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Optimum growth conditions for *Amphidinium carterae* Hulburt from eutrophic waters in Alexandria (Egypt) and its toxicity to the brine shrimp *Artemia salina*

AMANY ABDEL-HAMID ISMAEL, YOUSSEF HALIM and ABDEL-GHANY KHALIL

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Cultures of a strain of *Amphidinium carterae* Hulburt isolated from a eutrophic basin in Alexandria were investigated for the optimum growth conditions, including vitamin requirements, the assimilation index and the potential toxicity of this species. A division rate of 2.7 day^{-1} , was reached on the second and third day under optimum conditions, with and without addition of B_{12} and biotin. The rate of C_{14} assimilation per unit chlorophyll ranged from 0.2 to $5.4 \text{ mgC} \cdot (\text{mg Chla})^{-1} \cdot \text{h}^{-1}$ according to the growth conditions and to cell size. As the cell density increased in the cultures, the cell size decreased and their photosynthetic efficiency increased. Toxicity of *A. carterae* to brine shrimp resulted from grazing and not from membrane diffusion of extracellular toxins.

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Amphidinium carterae, a widely distributed species, reported from both temperate (Steidinger & Tangen 1995) and tropical waters (Larsen & Patterson 1990), is new for Egyptian Mediterranean waters.

The optimum growth conditions for *Amphidinium* spp. have been investigated by several authors, since the work of Braarud on salinity tolerance (Braarud 1951, 1961, McLachlan 1961). Investigations focused on the optimum temperature (Jitts et al. 1964, Morton et al. 1992), optimum irradiance (Sweeney & Hastings 1962, Jitts et al. 1964, Chan 1978, Humphrey 1983, Morton et al. 1992), nutrient requirements (Provasoli and McLaughlin 1963, Provasoli & Gold 1962), growth rate (Braarud 1951, Hellebust 1965, Parsons et al. 1961, Smayda 1997) and vitamin requirements (Droop et al. 1959, McLaughlin & Provasoli 1957, Provasoli & McLaughlin 1955, 1963; Carlucci & Silbernagel 1966, 1967), in addition to their physiology and biochemistry (Ryther 1956, Deane & O'Brien 1981, Hersey & Swift 1976). Sournia (1982) reviewed and discussed the relationship between various physiological parameters and cell size. McAllister et al. (1964) determined its carbon content; Williams (1965) the fatty acid composition; Parsons et al. (1961) the amino acid composition, and Parsons (1961), Parsons and Strickland (1963), Bunt (1964) determined the pigment pattern of *Amphidinium carterae*.

From 60 to 80 species of phytoplankton have been reported to be harmful in some way or other. Of these, 90% are flagellates, notably dinoflagellates (Smayda 1997). *A. carterae* was reported to be toxic as early as 1957 (McLaughlin & Provasoli). Ikawa and Taylor (1973), Ikawa and Sasner (1975) and Davin et al. (1988) stated that it produces toxic substances.

The present work was designed to investigate: a) The

optimum growth conditions of light, nutrient, temperature and the vitamin requirements of *A. carterae*; b) The assimilation index; c) Its potential toxicity to the brine shrimp, *Artemia salina*.

MATERIAL AND METHODS

A. carterae Hulburt cells were isolated from a eutrophic basin in Alexandria, the Eastern Harbour. The species was grown in enriched GPM medium (Loeblich 1975) at three irradiances; 1720 lux, 2900 lux and 3420 lux (cool-white fluorescent light) at an L:D cycle of 12:12 h and at four temperatures 20°, 23°, 25°, and 30°C. The cell counts were monitored daily using an inverted microscope. All experiments were triplicate. Total chlorophyll was determined spectrophotometrically (Parsons & Strickland 1963) and C_{14} assimilation determined according to Steemann Nielsen (1952) and Strickland & Parsons (1968) using liquid scintillation counter. Both total chlorophyll and carbon assimilation were measured during the logarithmic growth phase.

A two-way Anova with replications was applied to test the significance of the differences between measurements.

RESULTS AND DISCUSSION

Basic variables, experimentally tested before designing the growth experiments, were:

1. The relative volume of culture solution to the total volume of the flasks used. The optimum ratio was found to be 1: 3.
2. The most suitable initial cell density for the growth of *A. carterae* was determined to be 60-to100 cells ml^{-1} (Fig. 1).

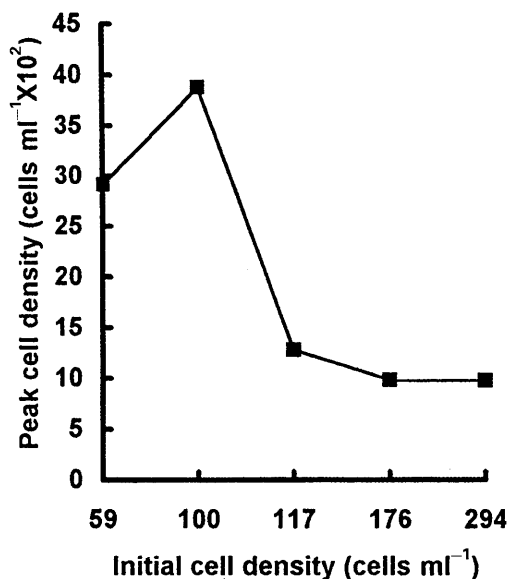


Fig. 1. Peak standing crop of *Amphidinium carterae* at the respective initial cell densities.

Optimum growth conditions

At each temperature and each of the three irradiation levels, four phosphate and nitrate enrichments were tested, respectively the GPM concentration (medium B) according to Loeblich (1975), 1.5 (medium C), 2 (medium D) and 2.5 (medium E) the GPM concentration. The N/P ratio was maintained constant in all cases at 6.1:1, as in the GPM medium. Further two additional concentrations were tested: 0.5 GPM and 3 GPM, but both showed low growth and were therefore disregarded (Figs. 2–3). Salinity was adjusted to 38‰.

It was found that the species tolerated a wide range of temperature, from 20 to 30°C, with the optimum temperature at 25°C. This is in agreement with Jitts et al. (1964), who found an optimum temperature of 24°C for *Amphidinium carterae*, with growth between 18°C and 33°C. Morton et al. (1992) found that the maximum growth for *Amphidinium klebsii* occurred at 27°C. The optimum nutrient requirement proved to be 2.5 times the nitrate and phosphate concentration of the GPM medium (medium E). The high nutrient requirement of this strain is likely to be due to the fact that it came from a eutrophic basin, the Eastern Harbour of Alexandria.

A light intensity from 2900 to 3420 lux was found to be optimal for *A. carterae*. It is difficult to compare studies in the literature concerning light because of the diverse systems of light units employed, the physical concepts involved and the instruments used (Guillard & Keller 1984). The optimum light intensity of a clone appears to reflect its habitat more than the phylogenetic class to which it belongs (Brand & Guillard 1981).

Vitamin requirements

Having established the optimum temperature, nutrient and light conditions, another set of experiments was carried out

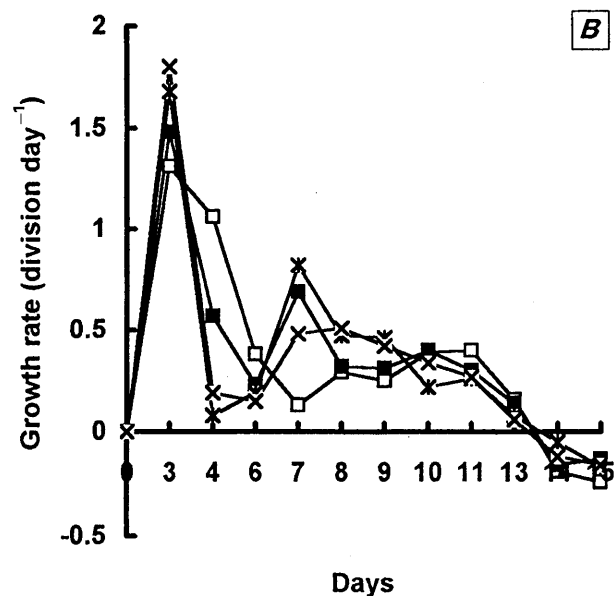
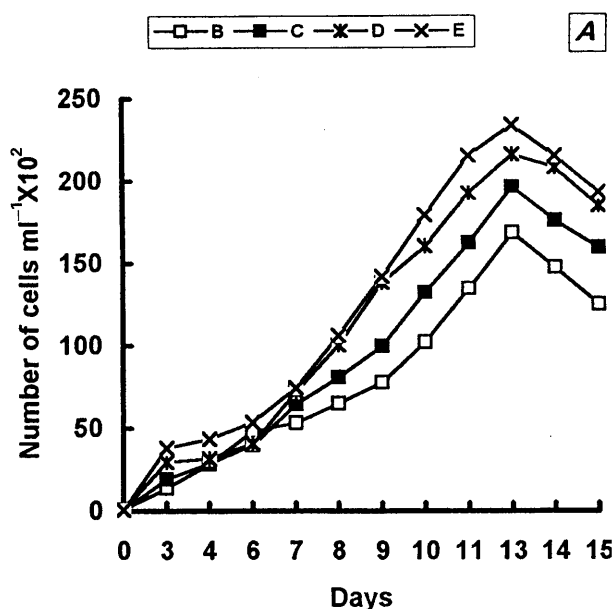


Fig. 2. Effect of different nutrient enrichments, GPM (B), 1.5 GPM (C), 2 GPM (D) and 2.5 GPM (E) on the growth of *Amphidinium carterae* at 25°C and 1900 lux.

to investigate the vitamin requirements of *A. carterae*. Three vitamins were tested at different concentrations, cyanocobalamin, thiamin HCl and biotin, singly and in combination. The cultures were grown in boiled aged seawater. The concentrations of vitamins used by different authors vary widely according to the respective requirements of the different species.

For cyanocobalamin, both Guillard & Ryther (1962) and Von Stosch (1964) used 1 µg l⁻¹. Throndsen (1978) used 1 µg l⁻¹, Guillard & Hargraves (1993) 0.5 µg l⁻¹.

For biotin, Aldrich & Wilson (1960) and Guillard &

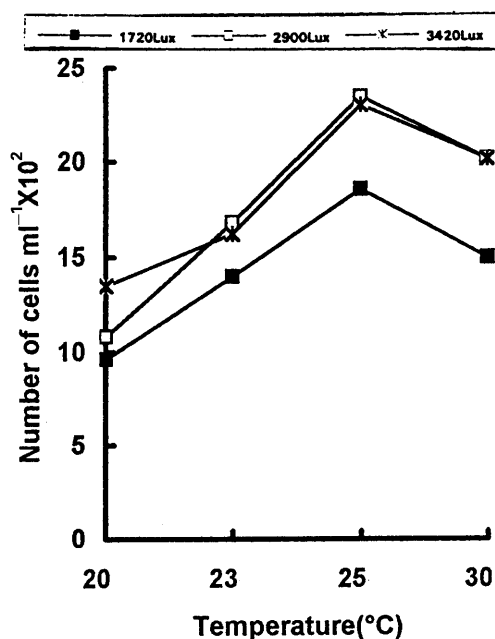


Fig. 3. Peak standing crop of *Amphidinium carterae* in relation to temperature at three light intensities in medium E.

Hargraves (1993) used a concentration of $0.5 \mu\text{g l}^{-1}$, while Loeblich (1975) used $2 \mu\text{g l}^{-1}$ and Guillard & Ryther (1962) $1 \mu\text{g l}^{-1}$.

For thiamin HCl, Gold (1973) used $1 \mu\text{g l}^{-1}$, while Aldrich and Wilson (1960) and Wilson and Collier (1955) used 1 mg l^{-1} .

Given this wide discrepancy in the concentrations used, it was deemed necessary to test a range of concentrations from $1 \mu\text{g l}^{-1}$ to 1 mg l^{-1} for cyanocobalamin, two concentrations $1 \mu\text{g l}^{-1}$ and 1 mg l^{-1} , for thiamin HCl and $0.5 \mu\text{g l}^{-1}$, 1 mg l^{-1} , and $2 \mu\text{g l}^{-1}$ for biotin.

Preliminary experiments showed that a concentration of $1 \mu\text{g l}^{-1}$ cyanocobalamin is preferable to 1 mg l^{-1} . Five intermediate concentrations of B_{12} therefore were tested: $50 \mu\text{g l}^{-1}$, $250 \mu\text{g l}^{-1}$, $500 \mu\text{g l}^{-1}$, $750 \mu\text{g l}^{-1}$ and 1 mg l^{-1} , in addition to the control (Fig. 4A). The optimum standing crop was obtained at the concentration of $500 \mu\text{g l}^{-1}$ cyanocobalamin. It is to be noted that no cobalt salts were added to the culture medium, which might explain the relatively high B_{12} requirement of this strain. No significant difference was observed between the growth of cultures with the addition of either $1 \mu\text{g l}^{-1}$ or 1 mg l^{-1} thiamin HCl and the control. Thiamin HCl, therefore, does not seem to be required by *A. carterae*. On the other hand, biotin strongly enhanced the growth of *A. carterae* at a concentration of $1 \mu\text{g l}^{-1}$ (Fig. 4B) in preference to $0.5 \mu\text{g l}^{-1}$ and $2 \mu\text{g l}^{-1}$. The synergistic effects of cyanocobalamin with biotin and then with thiamin HCl were tested. The vitamins were added at the optimum concentrations obtained earlier. The former two vitamins when combined significantly enhanced the standing crop above that obtained with the single addition of each of the two vitamins (Fig. 5A). The addition of thiamin HCl to the other two vitamins gave no significant

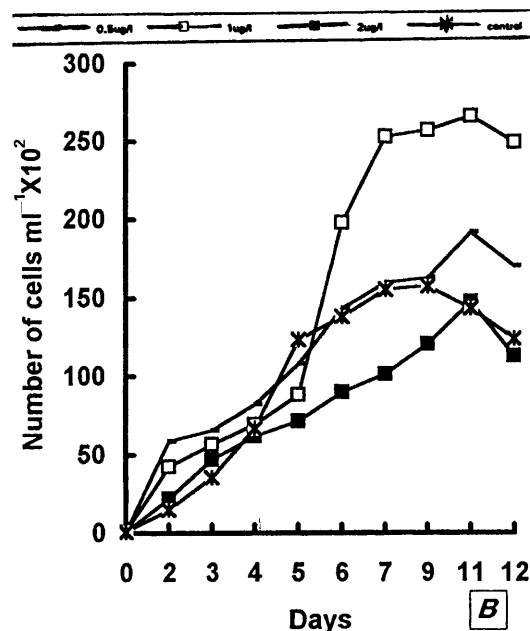
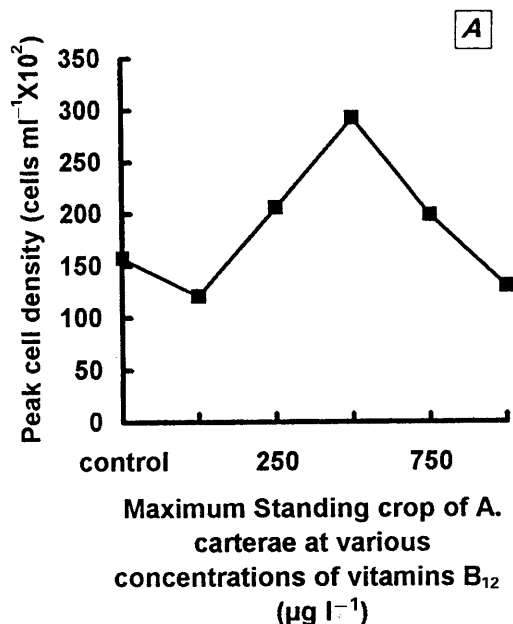


Fig. 4. Effect of different A) cyanocobalamin concentrations B) biotin concentrations on the growth of *Amphidinium carterae*.

difference from the latter cultures. Again, thiamin HCl does not seem to be required by this species (Fig. 5B).

Loeblich (1966) mentioned that the requirement for thiamin is not widespread among dinoflagellates. Ukeles (1976) mentioned that biotin is definitely required by a few dinoflagellates and chrysomonads. Biotin requirement has been demonstrated for *A. carterae* and *A. rhynchocephalum* (McLaughlin & Provasoli 1957). Carlucci and Silbernagel (1966, 1967) used *A. carterae* for assessing the biotin content of natural waters. The requirement for vitamin B_{12} appears to be more widespread than that for either biotin or thiamin. The survey of vitamin requirements made by Thomas (1968)

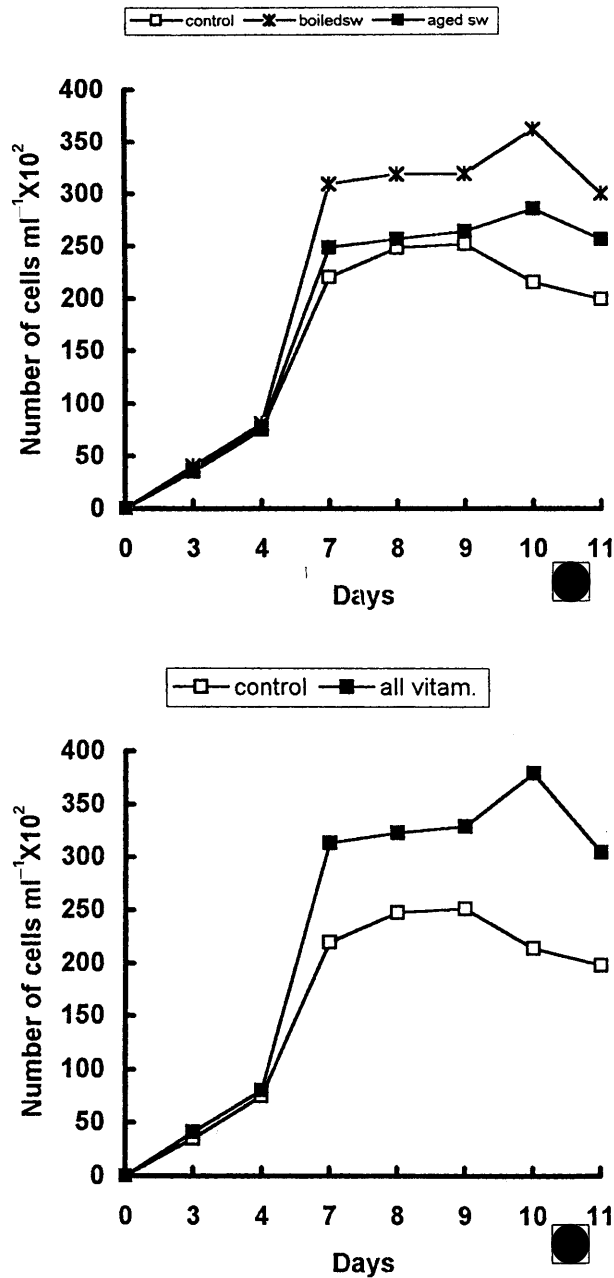


Fig. 5. Effect of A) cyanocobalamin (500 µg l⁻¹) in combination with biotin (1 µg l⁻¹), and B) Mixture of cyanocobalamin, biotin and thiamin—on the growth of *Amphidinium carterae*.

shows that vitamin B₁₂ is required by all dinoflagellates with the exception of *Gymnodinium simplex*.

The optimum biotin concentration, as found in the present work, 1 µg l⁻¹, is compatible with the concentration given by Guillard & Ryther (1962), Antia & Cheng (1970), Quraishi & Spencer (1971), but twice that given by Provasoli & McLaughlin (1963) for the growth of *A. carterae* (0.5 µg l⁻¹).

Growth rate

The growth rate of *A. carterae* was relatively high. The division rate is a function of light intensity, temperature and nutrients and increased with increasing nitrate and phosphate concentrations. Although the maximum standing crop was obtained with addition of combined vitamins, the maximum growth rate, 2.7 divisions day⁻¹, was obtained under the optimum temperature, nutrient and light conditions with and without vitamins. The combined addition of the three vitamins raised the standing crop to a maximum, but did not increase the division rate. (Table I). This result is compatible with the growth rates observed by Parsons et al. (1961) and Loeblich (1966), who found a growth rate of 2.67 d⁻¹ for *A. carterae* and Smayda (1997), 2.84 d⁻¹. The maximum division rate during the present work occurred during the first three days of the growth curve.

Chlorophyll content and the assimilation index

Although, as expected, the chlorophyll biomass of the culture increased in parallel with the numerical standing crop under optimum conditions, the chlorophyll content per cell decreased with the increase in cell density (Fig. 6). As the cell density increased in the culture, the biovolume of the cells decreased, and in consequence their content in chlorophyll decreased so that the number of cells per mg chlorophyll increased (Fig. 7B). The chlorophyll content per cell ranged from 10 × 10⁻³ to 87 × 10⁻³ µg chlorophyll per cell.

Nordli (1957), working on *Ceratium* species, found that the cell size in culture is always smaller than in natural populations and that there is an indirect relation between culture density and cell size. Nordli's conclusion is in complete agreement with the present results, as the size of *A. carterae* decreased significantly with increasing culture density.

The cell dimensions of *A. carterae* in culture decreased

Table I. Maximum standing crop per ml and maximum growth rate (division day⁻¹).

	Optimum conditions (without vitamins)	Vitamins			
		Single		Combined	3 vit.
		vit. B ₁₂	Biotin	B ₁₂ + Biotin	
Max. St. crop × 10 ³ ml ⁻¹	23.4	29.2	26.6	36.16	37.9
Day	13	10	7 to 11	10	10
Max. division rate per day	2.69	2.13	2.7	2.7	1.7
Day	2	3	2	3	3

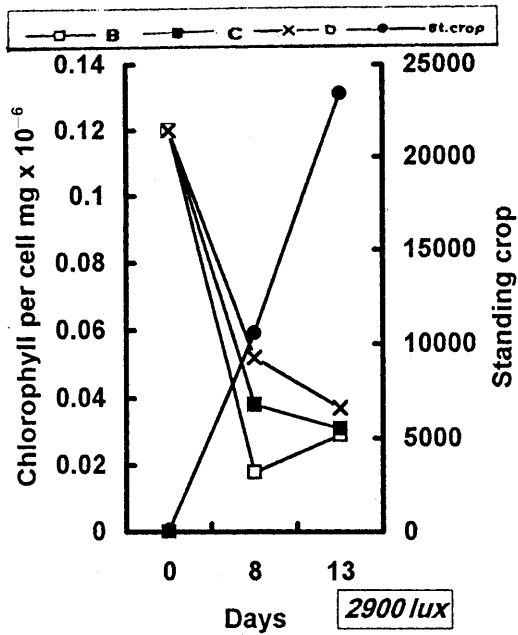


Fig. 6. Chlorophyll content of *Amphidinium carterae* per cell ($\text{mg} \times 10^{-6}$) at 25°C.

from 18 μm to 7 μm for the length and from 10 μm to 4 μm for the greatest diameter. The cell volume correspondingly varied in a wide range: from 1084 μm^3 at the beginning of the experiment to 398–478 μm^3 after 5 to 7 days and finally dropped to 81 μm^3 . Parsons (1961) found the biovolume of *A. carterae* to be about 740 μm^3 .

The assimilation index, that is the rate of carbon assimilation per chlorophyll content per hour, ranged from 0.26 to 5.4 according to the growth conditions. It was maximal under optimal growth conditions without vitamin addition, namely in medium E, at 25°C and 2900-lux light intensity. It was minimal in cultures grown in the GPM medium without enrichment, at a temperature of 20°C and light intensity of 1700-lux. There is a linear relation between this index and the number of cells per μg chlorophyll. With the decrease in cell size, as indicated by the increased number of cells per unit chlorophyll, the assimilation index increases. In other words, the photosynthetic efficiency increases as the cell size decreases (Fig. 7A), as also concluded by several authors for both natural and cultured populations (Paasche 1960, Throndsen 1976, Blasco et al. 1982, Parsons et al. 1984 and Dorsey et al. 1989).

Potential toxicity

Until present, HAB events in Egyptian waters were only caused by the dinoflagellate *Alexandrium minutum* Halim (Labib & Halim 1995), although many of the well known, potentially toxic dinoflagellate species occur in small numbers in the population. *Amphidinium carterae* is one of them, but no harmful blooms were produced by this species. McLaughlin and Provasoli (1957) found the toxin excreted by *A. carterae* and *A. rhynchocephalum* in the supernatant of the cultures to be toxic to two fish, *Lebistes*

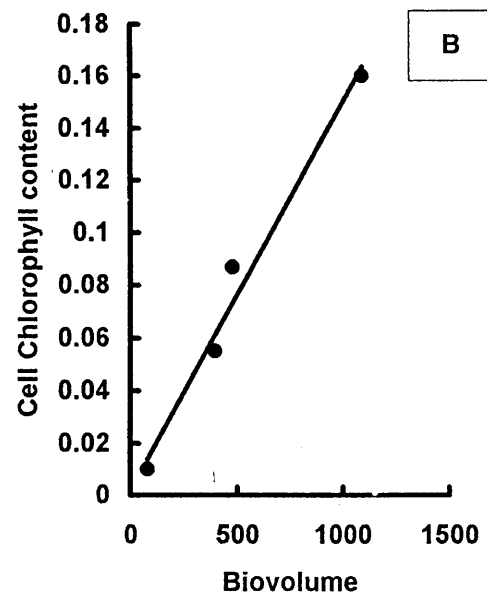
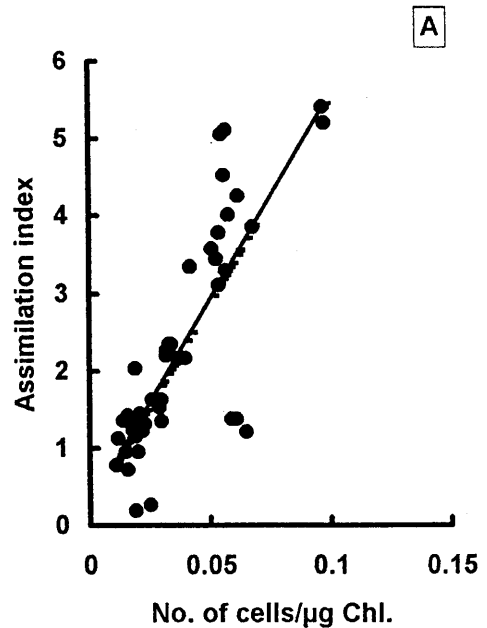


Fig. 7. Regression between: A) Number of *A. carterae* cells per μg chlorophyll and the assimilation index [$\text{mg C.}(\text{mg chl a})^{-1}.\text{h}^{-1}$], and B) Cell biovolume and cell chlorophyll content ($\text{mg} \times 10^{-6}$).

reticulatus and *Gambusia* sp. Loeblich (1966) mentioned that *Amphidinium* spp. and *Gymnodinium* spp. excrete their toxin into the culture medium. Ikawa and Taylor (1973), Ikawa and Sasner (1975), Nakajima et al (1981) and Davin et al. (1988) reported that *A. carterae* produced toxic substances. Yasumoto et al. (1987) found that *A. carterae* may produce five different haemolytic compounds which bioassays showed to be toxic to both mice and fish. Whether *A. carterae* is implicated in ciguatera is still uncertain (Juranovic & Park 1991), but Taylor et al. (1995) found that it can produce haemolytic compounds and may be

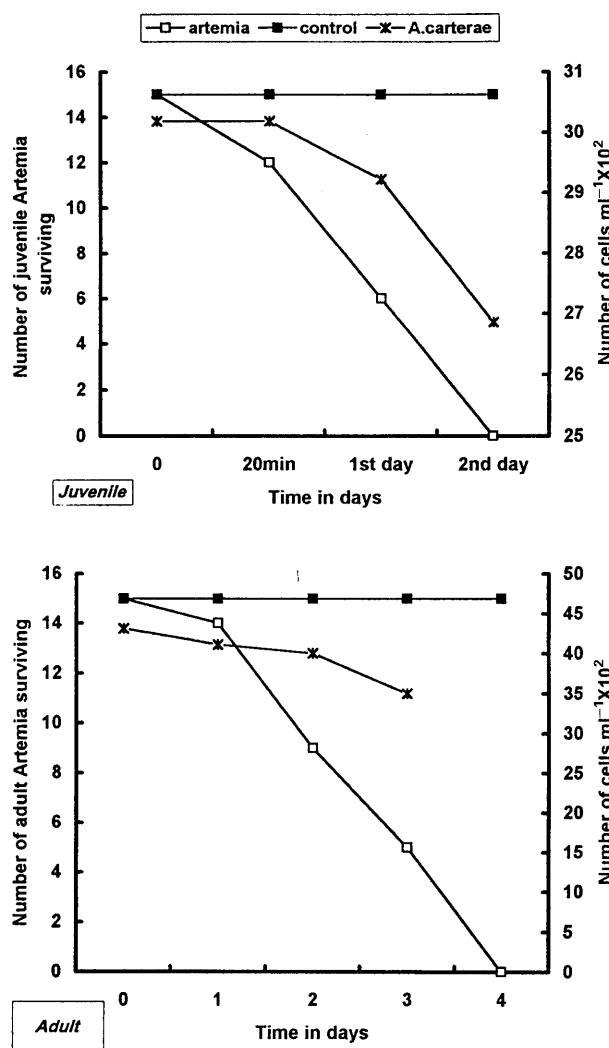


Fig. 8. Survival of juvenile (A) and adult (B) *Artemia salina* in *Amphidinium carterae* cultures.

implicated in ciguatera. The species is included on the list of harmful algae in relation to Norwegian fish farming and mussel fishery (IOC 1996).

In the present work, the toxicity of *A. carterae* was tested on the brine shrimp *Artemia salina*. While all earlier toxicity experiments were done on fish and mice, this is the first time its toxic effect is shown on a crustacean. Four types of culture waters were used for the toxicity test: a) One month culture. b) One year old cultures. c) Filtered water from a new culture (without cells). d) Filtered water from an old culture (without cells).

In all experiments both adult and juvenile *Artemia* (one day) were exposed. Every test was triplicate, with a control, and the number of *Artemia* in each test kept constant (15 organisms). All the toxicity tests are static.

It was found that the filtered medium of both old and young cultures had no effect on the brine shrimp. This is understandable given the very low permeability of the body membranes of the brine shrimp. On the other hand, younger cultures were shown to be toxic during their exponential

phase to both adult and juveniles, while older cultures remained harmless. The cell density in both young and old cultures became significantly depressed (by 20% to 60% in two to three days) as a result of active feeding by the brine shrimp on the cells.

It appears therefore that for the brine shrimp, the toxic effect results from grazing on *A. carterae* cells and not from membrane diffusion of extracellular toxins (Fig. 8).

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