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Raphael Kudela, Alice Roberts, and Meredith Armstrong.

Ocean Sciences Department, University of California Santa Cruz. 1156 High Street, Santa Cruz, CA 95064

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Laboratory Analyses of Nutrient Stress and Toxin Accumulation in *Pseudo-nitzschia* Species from Monterey Bay, California Raphael Kudela¹, Alice Roberts¹, and Meredith Armstrong¹. ¹Ocean Sciences Department, University of California Santa Cruz. 1156 High Street, Santa Cruz, CA 95064 kudela@ucsc.edu

Abstract. Here we present results from laboratory experiments using *Pseudo-nitzschia* spp. isolated from Monterey Bay, California grown under constant temperature and irradiance. We demonstrate substantial clonal variability and variable toxicity depending on harvest time and nutrients. We also show that Si limitation can directly affect photosynthetic performance and looks functionally like iron and nitrogen limitation as assessed by variable fluorescence (decreasing variable fluorescence with increasing stress). This response negatively correlates with domoic acid accumulation in batch and chemostat experiments.

Introduction. There have been at least two recent occurrences (1998 and 2000) of toxigenic strains of *Pseudo-nitzschia* spp. in Monterey Bay, California (Scholin et al., 2000). Studies conducted on these bloom events have shown evidence of trophic transfer of the phycotoxin domoic acid (DA) to marine seabirds and mammals (Gulland et al., 1999; Scholin et al., 2000). Despite the frequent occurrence of blooms and the large number of studies (both field and laboratory), there is still no conclusive link to a specific “trigger” of toxin production. This may be in part because of the enormous variability in the ambient oceanographic conditions but also in the range of physiological responses evident from natural isolates of *Pseudo-nitzschia* spp. Here we demonstrate that the natural range of responses to nutrient stressors and effects of culturing methodology may account for much of the observed variability in laboratory and field analyses.

Isolate	Clone ID	Isolation Date	Max Biomass (cells/mL)	Mean growth rate (d ⁻¹)	S.D.
<i>P. australis</i>	Au-211M	Feb-02	15727	0.76	0.26
<i>P. australis</i>	Au-211J	Feb-02	16167	0.66	0.05
<i>P. australis</i>	Au-211K	Feb-02	16991	0.33	0.18
<i>P. australis</i>	Au-211D	Feb-02	15287	0.61	0.16
<i>P. australis</i>	Au-211B	Feb-02	18146	0.54	0.07
<i>P. australis</i>	Au-211L	Feb-02	17321	0.40	0.24
<i>P. australis</i>	Au-1004V	Oct-01	19630	0.60	0.18
<i>P. australis</i>	Au-211A	Feb-02	13198	0.26	0.25
<i>P. australis</i>	Au-211C	Feb-02	17101	0.47	0.10
<i>P. australis</i>	Au-1004W	Oct-01	17266	0.58	0.14
<i>P. australis</i>	Au-1004A	Oct-01	17156	0.40	0.25
<i>P. australis</i>	Au-1004F	Oct-01	18036	0.46	0.16
<i>P. multiseriis</i>	Mu-411A	Apr-01	27436	0.60	0.23
<i>P. multiseriis</i>	Mu-411I	Apr-01	25567	0.35	0.13
<i>P. multiseriis</i>	Mu-411O	Apr-01	20949	0.48	0.03
<i>P. multiseriis</i>	Mu-411P	Apr-01	21994	0.59	0.12
<i>P. multiseriis</i>	Mu-420B	Apr-01	26776	0.48	0.24

Methods and Materials. Data presented represent growth of *Pseudo-nitzschia* spp. in f/2 medium enriched with silicic acid (batch cultures), Si-limited medium (continuous cultures) and synthetic seawater medium (semi-continuous batch cultures). For batch culture experiments, seventeen toxigenic strains of *Pseudo-nitzschia* (*P. multiseriis* and *P. australis*) isolated from Monterey Bay, CA in 2001/2002 were made available courtesy of M. Hughes (UCSC). Clones were grown in one liter of medium with f/2 enrichment, and were

maintained under 12 h light (ca. 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 15 °C. Cultures were monitored daily for cell density using *in-vivo* chl *a* fluorescence. Successive transfers were made in exponential growth. For testing DA accumulation in two strains of Mu-411P, eight transfers in log phase were made, and at the final transfer half the cells were harvested for DA content. The remainder was kept in the same conditions for 48 hours. The experiment was then terminated, and DA content was measured on these cells in early stationary phase. Cell numbers were calculated from a regression of *in-vivo* chl *a* fluorescence measurements and cell counts conducted by microscopy.

Chemostat runs were carried out using a uni-algal non-axenic strain of *P. australis* (Au-221A) and *P. multiseriis* (Mu-6 and Mu-411P). 750 mL plexiglas water-jacketed cylindrical chemostat vessels were maintained under 24 h light (ca. 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 15 °C, and monitored daily for cell density using fluorescence and cell counts. Dilution rates were calculated by measuring the effluent volume collected from the vessels daily. The growth media consisted of 0.2 μm filtered seawater augmented with L1 nutrient additions or f/2 nutrient additions limited by silicic acid. The chemostats were run at variable dilution rates (ca. 20-80% of μ_{max}). For analysis of harvest-date on toxin accumulation (Figure 1), one set of chemostats was harvested at 3 days steady-state (constant cell density), and the second 7 days later, using Mu-411P and Au-221A. Data in Figure 2 are for Mu-6 and Mu-411P; *P. australis* show similar trends (data not shown).

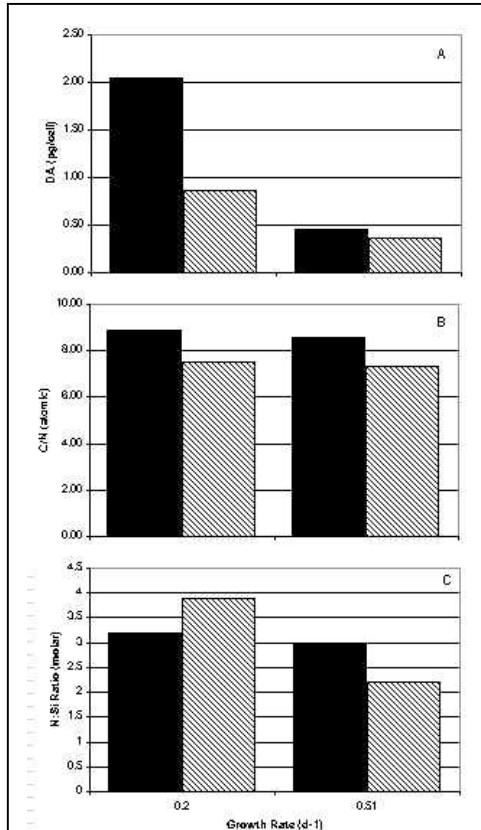


Figure 1. Biochemical composition of chemostats (Mu-411P; solid; Au-221A, striped) harvested early (3 days; solid) and late (10 days; striped) during steady-state. A: DA per cell. B: C:N composition. C: Si:N composition.

and irradiance (Table 1). Mean growth rates were similar for *P. multiseri* and *P. australis* (Table 1), although *P. australis* maximal cell densities were typically lower.

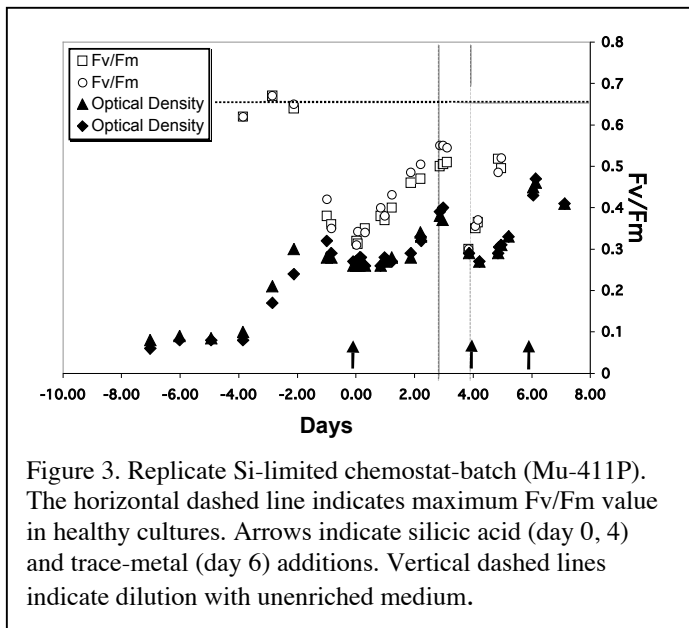
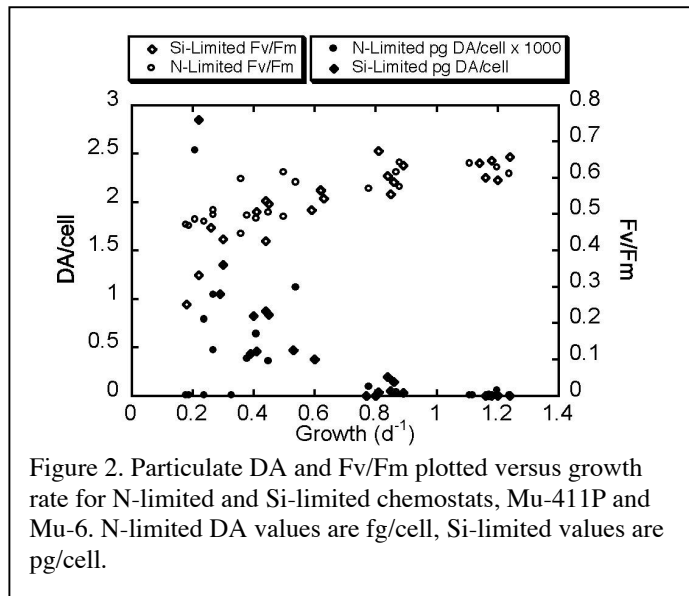
Domoic acid accumulation: Cellular DA content in semi-continuous cultures of *P. multiseri* after eight consecutive transfers show that Si-limitation can trigger up to a 30-fold increase in particulate DA concentrations from late exponential to early stationary phase (0.1-0.3 pg cell vs. 2.5-3.2 pg-cell). Strain and species variability is also pronounced, with typical early stationary particulate DA values ranging from < 0.1 to > 15 pg-cell for isolates grown under the same conditions. To determine whether the observed increase in DA content per cell in the semi-continuous batch transfers was a function of growth rate or acclimation, we conducted a series of chemostat experiments at low (0.2 d⁻¹) and high (0.51 d⁻¹) growth rates. By harvesting “early” (3 days in steady-state) vs. “late” (10 days), particulate DA again changed as a function of growth rate, with up to a 3-fold decrease in toxin accumulation (Figure 1). The chemostats exhibited essentially constant cell density, pigment per cell (not shown), and elemental composition (Figure 1). The dramatic changes in DA suggest that physiological adaptation (i.e. toxin production) takes much longer to stabilize than commonly used estimators of steady-state, such as density and biochemical composition (Figure 1).

Variable Fluorescence: Si-limited and N-limited chemostats (*P. multiseri* clone Mu-6) were maintained under a range of nutrient-limited growth conditions (Figure 2). Variable fluorescence was measured after harvesting, again using the criteria of 3 days with cell densities within one standard deviation, and showed a good relationship (negative correlation) for both growth rate and toxin accumulation under both N and Si limitation. Although N-limited cultures were ca. 1000x lower in DA accumulation, the general pattern (increasing accumulation with decreasing growth rates) was similar to Si-limitation. These results suggest that variable fluorescence could be a useful indicator for toxin accumulation. It also strongly suggests that Si-limitation of diatoms impacts photosynthetic performance, possibly due to regulatory feedback mechanisms (e.g. Lippemeier et al., 1998) or through inhibition of a carbon-concentrating mechanism (e.g. Milligan and Morel, 2002).

For chemostat/batch experiment presented in Figure 3, replicate semi-continuous cultures of *Pseudo-nitzschia multiseri* (Mu-411P) were maintained in exponential growth phase over a 29 day period in Guillard’s f/2 enrichment medium with 50 uM silicic acid (same as for the chemostats), but with no nutrient limitation (growth was controlled by washout). After reaching steady state, an aliquot was harvested for DA, variable fluorescence, and other parameters (below), and the pumps were shut off, allowing the cultures to enter batch (Si-limited) conditions. A subset of the sample was harvested daily for variable fluorescence, DA, and cell density. Cell numbers were determined using optical density (800 nm) with a subset of cell counts to validate the optical density measurements. Successive additions (4, 8, and 10 days from entering batch mode) of silicic acid (days 4 and 8) or f/2 trace-metal stock (day 10) were added to assess nutrient stress.

Samples collected for most experiments also include pigments, macronutrients, biogenic silica, and particulate CHN. Because of detection issues, dissolved DA was not available for every sample. When measured, dissolved concentrations were typically less than 1% of the total DA in chemostats; in batch cultures, dissolved DA was typically less than 10% of the total, but was more variable. For this contribution, particulate DA values are reported. Particulate DA concentrations were determined using the FMOC-HPLC method described by Pocklington et al. (1990). Variable fluorescence (Fv/Fm) may be considered an indicator of cell health, or impairment of the photosystem II system, and is a direct estimate of photosynthetic competency (Schreiber, 1986; Kolber et al., 1998). Estimates of variable fluorescence were conducted using a Walz PAM 101/102/103 instrument, and validated with DCMU-excitation fluorescence. Samples were dark-adapted >30 min. at 15°C for Fo estimates (Schreiber, 1986; Lippemeier et al., 1998).

Results and Discussion. Clonal Variability: In this study, 17 clonal isolates from Monterey Bay were maintained under identical growth conditions. There is a high degree of variability in growth of different clones of *Pseudo-nitzschia*, isolated from the same location on the same day, and reared under identical temperature



To determine whether Si-limitation was really affecting photosynthetic performance, duplicate chemostats of *P. multiseriis* clone Mu-6 were maintained until steady state was achieved, and then grown as batch cultures (Figure 3). The cells were allowed to become Si-limited, and were then pulsed (2x) with silicic acid additions (ca. 10 μ M), demonstrating that Si-limited Fv/Fm variability is inducible and reversible. Trace-metal additions (Figure 3) showed no response in any measured parameter, indicating that, although the Fv/Fm signal was similar to the response expected from iron limitation (Behrenfeld et al., 1996), there was no apparent metal stress.

Conclusions. In this study we demonstrated that there is variability in growth among different clones and species of *Pseudo-nitzschia*, isolated from the same area and grown under identical conditions. Si limitation correlates with increased DA accumulation in batch, semi-continuous, and continuous cultures, as expected. There was a surprising amount of variability in DA accumulation, however, depending on exactly when the cells were harvested, which was not well correlated with cell density or biochemical composition. Variable fluorescence is a good indicator of nutrient stress, and presumably DA production, with Fv/Fm declining as DA accumulation increases. We suggest that cellular toxicity may largely be dependent on the culturing methods, and may take much longer to stabilize relative to other physiological parameters. This highlights the importance of consistent methodology and careful intercomparisons when analyzing different strains and experiments.

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