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The Ecology of *Dunaliella salina* (Chlorophyceae, Volvocales): Effect of Environmental Conditions on Aplanospore Formation

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Abstract

The conditions leading to aplanospore formation in the green halophilic flagellate, *Dunaliella salina* (Dunal) Teodoresco, were studied using mixed cultures established from field collected samples. Aplanospore formation generally requires reduced salinity, (at salinities > 20% w/v NaCl aplanospores are rarely formed), nitrogen depletion and the presence of sulphate. Cool temperatures and short daylength may also promote spore formation, whereas pH and irradiance have no effect. Aplanospore formation takes place once the culture has reached stationary phase and, in such cultures, up to 36% of the total cells present can be aplanospores, although percentages of spores of less than 5% are most common. The only exceptions to this are high-phosphate cultures where aplanospore formation occurs early in the growth cycle and then ceases. However, not all cultures are competent to form aplanospores, and some mixed or unialgal cultures never formed aplanospores under conditions identical to those where aplanospore formation was observed in others. The factor(s) leading to competency are not known. Unlike free-living cells of *D. salina*, the aplanospores contain the ketocarotenoid, canthaxanthin.

Introduction

The life history of the green flagellate *Dunaliella* (Chlorophyceae, Volvocales) is little known other than for some early studies (Hamburger 1905, Labbé 1925, Lerche 1937). This is despite the fact that at least one species, *Dunaliella salina* (Dunal) Teodoresco, has achieved great commercial importance as a source of natural β-carotene (Borowitzka, L. J. and Borowitzka 1989). Sexual reproduction and zygote formation in *D. salina* were first noted by Teodoresco (1905) and have been studied in some detail by Lerche (1937); however, the formation of resting stages such as aplanospores and palmellas is much less known.

Vegetative cysts or aplanospores were first noted in *D. salina* by Hamburger (1905) who described them as spherical with a thick two-layered cell covering with small round bumps on the surface. Labbé (1925) mentions the formation of 'erythrocysts' in winter at low salinities, and Margulis *et al.* (1980) also report *Dunaliella* cysts in Laguna Figuera at low salinities. The formation of aplanospores at low salinity also has been observed by Liebetanz (1925) in the laboratory. We have studied *Dunaliella* in the field at Hutt Lagoon, Western Australia, and have observed aplanospores mainly in winter (Borowitzka, M. A. *et al.* 1982). Massyuk (1973) describes vegetative cysts (aplanospores) in *D. tertiolecta* Butcher, *D. viridis* Teodoresco, *D. terricola* Massyuk, *D. ruineniana* Massyuk, and *D. peircei* Nicolai *et* Baas-Becking, but gives no data on the conditions leading to cyst formation. Other workers on *Dunaliella*, such as Lerche (1937), Butcher (1959) and Cifuentes *et al.* (1992), have apparently never observed aplanospores despite their extensive studies of growth and reproduction in this genus. The most detailed information on aplanospore formation comes from the work of Loeblich (1969, 1972) who found that her cultures of *D. salina* formed aplanospores at salinities of 2–8% NaCl[1]) in the

[1]) Throughout this paper salinity is expressed as % w/v NaCl

stationary growth phase. She also showed that these cysts contained about the same amount of DNA as the presumably haploid motile vegetative cells and were therefore resting cysts, not zygotes.

This paper is a study of the environmental conditions leading to aplanospore formation in *Dunaliella salina*.

Material and Methods

Samples of *Dunaliella salina* were collected from the growth ponds of Wesfarmers Algal Biotechnology Pty Ltd at Hutt Lagoon, Western Australia, and were established and maintained at the collection salinity (25 to 30% NaCl) in 15 cm deep, 15 L mini-ponds in a glasshouse. These cultures were neither axenic nor unialgal and contained mainly *D. salina* as well as low numbers of *D. viridis*. The cultures were: M1-1 (collected 27 January 1985), K1-64 (collected 17 May 1985), H1-8 (collected 10 July 1985), H1-35 (collected 14 October 1985), H1-55 (collected 9 November 1985) and H2-20 (collected 20 March 1986).

The basic growth medium used was Modified Johnson Medium (Borowitzka, M. A. 1988) with either full strength nitrate and phosphate (J/1 in text), 1/2 the concentration of nitrate and phosphate (J/2), or 1/10th nitrate and phosphate (J/10). Johnson medium is normally unbuffered, but in the pH experiments the medium was buffered with 20 mM of the zwitterionic buffers MES (pH 6.0), PIPES (pH 6.5 and 7.0), TES (pH 7.5), HEPES (pH 8.0), BICINE (pH 8.5) or CHES (pH 9.0). Cultures were grown either in environmental cabinets under 'summer' (14 : $\overline{10}$ h L : $\overline{D}$ cycle and 35 °C : 25 °C day : night temperature), 'winter' (10 : $\overline{14}$ h L : $\overline{D}$ and 25 °C : 15 °C temperature), 'cold winter' (10 : $\overline{14}$ h L : $\overline{D}$, 20 °C : 10 °C temperature), 'short/cool' (8 : $\overline{16}$ h L : $\overline{D}$, 20 °C : 15 °C temperature) or 'long/warm' (12 : $\overline{12}$ h L : $\overline{D}$, 25 °C : 20 °C temperature) conditions at an irradiance of approximately 200 μE m^{-2} sec^{-1} supplied by cool-white fluorescent tubes, or in a glasshouse at ambient light and temperature conditions.

In order to examine the effects of medium composition 12 different media based on the chemical composition of Hutt Lagoon brines were developed (Table I). Medium 1 is similar in composition to the winter brines, and in media 2 and 3 the $MgSO_4$ content is increased; such media had given the best aplanospore formation in preliminary experiments. Media 4 to 6 had a slightly reduced Cl^- content, with some of the Cl^- replaced with SO_4^-. In media 7 to 9 the Cl^- content was decreased even further, and media 10 and 11 were identical to medium 1, except that the $CaCl_2$ content was increased. Medium 12 is a control at 25% NaCl. Two separate inocula were used: H1-55 and H2-20. The cultures were grown at a 12 : $\overline{12}$ h, 20 °C : 20 °C day:night cycle.

Cell counts were determined with a haemocytometer. For carotenoid analysis the motile cells were killed and extracted by stepwise addition of ethanol to a concentration of 25% (v/v) ethanol. The culture was then filtered through GF/A glass fibre filters and briefly washed with 100% acetone to remove any residual pigments. This treatment had no observable effect on the integrity of the aplanospores but completely disrupted and extracted the motile cells. The aplanospores remaining were then extracted in 90% acetone by grinding with acid-washed sand in a mortar and pestle, and the carotenoids were analysed by HPLC (Borowitzka, M. A. *et al.* 1990).

The effect of salinity on aplanospore formation in axenic unialgal cultures of several isolates of *D. salina*, *D. viridis* and *D. polymorpha* Butcher in the Murdoch University Algal Collection (MUR) was also examined. These algae were grown in J/2 medium, except for strain MUR-204 which was grown in f/2 medium

Table I. Composition of the media used to examine effects of Ca : Mg ratio and SO_4^- concentration on aplanospore formation. J/10 medium was used except for the variations listed in the table. (All concentrations in g L^{-1}; molarities are given in brackets).

Medium	$MgSO_4$ $7H_2O$	$CaCl_2$ $2H_2O$	$CaSO_4H_2O$	Na_2SO_4	NaCl
1	2.5 (0.01)	1.5 (0.01)	0	0	150 (2.57)
2	25.0 (0.1)	1.5	0	0	150
3	50.0 (0.2)	1.5	0	0	150
4	2.5	0	1.7 (0.011)	0	150
5	25.0	0	1.7	0	150
6	50.0	0	1.7	0	150
7	2.5	0	1.7	120 (0.85)	50 (0.850
8	25.0	0	1.7	120	50
9	50.0	0	1.7	120	50
10	2.5	15 (0.1)	0	0	150
11	2.5	30 (0.2)	0	0	150
12	2.5	1.5	0	0	250 (4.28)

(Guillard and Ryther 1962) but using synthetic seawater (APSW, *cf.* Borowitzka, M. A. 1988) instead of seawater, over a salinity range of 5 to 30% NaCl. The temperature was 25 °C with a photon flux density of approximately 240 μ Einsteins m^{-2} sec^{-1} with a 12 : $\overline{12}$ h L : $\overline{D}$ cycle.

Results

Aplanospores are recognised by their thick, rough cell wall (Fig. 1).

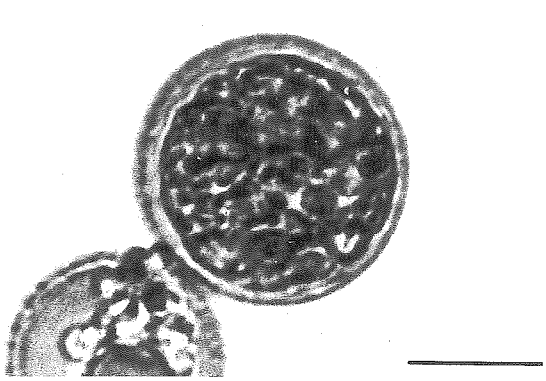

Fig. 1. Light micrograph of a *Dunaliella salina* aplanospore (Scale = 10 μm).

We first examined the effects of nitrate limitation, salinity and season (daylength and temperature) on growth and aplanospore formation. Cultures of 50 mL (M1-1) were set up at 15, 20 and 25% NaCl (w/v) salinity at 4 nitrogen concentrations (0.5, 0.25, 0.05 and 0.00 g L^{-1} KNO_3) and the cultures were grown under 'summer' and 'winter' conditions in growth cabinets. There was little difference in growth at the three salinities used, but growth was better at the higher nitrate concentrations (Figs 2, 3). Some aplanospore formation occurred in both the 'summer' and 'winter' conditions at almost all nitrate concentrations, but aplanospore formation was enhanced at the lower salinities and no aplanospores were observed in any of the 25% NaCl cultures. 'Winter' conditions further enhanced aplanospore formation, with the nitrate-free, 15% NaCl culture, having up to 15% of the cells present as aplanospores after 37 days (Fig. 3c); aplanospore production in most of the other cultures was less than 2% of total cell numbers.

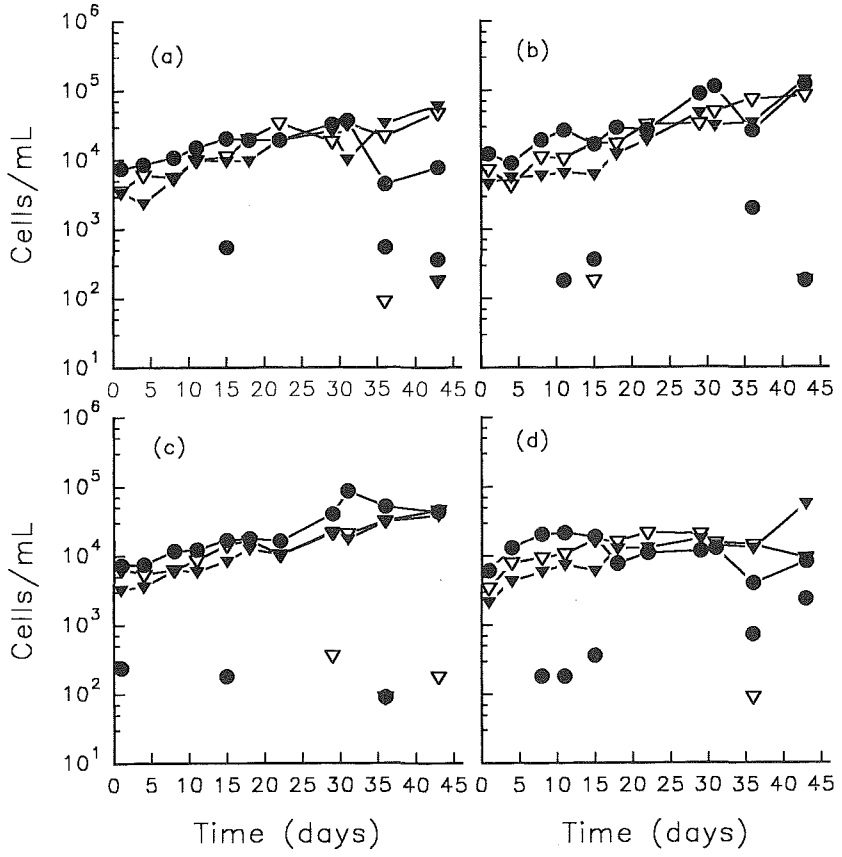

Fig. 2. Effect of salinity and nitrate conditions on aplanospore formation in *D. salina* under 'summer' conditions in growth cabinet. KNO_3 concentrations in g L^{-1} were: (a) 0.50; (b) 0.25; (c) 0.05; (d) 0.00. Salinities in % (w/v) NaCl were: (▼) 25%; (▽) 20%; (●) 15%. The points linked with lines indicate total cell numbers, individual points are aplanospore numbers.

When phosphate concentration was varied (0.035, 0.0175, 0.0035, 0.0000 g L^{-1} KH_2PO_4), higher phosphate concentrations stimulated aplanospore formation in the first 10–15 days of the culture, with spore numbers declining rapidly after that time. More spores formed in the 'winter' treatment and at the higher salinities.

The effect of pH on aplanospore formation was also examined by growing the cells in buffered media over the pH range of pH 6.0 to pH 9.0 in J/10 medium at 10% and 15% NaCl under 'winter' conditions. Rapid growth was observed from pH 6 to 7.5, whereas growth was greatly reduced at pH 8.5 and 9.0, possibly due to toxic effects of the buffers used. Very low numbers of aplanospores were observed at pH 7.5 and 8.0 after 23 days at 15% NaCl.

The effect of dilution as well as culture age was further examined by taking a sample collected 12 days before from Hutt Lagoon (K1-64) and diluting it gradually with distilled water over a period of 3 days from the original 29.5% salinity to 25%, 20%, 15% and 10% salinity. Replicates were placed under 'winter' and 'cold winter' conditions as well as in the glasshouse (temperature range 33 °C to 4 °C and maximum irradiance of approximately 1900 µEinsteins m^{-2} sec^{-1}). Another series was set up 10 days later, and a third, 31 days later. In the first series all cultures at < 25% salinity produced some aplanospores, but the yield did not exceed 2% of total cell numbers, with the 'winter' treatment producing slightly more aplanospores. The second and third series gave very similar results to the first series, indicating that culture 'age' appeared to have no effect on the capacity to form aplanospores.

Irradiance also appeared to have little effect as the glasshouse replicates did not form more aplanospores than the growth cabinet cultures.

Although the cultures contained mainly *Dunaliella salina*, low numbers of *D. viridis* were present also. The question arises, therefore, whether the aplanospores are formed by *D. salina* or by *D. viridis* or by both. To examine this we set up a field culture consisting mainly of *D. viridis* and a second culture made up by combining 15 different isolates of *D. viridis* from our culture collection. These cultures were set up at 5 and 15% salinity, either with no added nitrate and phosphate or with 1/10th Johnson nitrate and phosphate. The growth conditions were 'short/cool'

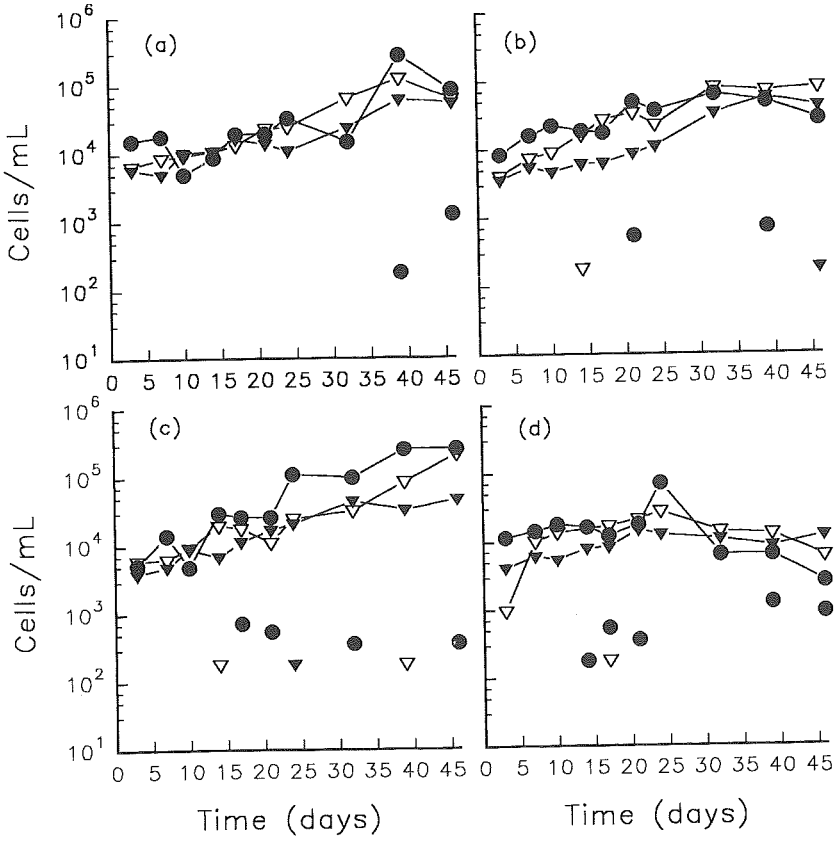

Fig. 3. Effect of salinity and nitrate conditions on aplanospore formation in *D. salina* under 'winter' conditions in growth cabinet. KNO_3 concentrations in g L^{-1} were: (a) 0.50; (b) 0.25; (c) 0.05; (d) 0.00. Salinities in % (w/v) NaCl were: (▼) 25%; (▽) 20%; (●) 15%. The points linked with lines indicate total cell numbers, individual points are aplanospore numbers.

and 'long/warm'. No aplanospores were observed in any of these cultures, suggesting that *D. viridis* is not the source of the aplanospores observed in mixed cultures.

In another experiment we concentrated the cells of *D. salina* by carefully pipetting off the red cells which accumulated at the surface of a 26% NaCl stock culture. These cultures were grown at a series of dilutions and aplanospore concentrations of up to 36% of the total cell number were observed. Such a high proportion of aplanospores was rarely observed in other cultures, and this further supports the contention that it is mainly *D. salina* which forms aplanospores in mixed cultures.

Finally, we also isolated 5 individual aplanospores from M1-1 by micropipetting, and used these to establish clonal cultures. All of these cultures were *D. salina* and in three of them aplanospore formation could be induced in culture by reducing the salinity to between 5 and 10% NaCl. We have also observed aplanospore formation at reduced salinity (generally below 10% NaCl) in some other isolates of *D.salina*, *D. viridis* and *D. polymorpha* from a range of localities, but not in other isolates cultured under identical conditions (Table II).

The above experiments show that, generally, reduced salinity, 'winter' conditions and low N all enhance aplanospore formation; however these conditions appear to be insufficient to induce aplanospore formation in all cultures. Two repeats of the experiment examining the effects of nitrate, salinity, temperature and season, using different inocula (H1-8 and H1-55), produced only very few aplanospores even after 50 days. As part of this study we inoculated 15 L mini-ponds (15 cm deep) containing J/2 medium at 26% NaCl salinity (the field salinity) with the various field collected samples. The mini-ponds were placed in a glasshouse and the cultures monitored for up to 17 months. The original salinity was maintained by regular topping-up with distilled water to make up for evaporative losses and no nutrients were added. Of the 7 cultures of different origin only one (M1-1 collected 25 January 1985; 22% salinity) produced aplanospores in appreciable numbers, and another (H1-8 collected 10 July 1985; 30% salinity) produced low numbers of aplanospores; in the rest low numbers of aplanospores were observed only intermittently. However, even in the aplanospore-producing cultures the number of spores produced varied seasonally (Fig. 4). Although this culture was established at the beginning of April, aplanospore formation did not commence until July (*i. e.* in winter). Around October the number of aplanospores began to decline, and from late December to June almost no aplanospores were observed.

Table II. Summary of the effects of salinity on aplanospore formation in unialgal cultures of *Dunaliella* spp. For growth conditions see Materials & Methods.

Species	Murdoch University Culture Collection No.	Origin	Other No.	Conditions under which aplanospores were observed
D. salina	MUR-8	Hutt Lagoon, W. A.		5% NaCl
	MUR-9	Hutt Lagoon, W. A.		5–10% NaCl
	MUR-22	Hutt Lagoon, W. A.		5–10% NaCl
	MUR-202	Death Valley, USA		5–10% NaCl
	MUR-10	Hutt Lagoon, W. A.		never
	MUR-23	Hutt Lagoon, W. A.		never
	MUR-89	?	Göttingen 19/4	never
	MUR-186	Salt Lake, USSR	CCAP 19/3	never
	MUR-192	Brackish Lake, North Sinai, Israel	CCAP 19/12	never
	MUR-194	?	CCAP 19/25	never
	MUR-200	Salt pond near Bardawil Lagoon, Israel	CCAP 19/30	never
D. viridis	MUR-196	Marine	CCAP 19/20	5% NaCl
	MUR-203	Loch Linnhe, Argyle, Scotland	CCAP 19/34	2.5–5% NaCl
	MUR-28	Hutt Lagoon, W. A.		never
	MUR-34	Salt Lake, South Australia		never
	MUR-41	Hutt Lagoon, W. A.		never
	MUR-60	Hutt Lagoon, W. A.		never
	MUR-61	Hutt Lagoon, W. A.		never
	MUR-197	Wad al Neifur, Egypt	CCAP 19/21	never
	MUR-201	?	CCAP 19/31	never
	MUR-208	Kuwait	KISR-K89	never
D. polymorpha	MUR-204	?	CCAP 19/24	<15% NaCl

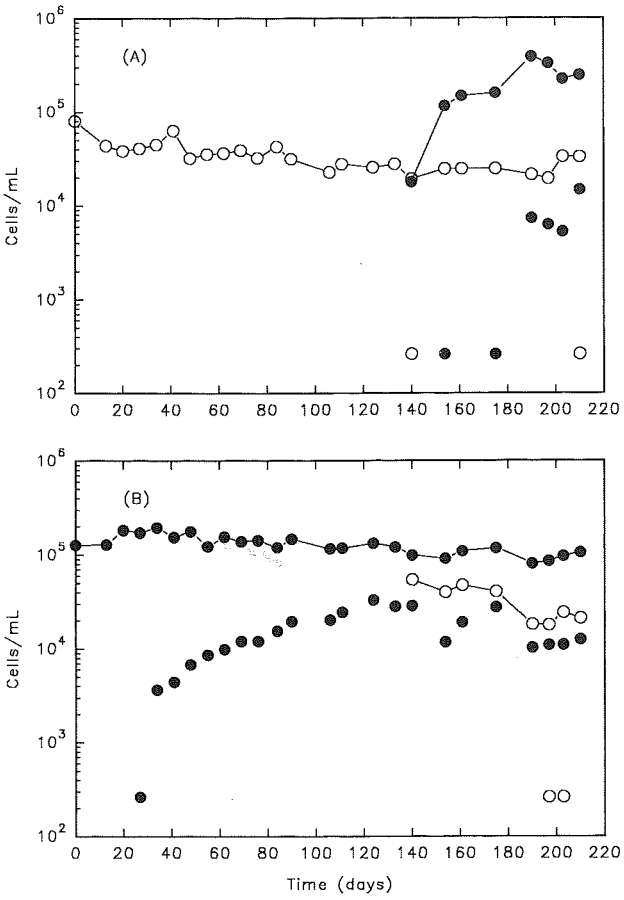

Fig. 4. Effect of dilution from 26% to 17% NaCl with deionised water with (●) or without (○) the addition of J/10 nutrients in 15 L mini-ponds in glasshouse. (A) Culture K1-55 – nutrients added to half the culture on day 140. (B) Culture H1-8 – duplicate stock culture diluted with deionised water set up on day 140. The points linked with lines indicate total cell numbers, individual points are aplanospore numbers.

Aplanospore formation could be induced in these cultures by dilution, as long as some nutrients were present. One of the replicate mini-ponds of culture M1-1 (at 26% salinity) was gradually diluted to 17% salinity with distilled water over two days. Over a period of 140 days following dilution no aplanospores were formed. At this time the culture was split into two, and J/10 nutrients were added to one replicate; the other remained unchanged. The addition of the nutrients resulted in growth and induced the formation of significant numbers of aplanospores once growth ceased (Fig. 5). A second culture (H1-8 at 26% NaCl) was also diluted to 17% salinity, but with J/10 nutrients added. After about 22 days aplanospore formation commenced and aplanospores were present over the whole 17 month period of monitoring (Fig. 5b). Some of the original 26% NaCl stock culture of H1-8 was diluted to 17% with distilled water only on day 140; this culture formed almost no aplanospores (Fig. 5).

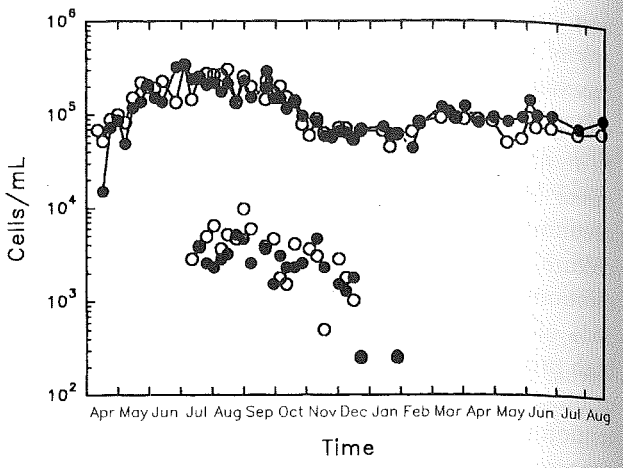

Fig. 5. Monitoring of long-term mini-pond cultures in glasshouse. J/2 medium inoculated with culture M1-1 and maintained at approximately 20% salinity. For clarity only 2 of 5 replicate ponds are shown; the other replicates gave similar results. The points linked with lines indicate total cell numbers, individual points are aplanospore numbers.

The composition of the natural brines at Hutt Lagoon varies greatly between summer and winter (Table III); the concentrations of magnesium and sulphate show the greatest variation, whereas sodium changes little and chloride not at all. Preliminary experiments also showed that sulphate was important for aplanospore formation and could not be replaced by chloride. It was therefore decided to examine the effects of medium composition, especially the effect of Mg^{2+}, Ca^{2+}, SO_4^- and NaCl concentration. Twelve different media (see Table I) and two different inocula (H1-55 and H2-20) were used.

Total cell numbers reached in all cultures were very similar and all cultures had reached stationary phase after about 15–20 days, although the cultures with a higher ionic strength grew slightly slower (Figs 6,

Table III. Composition of the brines in Hutt Lagoon in summer and winter (analyses curtesy of Wesfarmers Algal Biotechnology Pty Ltd). Concentrations are in g L^{-1} and the molarity is in brackets.

Ion	Summer (December)	Winter (August)
Na^+	58.00 (2.52)	107.50 (4.68)
K^+	8.50 (0.217)	1.00 (0.026)
Ca^{2+}	0.23 (0.006)	1.00 (0.025)
Mg^{2+}	29.00 (1.193)	3.13 (0.129)
Ca^{2+} : Mg^{2+} molar ratio	*0.005*	*0.193*
Cl^-	186.70 (5.27)	171.80 (4.85)
HCO_3^-	0.73	0.14
SO_4^-	39.05 (0.406)	6.91 (0.072)
Cl^- : SO_4^- molar ratio	*12.85*	*67.36*
pH	7.15	7.33

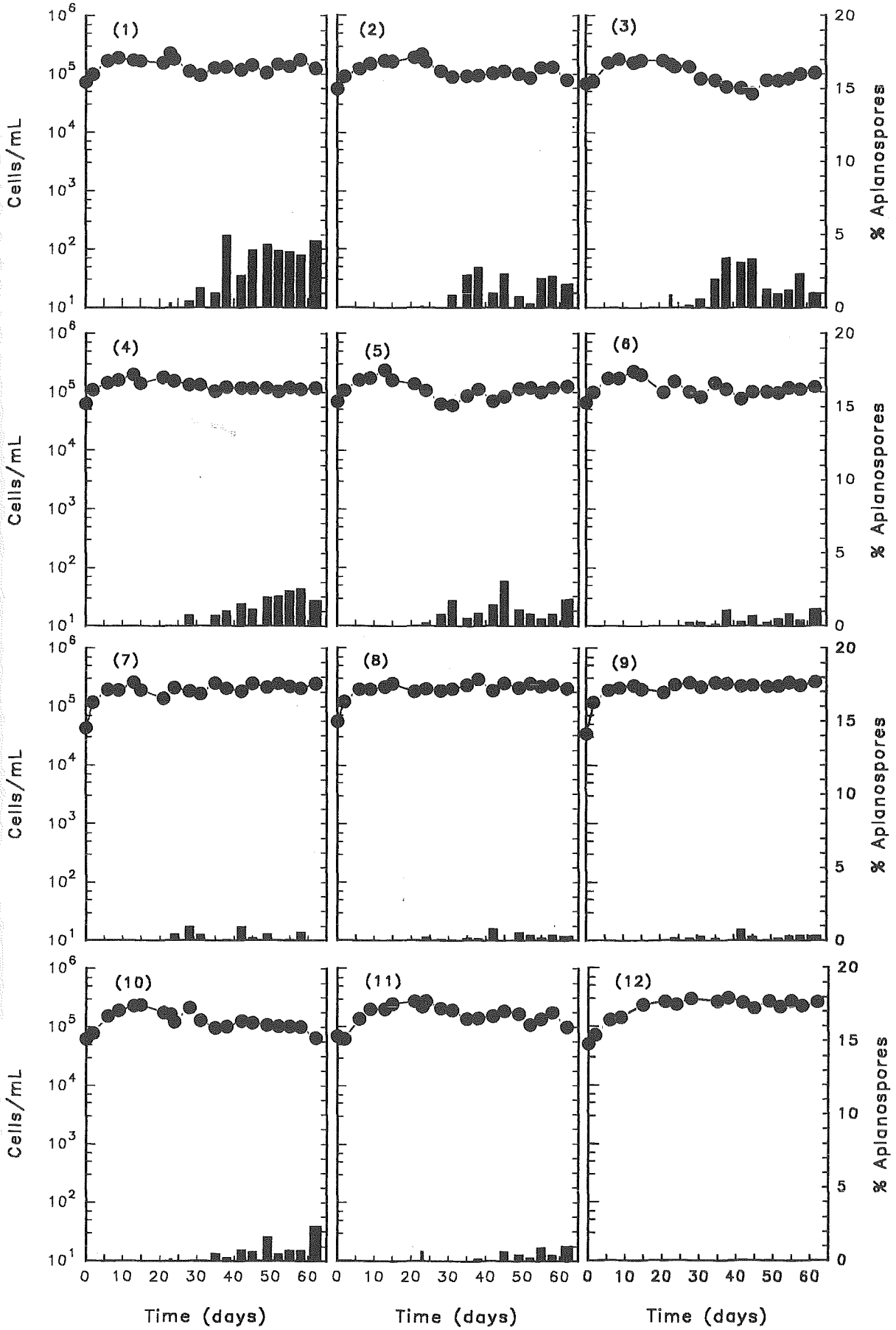

Fig. 6. Culture H1-55. Effect of medium composition on growth and aplanospore formation under 'long/warm' conditions in growth cabinet. • = total cell numbers; bars = aplanospores as % of total cell numbers. Numbers refer to the medium shown in Table I.

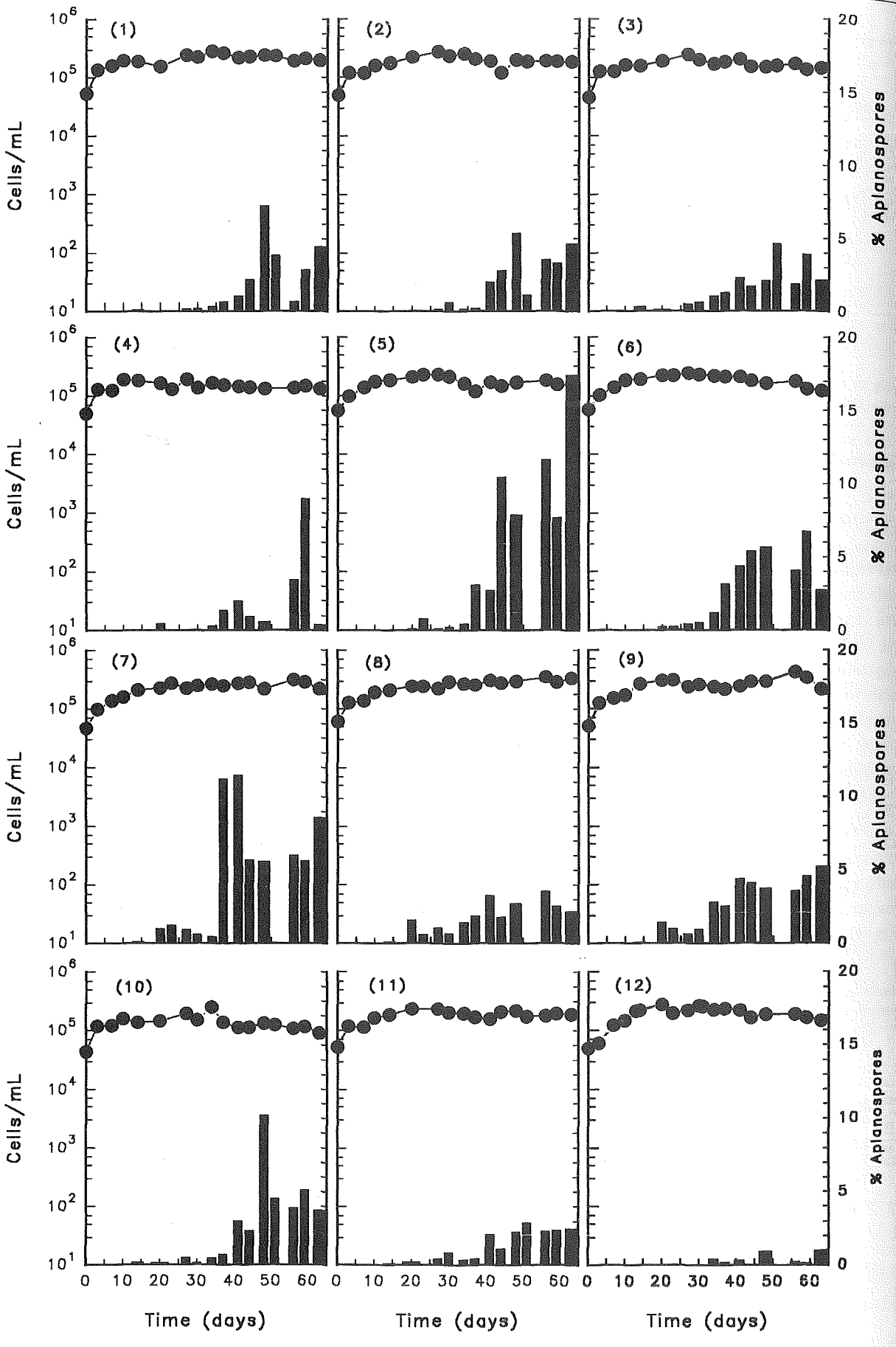

Fig. 7. Culture H2-20. Effect of medium composition on growth and aplanospore formation under 'long/warm' conditions in growth cabinet. ● = total cell numbers; bars = aplanospores as % of total cell numbers. Numbers refer to the media shown in Table I.

7). Both inocula produced almost no aplanospores in the high salinity 25% NaCl medium (medium 12) (Figs 6, 7), but the response of the two inocula differed for the other media. Overall, the H1-55 culture produced fewer aplanospores than the H2-20 culture. In culture H1-55, the 'winter' medium (medium 1) gave the highest aplanospore numbers, whereas in culture H2-20 the highest numbers were found in media 5 and 7 which had an intermediate sulphate concentration (Figs 6[*1*], 7[*5*], 7[*7*]). Increasing the $MgSO_4$ concentration reduced aplanospore number slightly in both cultures (Figs 6[*1-3*], 7[*1-3*]), as did increasing the $CaCl_2$ concentration (Figs 6[*1, 10, 11*], 7[*1, 10, 11*]). However, the two cultures differed in their response to other medium changes. Thus, in H1-55, increasing the SO_4^- concentration resulted in decreasing aplanospore numbers, whereas in H2-20, the highest aplanospore numbers were observed at intermediate sulphate concentrations. The trends observed are summarised in Table IV.

The carotenoid content of the aplanospores was also analysed and is shown in Table V, where it is compared with the red *D. salina* and the green *D. viridis*. The aplanospores contain appreciable amounts of the ketocarotenoid canthaxanthin which is not detectable in motile, free-swimming, cells.

Table IV. Summary of the effects of changing medium composition on aplanospore formation as shown in Figures 6 and 7. The composition of the media is shown in Table I. (↑) = increased aplanospore formation; (↓) = decreased aplanospore formation.

	Media	Effect on aplanospore formation	
		H1-55	H2-20
Increasing $MgSO_4$			
(+0.0 M SO_4^-)	1→2→3	↓	↓
(+0.11 M SO_4^-)	4→5→6	↓	↑→↓
(+0.96 M SO_4^-)	7→8→9	↓	↓
Increasing SO_4			
(0.01 M Mg^{2+})	1→4→7	↓	↓→↑
(0.10 M Mg^{2+})	2→5→8	↓	↑→↓
(0.20 M Mg^{2+})	3→6→9	↓	↑ (slight)
Increasing $CaCl_2$	1→10→11	↓	↓
Increasing NaCl	1→12	⇓	⇓

Table V. Analysis of the content of the major carotenoids in *Dunaliella* aplanospores compared with motile cells (in μg carotenoid 10^6 cells^{-1}).

	D. viridis	*D. salina*	Aplanospores
Lutein	0.3	1.0	1.0
Zeaxanthin	0.08	0.8	0.2
β-Carotene	0.15	74.0	0.7
Canthaxanthin	<0.1	<0.1	13.0

Discussion

The aplanospores of *Dunaliella salina* have a markedly different carotenoid composition from the vegetative cells, in that the main carotenoid is the ketocarotenoid canthaxanthin, and not β-carotene. Loeblich (1972) was the first to observe canthaxanthin in *D. salina* aplanospores, and her observations are confirmed here. Canthaxanthin has also been reported for motile cells of *D. pseudosalina* Massyuk *et* Radchenko (Massyuk and Radchenko 1973); however this report remains to be confirmed independently. The motile cells of all other *Dunaliella* species, especially *D. salina* and *D. parva*, accumulate mainly β-carotene (Aasen *et al.* 1969, Massyuk 1973). The β-carotene of *D. salina* is located in droplets in the intrathylakoid spaces of the chloroplast (Borowitzka, M. A. and Borowitzka 1988), whereas the canthaxanthin is apparently cytoplasmic (Loeblich 1972). The aplanospores of *D. salina* therefore are similar to the aplanospores of *Haematococcus pluvialis*, which accumulate large amounts of astaxanthin in the cytoplasm (Santos and Mesquita 1984, Grung *et al.* 1992). The cysts of other green algae have also been reported to accumulate carotenoids [*e.g.* the akinetes of some species of *Chlamydomonas* (Ettl 1976)] but no analyses of their carotenoid composition are available.

The induction of aplanospore formation in *Dunaliella salina* is clearly a complex process and the experiments presented here provide information on the major environmental factors leading to aplanospore formation. One important observation is that *Dunaliella* cultures are not always competent to form aplanospores, and that treatments which produce appreciable numbers of spores in some cultures will not do so in others. What determines this competency is not known, however these observations may explain why many workers with *Dunaliella* (*e.g.* Lerche 1937, Butcher 1959) never observed aplanospores despite intensive studies. It may also explain why unialgal laboratory cultures of *D. salina* have rarely been observed to form aplanospores.

The main requirement for aplanospore formation is reduced salinity (ionic strength); at high salinities (greater than about 20% NaCl) no aplanospores are formed. This is in accord with the observations of Hamburger (1905) and Loeblich (1972). Before aplanospore formation can take place, however, the cultures must be in stationary phase; if sufficient nutrients are present to sustain growth, aplanospores are generally not formed. Light intensity seems to play no role, although there are some indications that short daylength and cooler temperatures may enhance aplanospore formation. The ionic strength and com-

position of the brines in which *D. salina* normally occurs are very variable (*cf.* this paper, Post 1977), and it is not surprising that the ionic composition of the medium also has an effect on aplanospore formation. Of the major ions present, sulphate seems to have the greatest effect. Sulphate is required for growth (Massyuk 1965) and sulphate deficiency in *D. parva* inhibits cell division, amino-acid and protein formation and thus also photosynthesis (Gimmler and Weiss 1987), whereas high sulphate has little effect on growth. The results shown here indicate that, in at least one of the cultures, aplanospore formation is favoured by quite high concentrations of sulphate (between 0.1 and 0.9 M) and replacing some of the sulphate with chloride inhibits spore formation.

The aplanospores are a temporary resting stage which can survive extreme dilution. In the normal shallow hypersaline environment of *D. salina*, dilution by rain can normally be expected in winter. For example, at Hutt Lagoon almost all of the annual rainfall occurs between June and August. In most salt lakes rainfall not only causes dilution of the brines, but also changes their composition and ionic ratios. This probably accounts for the stimulatory effects of sulphate on aplanospore formation.

However, dilution alone is insufficient to cause aplanospore formation. For example, in our long-term culture kept at constant salinity and ionic composition, aplanospore formation only occurred in winter. With the onset of spring the number of aplanospores in this culture declined and none were produced in summer.

An important question raised by this study is that of competence. All the cultures used here came from the same location and therefore can be considered to be a fairly similar mixture of genotypes. The trigger inducing the competence to form aplanospores therefore appears most likely to be environmental. From our results it is unlikely that this trigger could be a physical factor such as light, daylength or temperature. A more likely hypothesis is that the factor leading to competence is some trace nutrient which may be stored in the cells for some time, but which is missing or in inadequate supply at certain times in the natural brines and in some laboratory media. This hypothesis remains to be tested.

The present study has provided some information as to the conditions which lead to aplanospore formation in *D. salina*, however further studies are required to identify clearly the key trigger(s) leasing to aplanospore formation.

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