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Intraspecific variation in the selenium requirement of different geographic strains of the toxic dinoflagellate Gymnodinium catenatum

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Abstract. The requirement for selenium (IV) was assessed in five strains of the toxic dinoflagellate Gymnodinium catenatum Graham, representing three populations from Tasmania (Australia), as well as one each from Japan and Spain. Strains were grown in nutrient-enriched sea water medium with 10–9 M selenium added as selenite (H2SeO3), or with no added selenium, and monitored for growth and cell yield. Strains exhibited different selenium (Se) requirements, as evidenced by (i) a decrease in exponential growth rate (10–20%) and cell yield (up to 80%) (Japanese strain); (ii) a decrease in cell yield only (Tasmania Derwent Estuary 1987, Tasmania Huon Estuary and Spanish strains); and (iii) no decrease in growth or cell yield (Tasmania Derwent Estuary 1993 strain). Variation in the response to Se deficiency was greatest between the two strains isolated from the Derwent Estuary, Tasmania, in different years (1987 and 1993) and less between Tasmanian strains from different localities (Huon and Derwent Estuaries are 50 km apart) or between Tasmanian and Japanese or Spanish strains. Strain variability in micro-nutrient responses such as described here may provide a partial explanation for different bloom patterns exhibited by the same dinoflagellate taxon under apparently similar environmental conditions.

Introduction

Blooms of the toxic, paralytic shellfish poisoning (PSP), chain-forming dinoflagellate Gymnodinium catenatum Graham pose a serious risk to human health, aquaculture developments and the coastal environment. During the last 20 years, G. catenatum has been found in an increasing number of locations worldwide, with vegetative cells being identified in both temperate and tropical waters, e.g. northwest Spain, Mexico, Japan, Portugal, Venezuela, Thailand, Philippines, Palau, Uruguay, Morocco, Malaysia and south-east Tasmania, Australia [see (Hallegraeff and Fraga, 1998) for review].

Several hypotheses have been put forward to explain G. catenatum bloom development. Bloom formation in the Huon Estuary, Tasmania, appears to be autochthonous and linked to the incidence of freshwater input after rainfall (contributing organic and inorganic growth factors) and is associated with extended periods of low wind-stress (Hallegraeff et al., 1995). In contrast to blooms in Tasmanian waters, G. catenatum outbreaks in the Spanish Rias may be the result of either: (i) shoreward transport of allochthonous oceanic seed populations into the Rias (Fraga et al., 1988); or (ii) germination of resting cysts from Rias sediments (Figueiras and Pazos, 1991; Blanco, 1995). In addition, Spanish
blooms are associated with upwelling relaxation which results in a deepening of the nutricline and selection for strongly migratory species such as *G. catenatum* (Fraga and Bakun, 1993). These bloom formation mechanisms involve changes in environmental conditions (e.g. before and after run-off events, transition from sediment to water column or offshore to inshore waters), so that cysts or motile vegetative cells are subject to different salinity, temperature, nutrient and turbulence regimes. Phenotypic and genetic diversity in nutritional and physiological requirements within and between populations may therefore comprise a successful ecological strategy for *G. catenatum*.

Micro-nutrients such as iron (Fe) and cobalt (Co) have been implicated in the incidence of harmful algal blooms of other species [e.g. Fe: dinoflagellate *Gymnodinium breve*, southern Florida (Ingle and Martin, 1971); Co: prymnesiophyte *Chrysochromulina polylepis*, Kattegat, south-east Sweden (Granéli and Risinger, 1994)]. The trace element selenium has also been identified as potentially important in *Gymnodinium nagasaktiense* dinoflagellate blooms in Japanese waters off Wakayama, where in 1988, a good correlation between selenium concentrations and cell density was reported [see (Boyer and Brand, 1998)]. Furthermore, addition of selenium to Temma Bay water stimulated growth of *G. nagasakiense* test cultures (Ishimaru *et al.*, 1989). Other harmful algae with a selenium requirement, or those which have shown an increase in growth or biomass yields upon addition of nanomolar concentrations of selenium, include the marine chrysophyte *Aureococcus anophagefferens* (Cospé *et al.*, 1993), raphidophyte *Chlotocilla verruculosa* (Imai *et al.*, 1996), dinoflagellate *Pyrodinium bahamense* (Usup and Azanza, 1998) and several *Chrysochromulina* prymnesiophyte species (Pintner and Provasoli, 1968; Wehr and Brown, 1985). Data presented elsewhere demonstrate that *G. catenatum* (strain GCDE08) also has a selenium requirement, with addition of selenite (Se IV) at concentrations from 1-100 nM, causing an increase in both growth rate and biomass yields (Doblin *et al.*, 1999a).

In the present work, we report on the variability in selenium (IV) requirement in five strains of *G. catenatum*, representing different regional and globally distributed populations. Comparisons were made between strains collected from the same location (Derwent Estuary, Tasmania) in different years (1987 and 1993), and between strains from adjacent (Derwent and Huon Estuaries, Tasmania, located 50 km apart) and widely separated areas (Tasmania, Japan and Spain).

**Method**

*Strain history and identification*

The strains used in this study were all provided by the CSIRO Collection of Living Microalgae (Australia). *Gymnodinium catenatum* strains were isolated from two locations in Tasmania, Australia: the Derwent Estuary in 1987 and 1993 (GCDE08 and GCDE9305, respectively), and the Huon Estuary in 1986 (GCHU02). Other strains were obtained from Ria de Vigo, Spain in 1985 (GCSP01) and Senzaki Bay, Japan in 1986 (GCJP10). Species identification of all
strains was confirmed by light and electron microscopy, and was also indepen-
dently verified by rRNA and RAPD molecular signatures (Bolch et al., 1999; 
Bolch, unpublished data). Further information about these strains is provided in 
Table I.

Culture conditions

Since being lodged with the CSIRO Collection of Living Microalgae, these strains 
have been maintained in enriched sea water medium (salinity 28 ppt) with GPM 
nutrients (Loeblich, 1975), soil extract and 10⁻⁸ M selenium (referred to as GSe 
medium in our laboratory). Experimental cultures were grown in activated char-
coal-treated, 0.22 µm filtered, Teflon-autoclaved oceanic sea water (collected off 
Maria Island, south-east Tasmania), with salinity adjusted to 28 ppt using sterile 
Milli-Q water. Nutrients were added to yield GPM medium at a concentration of 
1/10 of the original recipe [GSe/10; NO₃ = 200 µM; PO₄ = 20 µM (Loeblich, 1975)], 
with the addition of selenium as selenite (10⁻⁹ M H₂SeO₃; Aldrich Chemical 
Company, Lot no. 1124KH) and no added soil extract. Cultures were grown in 
acid-washed, 50 ml Pyrex or KIMAX tubes (loosely-capped, Teflon-lined lids) at 
a temperature of 18°C with bottom illumination at 180 µmol photons PAR 
m⁻² s⁻¹ from Philips Deluxe cool white fluorescent lights on a 12:12 h L:D cycle.

Growth measurements

Growth rates of cultures were calculated from changes in in vivo fluorescence 
(Brand et al., 1981a), measured at the same time each day (3 h after the start of 
the light period):

\[ k(\text{day}^{-1}) = \frac{\ln \left( \frac{F_1}{F_0} \right)}{(t_1 - t_0)} \]  

(Guillard, 1973).

Each treatment had four replicates plus at least one blank (medium containing 
no microalgae); fluorescence readings \( F_1, F_0 \) at each time period \( t_1, t_0 \) were 
corrected for fluorescence of blank tubes at the same time.

| Table I. Isolation history of Gymnodinium catenatum strains used in this study |
|-------------------|-----------------|-------------|-------------|-------------|
| Strain            | CS-code | Source                  | Date of isolation | Isolator   | Clonal       | Toxic       |
| GCDE08            | CS-301  | Derwent Estuary, Tasmania | 15 June 1987     | S.Blackburn | +           | +           |
|                   |         |                         |                  |            | (8 celled chain) |             |
| GCDE9305         | no CS # | Derwent Estuary, Tasmania | 13 May 1993     | H.Ling     | +           | ND          |
| GCHU02            | CS-302  | Huon Estuary, Tasmania   | 6 June 1986      | S.Blackburn | +           | +           |
|                   |         |                         |                  |            | (8 celled chain) |             |
| GCJP10           | CS-305  | Senzaki, Japan          | 1986             | T.Ikeda    | unknown     | +           |
| GCSP01           | CS-306  | Ria de Vigo, Spain      | 5 November 1985  | I.Bravo    | 4 chains of 16–30 cells | + |

ND, not determined.
Yield estimates

Cultures were sampled at the end of exponential phase for cell density and chlorophyll *a* (Chl *a*). Duplicate 1 ml samples were taken for cell counts and immediately fixed with acid Lugol's solution [0.2% (Lovegrove, 1960)]. Depending on cell concentration, samples were diluted with sterile culture medium and counted with a Sedgwick-Rafter counting chamber (Rigosha, Japan) under bright field optics using a Zeiss Axioplan microscope. When possible, estimates were based on counting a minimum of 400 cells [-10% error (Guillard, 1973)]. Water mounts of unpreserved cultures were also made for observations of living cells. Subsamples were also filtered through 25 mm GF/F filters under dim light and filters stored at –20°C for later Chl *a* analysis. Pigments on filters were extracted with 100% acetone and the mixture sonicated for 45 s. Extracts were left on ice in the dark overnight, centrifuged (2000 g for 5 min), and Chl *a* in the supernatant fluid determined spectrophotometrically, using the appropriate equations of Jeffrey and Humphrey (Jeffrey and Humphrey, 1975).

**Gymnodinium catenatum** growth and biomass production without and with 10⁻⁹ M added selenium

**Gymnodinium catenatum** strains were inoculated from GSe medium into GSe/10 medium containing no added selenium (–Se) or GSe/10 with 10⁻⁹ M selenium (IV) as H₂SeO₃ (+Se). To ensure that differences in growth rates and biomass yields were not the result of different stages of acclimation [see (Brand et al., 1981a)], experimental cultures were transferred three times (>eight generations), with the inoculum (1 ml) being diluted 1:30 (the minimum carry over for sustained growth of *G.catenatum* in culture tubes). We are therefore confident that the observed phenotypic differences in selenium requirement between strains are due to genotypic variation. Cultures were not axenic but precautions were taken to minimize bacterial levels by carefully timed transfers (late exponential phase). Growth of cultures was monitored by *in vivo* fluorescence, and at the end of exponential phase during the third transfer, cultures were subsampled for cell counts and Chl *a*.

**Statistical analyses**

Growth rates were determined by least squares linear regression using a minimum of four successive fluorescence values. These points were selected from early exponential phase and yielded the greatest *R*² value. All slopes were significantly greater than zero. Differences in growth rates, cell yields and Chl *a* content between strains or Se treatments were tested using ANOVA and when appropriate, planned comparisons were used to test for differences between strains isolated from (i) the same location in different years (Tasmania Derwent Estuary 1987 and 1993); (ii) locations in close proximity (Tasmania Huon and Derwent Estuaries); and (iii) widely separate locations (Tasmania, Japan and Spain). The analyses were calculated using Systat 5.1 at a significance level of *α* = 0.05.
Results

Gymnodinium catenatum growth and biomass production without and with $10^{-9}$ M added selenium

Despite similar cell morphology, strains differed from one another in their growth habit and rate of exponential growth (Figure 1). The Japanese strain (GCJP10) typically formed long chains of 16 cells, while the Huon strain (GCHU02) formed mostly single cells. The remaining strains (GCDE08, GCDE9305 and GCSP01) formed chains of intermediate length (4–8 cells). Long-term observations on these clones in culture confirm that the observed differences in chain length are consistent between transfers and are not related to nutrient deficiency or growth stage (Blackburn et al., 1989). Furthermore, since the strains used in this study have been unambiguously identified using rRNA and RAPD signatures (Bolch et al., 1999; Bolch, unpublished data), we are confident that the single-celled strain is *G. catenatum* and not the recently described *G. nollerii* (Ellegaard and Moestrup, unpublished data).

Growth rates of different *G. catenatum* strains were variable, ranging from 0.19–0.34 day$^{-1}$ (Figure 1 and Figure 2a). In Se-replete medium, the Spanish (GCSP01) and Japanese (GCJP10) strains exhibited the lowest growth rates ($k = 0.24$ day$^{-1}$; $n = 4$, $F = 187$, $P < 0.001$). Of the Tasmanian strains, the Huon strain (GCHU02) grew more slowly ($k = 0.28$ day$^{-1}$) than the two Derwent strains,
which had comparable growth rates ($k = 0.34 \text{ day}^{-1}; n = 4, F = 40, P < 0.001$). Strain GCJP10 showed the greatest decrease (from 0.24 to 0.19 day$^{-1}$) in growth in Se-deplete medium. However, Se deficiency had a minimal effect on growth of the other strains (Figure 1a). Intraspecific variability in exponential growth rates of *G. catenatum* strains in the presence and absence of Se resulted in no significant difference in growth between all strains as a result of Se deficiency ($n = 4, F = 3.2, P = 0.084$).

Strains GCDE08 and GCSP01 showed the highest cell yields when grown in Se-replete medium (~6.0 × 10$^5$ cells l$^{-1}$; Figure 2b). Cell yields of GCJP10 and GCHU02 were slightly lower (4.09 × 10$^5$ cells l$^{-1}$ and 4.65 × 10$^5$ cells l$^{-1}$, respectively), while those of strain GCDE9305 were lowest (1.16 × 10$^5$ cells l$^{-1}$). Chlorophyll *a* concentrations showed a similar pattern (Figure 2c), with highest levels found in GCDE08 (74 µg l$^{-1}$), intermediate levels in GCSP01, GCJP10 and GCHU02 (22–25 µg l$^{-1}$) and lowest levels in GCDE9305 (11 µg l$^{-1}$). All strains

**Fig. 2.** (a) Exponential growth rates ($\mu$), (b) cell yields, (c) Chl *a* biomass and (d) Chl *a* quota of *G. catenatum* strains after ~50 days (>eight generations) in nutrient-enriched sea water medium with 10$^{-9}$ M selenium added as selenite (H$_2$SeO$_3$) or no added selenium (–Se); $n = 4$, error bars = SE.
except GCDE9305 exhibited reduced yields when cultured in the absence of selenium (Figures 2b and 2c; cell abundance: \( n = 4, F = 13, P = 0.001; \) Chl \( a: n = 4, F = 15, P < 0.001 \)). The Spanish, Japanese and Tasmanian Huon strains (GCSPO1, GCJP10 and GCHU02, respectively) showed the greatest decrease (\( \approx 80\% \)) in cell abundance and Chl \( a \) after three transfers in Se-deficient medium. Cell yields decreased from \( \approx 5.99 \times 10^5 \) to \( 1.18 \times 10^5 \) cells l\(^{-1} \), \( 4.09 \times 10^5 \) to \( 7.67 \times 10^4 \) cells l\(^{-1} \) and \( 4.65 \times 10^5 \) to \( 9.7 \times 10^4 \) cells l\(^{-1} \) (respectively) with no addition of selenium. Similarly, Chl \( a \) decreased from 23.1 to 4.6 µg l\(^{-1} \) in GCSPO1, from 22.3 to 9.3 µg l\(^{-1} \) in GCJP10 and 24.5 to 10.2 µg l\(^{-1} \) in GCHU02. In contrast, there was only a 10\% reduction in cell yield in the Tasmanian Derwent strain (GCDE08: from \( 6.09 \times 10^5 \) to \( 5.47 \times 10^5 \) cells l\(^{-1} \)) and a 45\% decrease in Chl \( a \) (from 74.2 to 41.3 µg l\(^{-1} \)) in Se-deficient medium. The other Tasmanian Derwent strain (GCDE9305) showed no difference in cell yields or Chl \( a \) in Se-replete and Se-deficient medium (Figures 2b and 2c).

Planned comparisons showed that the greatest variability in cell yield was between strains isolated from the Derwent Estuary at different times (GCDE08 and GCDE9305; \( n = 4, F = 23, P < 0.001 \)), and not between Tasmanian strains from adjacent areas (\( n = 4, F = 0.03, P = 0.96 \)) or between Tasmanian and Japanese or Spanish populations (\( n = 4, F = 0.21, P = 0.65 \)). Similarly, for Chl \( a \), the greatest variability between strains was between those isolated from the Derwent Estuary in different years (GCDE08 and GCDE9305; \( n = 4, F = 23, P < 0.001 \)), but also between Tasmanian strains and those isolated from Japanese or Spanish populations (\( n = 4, F = 15, P = 0.001 \)).

Chl \( a \) quotas were different between strains (\( n = 4, F = 5.7, P < 0.01 \)), with the Spanish strain having a lower quota (\( \approx 40 \) pg) compared with Tasmanian and Japanese strains (\( \approx 85–160 \) pg). However, they did not change as a result of selenium deficiency (\( n = 4, F = 0.19, P = 0.67 \)). Interpretation of these results is confounded by the absence of cell volume data which would distinguish between a change in cell size or cellular Chl \( a:C \).

**Discussion**

**Selenium requirement of G.catenatum**

This is the first study which has attempted to characterize the response of different strains of *G.catenatum* to the micro-nutrient, selenium. Previous physiological investigations on other phytoplankton species [e.g. the dinoflagellate *Peridinium cinctum* fa. *westii* (Lindström and Rodhe, 1978); diatom *Thalassiosira pseudonana* (Price *et al*., 1987); dinoflagellate *Gymnodinium sanguineum*, prymnesiophyte *Chrysochromulina polylepis*, diatom *Chaetoceros gracilis* and others (Harrison *et al*., 1988)] have all examined selenium requirements using only a single clonal isolate and then made comparisons with other species. However, proper evaluation of interspecific differences is only possible when the magnitude of within-species variation is defined (Wood and Leatham, 1992).

The present investigation demonstrated a variable requirement for selenium between *G.catenatum* strains, as shown by the three different responses to Se deficiency: (i) a reduction of exponential growth rate and biomass (cell density...
and Chl $a$) yields (GCJP10); (ii) a reduction in biomass yields only (GCDE08, GCSP01 and GCHU02); and (iii) no reduction in growth or biomass yields (GCDE9305). Due to the variability between strains, the ANOVA detected no effect on $G$.catenatum growth as a result of Se deficiency. There was, however, a clear reduction in $G$.catenatum biomass yields in Se-deficient medium, with the Japanese (GCJP10) and one Tasmanian Derwent strain (GCDE08) showing a more marked response to Se deficiency. Such effects of Se on $G$.catenatum cellular processes may be important during bloom development, when biomass accumulation (through favourable meteorological and hydrological conditions) could create a scenario where $G$.catenatum cell yield (but not growth rate) may be Se-limited.

The metabolic function of selenium in phytoplankton involves the selenoenzyme, glutathione peroxidase, which protects cells against the harmful effects of oxidants such as hydrogen peroxide (Price and Harrison, 1988). Gennity et al. have also demonstrated that the amino acid, selenocysteine, can degrade peroxides non-enzymatically (Gennity et al., 1985). Furthermore, selenium may be important in cell division processes and in maintaining internal membrane integrity (Doucette et al., 1987). Selenium cannot be replaced with any other trace metals, at least for the diatom Thalassiosira pseudonana (Price and Harrison, 1988), and as such is considered essential. There is an increasing body of evidence which indicates that the addition of nanomolar quantities of selenium can stimulate or influence growth of a wide variety of marine and freshwater phytoplankton, including harmful species [see (Doblin et al., 1999a) Table III]. There does not appear to be a greater requirement for selenium amongst neritic compared with oceanic species, although further testing with a larger number of species from different algal classes is necessary to confirm this (Harrison et al., 1988).

The concentration of selenium added to cultures (1.0 nM) was approximately equal to levels commonly found in coastal waters, including Tasmania [0.4–1.8 nM l$^{-1}$ (Wrench and Measures, 1982; Cutter and Bruland, 1984; Takayanagi and Wong, 1984; Doblin et al., 1999a)]. In previous selenium bioassays with $G$.catenatum, we have observed no change in bacterial abundance in cultures with 0–10$^{-7}$ M added selenium, even with addition of natural dissolved organic matter (Doblin et al., 1999b), indicating that the decrease in $G$.catenatum growth in the absence of selenium is unlikely to be related to a selenium–bacteria interaction.

The phenotypic variation in selenium requirement of $G$.catenatum found in this study adds to previous reports on strain variation for other characters within and between $G$.catenatum populations. For example, Oshima et al. compared the relative abundances of saxitoxin derivatives in a number of $G$.catenatum strains, including three used in this study (GCDE08, GCHU02 and GCSP01), and found that there were small but consistent differences between Tasmanian, Japanese and Spanish populations (Oshima et al., 1993). Furthermore, Tasmanian strains were unique in their production of deoxy-decarbamoyl-STX toxins, suggesting that this could be used as a biochemical marker to distinguish different geographic populations. Bolch et al., using RAPD-PCR (Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction) analyses, found that the Japan-
ese (GCJP10) and Spanish (GCSP01) strains have approximately equal genetic similarity compared with the Tasmanian Derwent (GCDE08, GCDE9305) strains (Bolch et al., 1999). These authors also identified a greater genetic distance between Tasmanian Huon and Derwent strains compared with Tasmanian Huon and Japanese/Spanish strains, indicating relatively high genetic diversity within a small area.

Comparisons of the Se requirement between strains in this study revealed that there was an equivalent or greater amount of phenotypic variation between strains isolated from the Derwent Estuary, Tasmania, on different dates compared with the variation between strains isolated from adjacent areas in Tasmania, and between Tasmanian and Spanish or Japanese strains. Other investigations have shown both an unexpectedly small amount of genetic variation between micro-algal clones from widely separated locations (Brand et al., 1981b; Hayhome et al., 1989; Chinain et al., 1997) and a high degree of genetic variation between strains from the same or adjacent areas (Gallagher, 1980; Bolch et al., 1999). Other phytoplankton species also exhibit intraspecific variability in their nutrient requirements. For example, selected strains (Narragansett Bay and Osaka Bay) of the ichthyotoxic raphidophyte, *Heterosigma akashiwo*, cannot synthesize alkaline phosphatase required for organic P assimilation (Nakamura, 1985). This suggests that considerable phenotypic and genotypic variability exists within and between populations (on different temporal and spatial scales), highlighting the need for testing multiple strains in ecophysiological investigations.

The reasons behind the observed intraspecific differences in *G. catenatum* selenium requirement remain to be determined. It is also unclear why the GCDE08 strain showed a stronger Se requirement in a previous study (with a greater reduction in growth in Se-deplete medium), despite being cultured under identical conditions (Doblin et al., 1999a). The latter may be related to the use of different sea water batches (with potentially different background selenium concentration) for the two experiments. Such problems would be avoided by using a chemically defined medium, but despite several attempts at growing *G. catenatum* in Aquil (Price et al., 1988) and in ESAW (Harrison et al., 1980), we have not been successful. Although it is likely that populations from widely separated locations have evolved different physiological and nutritional requirements as a result of natural selection under different ‘selenium regimes’, the variability in selenium requirement between *G. catenatum* strains demonstrated in this study suggests that these selection pressures may operate over much smaller spatial and temporal scales. Furthermore, it is not clear how rapidly the selenium requirement of a population can shift as a result of changing selenium bioavailability, but populations with higher genetic (and hence phenotypic) diversity may have an advantage over less diverse populations. Alternatively, selenium availability may play a minor role in structuring *G. catenatum* populations—the demonstrated diversity may be due to chance (such that all variations in selenium requirement are possible). Information about the variability in selenium concentrations in coastal waters, and assessment of the selenium requirement of a larger number of strains, will provide a better understanding of the role of such micro-nutrients in determining *G. catenatum* population structure.
This study has demonstrated that the toxic dinoflagellate, *G. catenatum*, has a variable selenium requirement and that populations of this species exhibit high phenotypic diversity in their response to this trace element. Subtle strain variability in micro-nutrient requirements of phytoplankton species, as documented in this work, may therefore provide a partial explanation for the perplexing complexity of bloom patterns exhibited by the same taxon in different geographic regions.

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