentation, photosynthesis and growth of the mixotrophic dinoflagellate *Gyrodinium galatheanum*. Aquatic Microbial Ecology 19 (2), 163–176.

Li, A.S., Stoecker, D.K., Coats, D.W., 2000a. Mixotrophy in *Gyrodinium galatheanum* (Dinophyceae): Grazing responses to light intensity and inorganic nutrients. Journal of Phycology 36 (1), 33–45.

Li, A.S., Stoecker, D.K., Coats, D.W., 2000b. Spatial and temporal aspects of *Gyrodinium galatheanum* in Chesapeake Bay: distribution and mixotrophy. Journal of Plankton Research 22 (11), 2105–2124.

Li, A.S., Stoecker, D.K., Coats, D.W., 2001. Use of the 'food vacuole content' method to estimate grazing by the mixotrophic dinoflagellate *Gyrodinium galatheanum* on cryptophytes. Journal of Plankton Research 23 (3), 303–318.

Loret, P., Tengs, T., Villareal, T.A., Singler, H., Richardson, B., McGuire, P., Morton, S., Busman, M., Campbell, L., 2002. No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. Journal of Plankton Research 24 (7), 735–739.

Mansour, M.P., Volkman, J.K., Blackburn, S.I., 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 (2), 145–153.

Nakamura, Y., Suzuki, S.Y., Hiromi, J., 1995. Growth and grazing of a naked heterotrophic dinoflagellate. Gyrodinium dominans. Aquatic Microbial Ecology 9 (2), 157–164.

Nascimento, S.M., Purdie, D.A., Morris, S., 2005. Morphology, toxin composition and pigment content of Prorocentrum lima strains isolated from a coastal lagoon in southern UK. Toxicon 45 (5), 633–649.

Nejstgaard, J., Tang, K., Steinke, M., Dutz, J., Koski, M., Antajan, E., Long, J., 2007. Zooplankton grazing on *Phaeocystis*: a quantitative review and future challenges. Biogeochemistry 83, 147–172.

Paz, B., Riobo, P., Ramilo, I., Franco, J.M., 2007. Yessotoxins profile in strains of *Protoceratium reticulatum* from Spain and USA. Toxicon 50 (1), 1–17.

Putt, M., 1990. Metabolism of photosynthate in the chloroplastretaining ciliate *Laboea strobila*. Marine Ecology Progress Series 60, 271–282.

Raven, J.A., 1997. Phagotrophy in phototrophs. Limnology and Oceanography 42 (1), 198–205.

Reuss, N., Poulsen, L.K., 2002. Evaluation of fatty acids as biomarkers for a natural plankton community. A field study of a spring bloom and a post-bloom period off West Greenland. Marine Biology 141, 423–434.

Rynearson, T.A., Armbrust, E.V., 2000. DNA fingerprinting reveals extensive genetic diversity in a field population of the centric diatom *Ditylum brightwellii*. Limnology and Oceanography 45, 1329–1340.

Ruiz, J., Antequera, T., Andres, A.I., Petron, M.J., Muriel, E., 2004. Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. Analytica Chimica Acta 520 (1–2), 201–205.

Sanders, W.R., 1991. Mixotrophic protists in marine and freshwater ecosystems. Journal of Protozoology 38, 76–81.

Sheng, J., Malkiel, E., Katz, J., Adolf, J.E., Place, A.R., 2010. A dinoflagellate exploits toxins to immobilize prey prior to ingestion. Proceedings of the National Academy of Sciences 107 (5), 2082–2087.

Skovgaard, A., 1996. Mixotrophy in *Fragilidium subglobosum* (Dinophyceae): growth and grazing responses as function of light intensity. Marine Ecology Progress Series 143, 247–253.

Skovgaard, A., Hansen, P.J., Stoecker, D.K., 2000. Physiology of the mixotrophic dinoflagellate *Fragilidium subglobosum*. I. Effects of phagotrophy and irradiance on photosynthesis and carbon content. Marine Ecology Progress Series 201, 129–136.

Stickney, H.L., Hood, R.R., Stoecker, D.K., 2000. The impact of mixotrophy on planktonic marine ecosystems. Ecological Modelling 125, 203–230.

Stoecker, D.K., Li, A., Coats, D.W., Gustafson, D.E., Nannen, M.K., 1997. Mixotrophy in the dinoflagellate *Prorocentrum minimum*. Marine Ecology Progress Series 152, 1–12.

Straile, D., 1997. Gross growth efficiencies of protozoan and metazoan zooplankton and their dependence on food concentration, predator-prey weight ratio, and taxonomic group. Limnology Oceanography 42 (6), 1375–1385.

Strom, S.L., Buskey, E.J., 1993. Feeding, growth, and behavior of the thecate heterotrophic dinoflagellate *Oblea rotunda*. Limnology and Oceanography 38, 965–977.

Strom, S.L., Morello, T.A., 1998. Comparative growth rates and yields of ciliates and heterotrophic dinoflagellates. Journal of Plankton Research 20 (3), 571–584.

Terlizzi, D.E., 2000. *Gyrodinium galatheanum*: a threat to estuarine aquaculture. responsible aquaculture in the new millenium. In: Flos, R., Creswell, L. (Eds.), Abstracts of contributions presented at the International Conference AQUA 2000. Nice, France, May 2–6, 2000. European Aquaculture Society Special Publication No 28 Oostende, Belgium.

Tillmann, U., Alpermann, T.L., da Purificacao, R.C., Krock, B., Cembella, A., 2009. Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. Harmful Algae 8 (5), 759–769.

Vaqué, D., Felipe, J., Sala, M.M., Calbet, A., Estrada, M., Alcaraz, M., 2006. Effects of the toxic dinoflagellate *Karlodinium* sp. (cultured at different N/P ratios) on micro and mesozooplankton. Scientia Marina 70 (1), 59–65.

Waggett, R.J., Tester, P.A., Place, A.R., 2008. Anti-grazing properties of the toxic dinoflagellate *Karlodinium veneficum* during predator–prey interactions with the copepod *Acartia tonsa*. Marine Ecology Progress Series 366, 31–42.

Wang, D.Z., Zhang, S.G., Gu, H.F., Chan, L.L., Hong, H.S., 2006. Paralytic shellfish toxin profiles, and toxin variability of the genus *Alexandrium* (Dinophyceae) isolated from the Southeast China Sea. Toxicon 48 (2), 138–151.

Yoo, Y.D., Jeong, H.J., Kang, N.S., Song, J.Y., Kim, K.Y., Lee, G., Kim, J., 2010. Feeding by the newly described mixotrophic dinoflagellate *Paragymnodinium shiwhaense*: feeding mechanism, prey species, and effect of prey concentration. Journal of Eukaryotic Microbiology 57 (2), 145–158.