LIMNOLOGY
and
OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 7, 2009, 21–32
© 2009, by the American Society of Limnology and Oceanography, Inc.

A sea-going continuous culture system for investigating phytoplankton community response to macro- and micro-nutrient manipulations

Lisa D. Pickell[1], Mark L. Wells[1], Charles G. Trick[2] and William P. Cochlan[3]*
[1]Darling Marine Center, University of Maine, 193 Clarks Cove Rd, Walpole, ME 04573, USA
[2]Schulich School of Medicine and Dentistry, and Departments of Biology and Microbiology & Immunology, University of Western Ontario, London, Ontario, N6A5B7, Canada
[3]Romberg Tiburon Center for Environmental Studies, San Francisco State University, 3152 Paradise Drive, Tiburon, CA, 94920-1205, USA

Abstract

Continuous cultures can provide refined insights on the response of phytoplankton communities to small changes in nutrient flux, however are logistically more challenging to perform at sea than batch cultures. Here we describe the design and successful testing of a new continuous culture system for shipboard experiments using natural phytoplankton communities. Using this system, we studied the effects of nitrate amendments in coastal waters of the Pacific Northwest, and low-level iron additions in High Nitrate, Low Chlorophyll (HNLC) waters of the subarctic Pacific. With nitrate amendments the coastal phytoplankton community showed proportional increases in chlorophyll biomass and appeared to achieve dynamic steady state as biomass, nutrient drawdown, and photo-physiology stabilized after 4 d. In contrast, biomass did not reach steady state with iron amendment in the 10-d HNLC experiment, however a major transition in dominant phytoplankton from small autotrophic flagellates to the toxigenic diatom *Pseudo-nitzschia* was observed. This new continuous culture design demonstrated high precision in flow rates, good mixing within culture vessels, and was simple to operate at sea. This system provides an effective platform for investigating small changes in macro- and micro-nutrient flux on the growth of individual phytoplankton species and, in turn, the trajectory of planktonic ecosystems.

Introduction

Our current understanding of the interactions among phytoplankton and their natural environment stems largely from deck-board batch incubation experiments using communities collected from surface waters. While logistically simple, the batch culture approach creates continually changing chemical conditions over time that can generate many variable stressors on growth rates and competitive interactions (Rhee 1980). Continuous cultures provide a constant growth environment, where cells are maintained in exponential growth phase independent of time and are more useful for investigating the effects of one environmental parameter while holding all others constant (Herbert et al. 1956; Rhee 1980). Additionally, in a natural community study, continuous cultures can provide an effective means to study the complex adaptive mechanisms of phytoplankton and how they interact to shape the community composition in response to the limiting environmental stressor (Dunstan and Menzel 1971; Thomas et al. 1980; Hutchins et al. 2003; Hare et al. 2005, 2007a). Until recently however, the considerable challenges of conducting continuous culture experiments onboard research vessels have prevented their application at sea.

Continuous cultures are highly controlled systems that use simple mathematical relationships to study the biochemical and physiological response of organisms to a single limiting factor. Monod (1950) and Novick and Szilard (1950) were the first to apply growth relationships to these well-defined systems for

*Corresponding author: E-mail: lisa.pickell@umit.maine.edu
Tel: 1-207-563-3146; fax: 1-207-563-3119

Acknowledgments

We thank the officers and crew of the R/V *Atlantis* (ECOHAB-PNW III, 2004) and R/V *Thomas G. Thompson* (PAPA-SEEDS, 2006) for their assistance during these field studies. We are grateful to Machine Services at The University of Western Ontario for their engineering and design expertise. For their help in the analysis and conduction of these experiments, we thank J. Herndon, B. Bill, R. Radan and M. Auro (RTC/SFSU), E. Lessard, M. Foy and M. Bernhardt (UW), B. Beall, B. Bjornsson and L. McClintock (UWO), A. Drzewianowski, K. Hardy and E. Roy (UMaine) and P. Hughes (UCSC). This work was funded by the NSF ECOHAB project OCE-0234587 and DOE OCE-0241752 to MLW, CGT and WPC. This is ECOHAB publication #302 and ECOHAB-PNW publication #24.

cultures in steady state under nutrient-limited conditions. In a continuous culture, new medium is continuously pumped into a sealed culture vessel at a constant flow rate, F (mL d^{-1}), establishing an equal volume outflow such that a constant volume, V (mL) is maintained in the culture vessel. The resultant dilution rate, D (d^{-1}) is defined as:

$$D = F/V \quad (1)$$

The dilution rate controls the limiting nutrient supply, which along with the specific growth rate of the organism determines the biomass concentration in the culture. The net rate of change in biomass, X (cells mL^{-1} or Chlorophyll *a* mL^{-1}) over time therefore, is the difference between specific growth rate, μ (d^{-1}) and dilution rate, D (d^{-1}), whereby:

$$dX/dt = X(\mu - D) \text{ (change = growth – output)} \quad (2)$$

If growth and dilution rates are balanced, resulting in a constant biomass over time (i.e., $dX/dt = 0$), the system is in steady state and

$$\mu = D \quad (3)$$

In this case, the continuous culture is considered a chemostat, where growth rate is equal to the dilution rate set by the experimenter (Herbert et al. 1956; Veldkamp 1976; Rhee 1980; Rhee et al. 1981). When growth is nutrient limited (in contrast to light or temperature), biomass in the culture is governed by the concentration of limiting nutrient (S), consistent with Liebig limitation, whereas the physiological adjustment of the cells to the supply of limiting nutrient reflects the degree of imposed Blackman limitation (MacIntyre and Cullen 2005). At steady state, the concentration of limiting nutrient coming into the vessel (inflow) should equal the sum of the outflow plus consumption concentrations, such that there is no change in limiting nutrient concentration with time (i.e., $dS/dt = 0$) (Herbert et al. 1956; Veldkamp 1976; Rhee 1980; Bazin 1981).

A chemostat is one type of continuous culture, and the two terms are not equivalent (Jannasch 1974). As described above, chemostats are strictly controlled systems where biomass reaches steady levels determined by a limiting substrate, and all other conditions are held constant independent of time. Conditions are less uniform in other types of continuous cultures, for instance a cyclostat, in which cultures experience a 24-h light/dark photocycle, or continuous cultures using a natural community assemblage, where effects such as microzooplankton grazing and viral lysis can influence growth and species selection (Rhee 1980; Rhee et al. 1981; Hutchins et al. 2003; Hare et al. 2007a). Though a cyclostat is not a 'true' chemostat, steady state can be achieved if the photocycle is constant and growth rate is integrated (μ_T) over the entire photoperiod (T) (Jannasch 1974). Similarly, balanced growth in a mixed community continuous culture can be achieved between dilution rate and net growth rate of the whole community, rather than specific growth rate of one species. In these cases, strict application of chemostat mathematics becomes problematic, and growth is considered to be in dynamic, rather than simple, equilibrium where continuous changes are occurring yet relatively uniform conditions prevail (MacIntyre and Cullen 2005).

Natural surface waters can display properties of both batch and continuous culture systems. Whereas blooms associated with spring transitions in coastal temperate and upwelling areas may be more analogous to batch-type cultures (i.e., large pulses of nutrients), conditions in oceanic subtropical and tropical environments may be more typical of continuous cultures, where low-level nutrient inputs balanced by loss (i.e., grazing), maintain a fairly constant and low biomass (Jannasch 1974; Chavez et al. 1991; Frost and Franzen 1992; Hutchins et al. 2003), however see Riser and Johnson (2008). Hutchins et al. (2003) were the first to apply shipboard continuous cultures in the field and argue that these systems are a more effective analogue to natural processes for determining nutrient-limited growth and community response. They termed their system an "Ecostat," in that a natural mixed community is used and experiences a 24-h photocycle, and promote its use for the study of nutrient effects on phytoplankton species succession and dominance (Hutchins et al. 2003; Hare et al. 2005, 2007a). For instance, based on competitive displacement, species well adapted to the imposed growth conditions will flourish, whereas less adapted organisms either will be restricted to low abundance or be flushed entirely from the system.

Here we describe the development and application of an improved sea-going Ecostat that addresses some of the logistical problems in the device presented by Hutchins et al. (2003). In particular, we have adopted a substantially modified approach for achieving constant flow rates and well-mixed cultures, parameters that are essential to successful continuous culture operations. Our goals for this method development were to expand on the pioneering efforts of Hutchins and colleagues (2003), and to assess our modified Ecostat capabilities for studying macro- and micro-nutrient (Fe) limiting effects on natural planktonic communities in coastal and oceanic waters.

Materials and procedures

Continuous culture apparatus and operation—A schematic of the continuous culture apparatus and culture set-up (Fig. 1A and 1B), illustrates the specialized mechanics developed for maintenance of continuous culturing criteria during outdoor, shipboard operations. Our system consists of 14, 2-L custom cylindrical culture vessels supported in cradles that are mechanically rotated ~30° left then right by a 1/6 horse-power motorized arm (Lesson Electric motor) that extends down the center of the incubator. Dye tests indicate that the rocking cycle, on a frequency of ~1 s^{-1}, maintains well-mixed, homogeneous conditions within the culture vessels, a conclusion supported by observations of cell biomass during seagoing tests. Culture vessels are fabricated of clear polycarbonate with

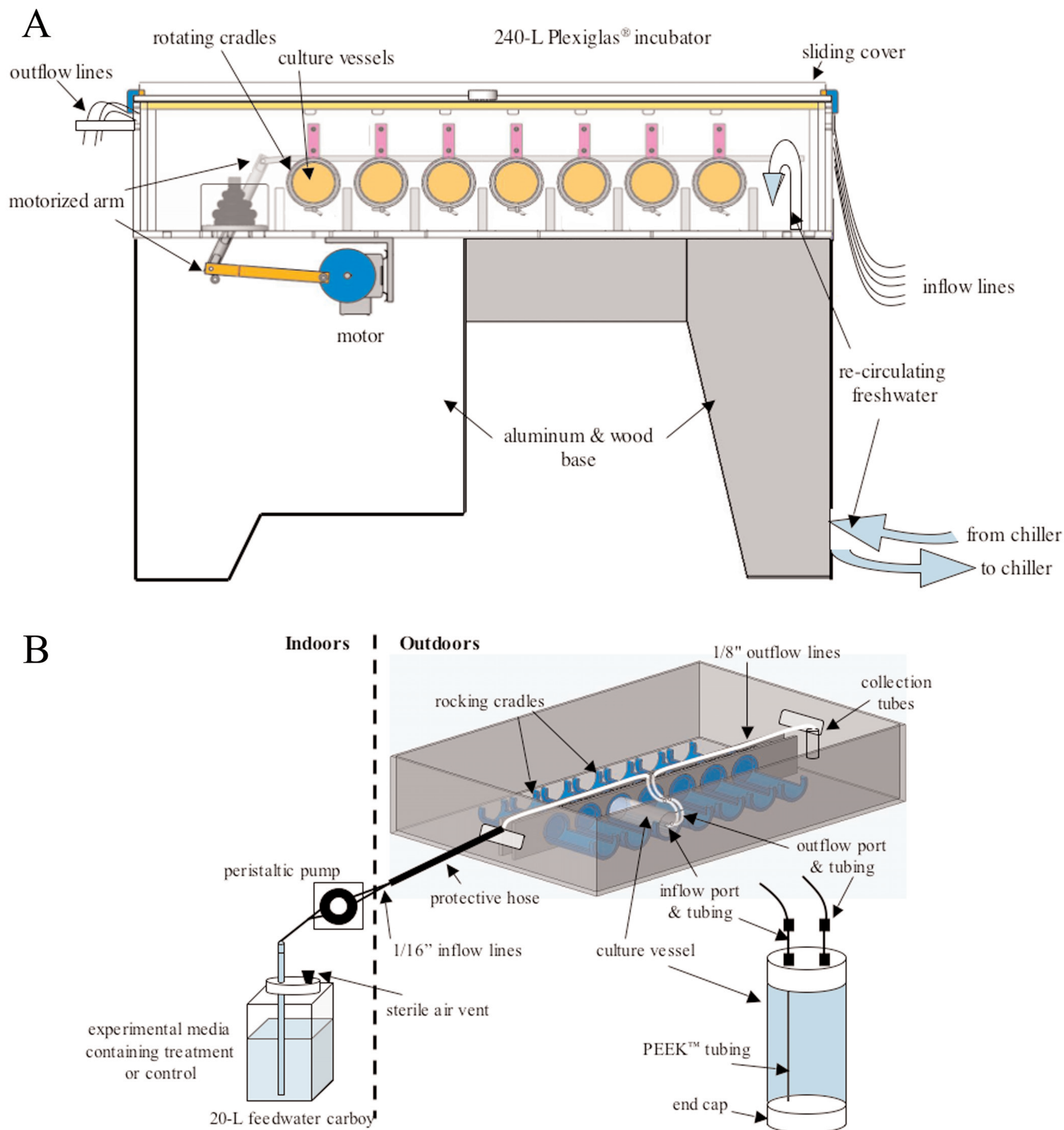

Fig. 1. Design and set-up schematics of the sea-going continuous culture system. (A) Side-view of apparatus; transparent 240 L Plexiglas® incubator mounted on a raised base constructed of aluminum and wood. Within the incubator, 14 rotating cradles (7 on either side of motorized arm) securely hold 2-L culture vessels in a temperature-controlled freshwater bath. The bath is recirculated by centrifugal pump through a chiller set-up in a protected area. (B) Incubation and culture vessel set-up. The 20-L feed-water carboys containing the amended filtered seawater are housed indoors where an adjustable 14-channel peristaltic pump continuously delivers new media to the attached duplicate culture vessels through clean 1/16 inch inflow lines. All tubing is bundled within a protective hose running across deck to the incubator. Outflow from the culture vessels is through clean 1/8 inch tubing directed out the opposite side of the Plexiglas® incubator, where samples are collected or waste flows out from each line. The inset illustrates culture vessel set-up containing the natural community; one end cap is fitted with the in- and outflow ports, where PEEK tubing extends the inflow media stream to the bottom of the culture vessel. O-rings in the end caps seal tightly to establish positive pressure and maintain constant volume within the bottles.

polyethylene end-caps sealed airtight with silicone O-rings (A.C. DePuydt); one end-cap is fitted with both the inflow and outflow ports (Fig. 1B). Viton O-rings used during initial experiments proved to be toxic to phytoplankton. Polyetheretherketone (PEEK) tubing (1/16-inch outer diameter (OD); Upchurch Scientific) extends the inflow stream to the opposite end of the culture vessel to ensure nutrients are further homogenized within the bottles and the constant inflow of new media establishes an equal volume outflow through the other port via positive pressure. The 14 cradle positions allow for the simultaneous operation of duplicate controls and six duplicate treatments per experimental run.

The bottles, cradles, and part of the rocking assembly are contained within a transparent, enclosed ~240 L Plexiglas® incubator mounted on a raised base constructed of aluminum and wood (Fig. 1A). The sliding Plexiglas® cover rides on polyethylene tracks, permitting access to either one or the other bank of culture vessels at a time (7 vessels on each side). Because uniform temperature is important for successful culturing, flowing seawater is not ideal for temperature control as ship tracks often cross frontal zones having significant temperature fluctuations. Additionally, the metallic parts within the incubator would be subject to rapid degradation if seawater was used. Therefore instead, a recirculating freshwater bath was maintained at ambient sea surface temperatures using a 1/2 HP Aquarium Chiller (GenX C-500; Pacific Coast Imports) and circulated at ~70 L min^{-1} from chiller to incubator through thick fiberglass-reinforced plastic hoses using a magnetically driven centrifugal pump (Iwaki). The chiller and pump are located within 15 m of the incubator, housed within the protective superstructure of the research vessel. The main cradle support in the center of the incubator is perforated with large openings to facilitate the circulation of incubator water, and small articulating paddles at the base of each cradle serve to circulate the water clockwise as the cradles rock.

During operation, the incubator is secured on a suitable deck (often aft main deck) with the main cradle support orientated fore and aft to minimize incubator water loss due to the ship's roll. In addition, the incubator is positioned to minimize shading from the ship's superstructure and cranes, and to ensure maximum and equal irradiance reaches each culture vessel. We typically locate duplicate treatments and controls on either side of the main cradle support in a further effort to normalize variations in light conditions. As the incubator is housed on deck, the cultures experience the natural light/dark photocycle.

Culture media used as the feed-water, in this case filtered (0.2 µm) ambient seawater, is stored in 20-L polycarbonate reservoir carboys and pumped continuously to the culture vessels through 1/16-inch OD Teflon perfluoroalkoxyalkane (PFA) high-purity (HP) tubing (Upchurch) (one for each of the 14 culture vessels) (Fig. 1B). The 14 tubing channels are bundled within an industrial strength garden hose for protection and run across the deck under custom-fabricated wood covers. The reservoir carboys containing the control and various treatments are maintained indoors in the dark, where media is pumped to the culture vessels using an adjustable 14-channel high precision peristaltic pump (Watson-Marlow). The continuous input of media creates positive pressure in the culture bottles, establishing an equal volume outflow that results in a constant volume at all times. Feed-water for duplicate culture vessels is drawn from a single treatment (or control) carboy using platinum-cured silicone pump tubing (Ismatec) attached directly to the Teflon PFA lines attached to each culture vessel. Each reservoir carboy lid is fitted with a sterile 0.2 µm polycarbonate syringe filter (Acrodisc) serving as a sterile air vent. Outflow from each culture vessel is carried by 1/8-inch OD Teflon PFA HP tubing to a central point outside the water bath where flow rates were measured and samples collected daily. Water temperature in the incubator was measured continuously using a data logger (Tidbit v. 2 HOBO), and external and internal light levels monitored daily with a light sensor (Biospherical Instruments) using PalmLight digital output software (PocketPC). At a dilution rate set at 0.5 d^{-1}, this system could operate for up to 10 consecutive days, however other shipboard experiments have been conducted for up to a month by periodically refilling carboys with extra filtered seawater collected at time of inoculation or by using larger (e.g., 50 L) carboys (Hutchins pers. comm.).

Experimental conditions—Seawater for the continuous culture experiments was collected while underway at ~2 knots using a trace metal (TM) clean, all-Teflon pumping system comprising a Kevlar-reinforced 1.27-cm OD polyethylene tube, and a weighted tow-fish suspended ~ 7 m outboard from the vessel to avoid contamination (see Bruland et al. 2005). Water was pumped from 5-8 m depth (approximating the 50% light penetration depth) using an air-driven, double-diaphragm pump (American Pumps) at 3-4 L min^{-1}. Water was pumped directly into a positive pressure clean room fabricated from plastic sheeting and wood framework and multiple downward-directed HEPA (class-100) filter fan modules (Envirco). All water dispensing and sample manipulations were conducted within these laminar-flow hoods. The sampling system was cleaned rigorously with 10% (v/v) TM-grade HCl acid prior to the cruise, and seawater was pumped though the system for several hours to thoroughly flush lines prior to sample collections. Shipboard dissolved iron measurements by flow injection chemiluminescent analysis (Roy et al. 2008), confirmed the sampling system was free of iron contamination. Seawater for the continuous culture feed reservoirs was filtered in-line using TM-cleaned 0.2 µm cartridge filters (Polycap) and collected into the control and six treatment carboys, which were thoroughly rinsed. Macro- or micro-nutrient amendments were made to the feed media carboys, which were then sealed and moved from the clean room to the feed station.

Unfiltered seawater containing the natural phytoplankton assemblage was used to initiate the experiments. The culture water was pumped onboard and through a 200 µm nylon

mesh (Nitex) to remove macrograzers, and homogenized in a 50-L low-density polyethylene carboy to ensure a uniform community composition before dispersing into the culture vessels. All macronutrients, when added, came from individual TM-clean stocks previously processed by ion exchange (Chelex 100, Biorad) according to Price et al. (1989/90). Following direct additions of macronutrients or metals, the culture vessels were sealed and transported under black plastic sheeting to the incubator. The inflow and outflow lines were immediately attached, the vessels laid in their respective cradles and the mixing initiated. When filled, a very small (~2 mL) air space remained in the culture vessels that did not cause foaming or excessive sloshing.

Experimental protocols—Control and treatment vessels were sampled daily (mid-morning) for determination of flow rates, concentrations of total Chl *a* biomass, macronutrients, and cellular fluorescence capacity (F_V/F_M). Flow rates were determined by measuring effluent volume over a timed interval and were corrected as needed by adjusting the individual channel pressures applied to the pump tubing to keep flow constant. Darkened 50 mL polypropylene centrifuge tubes (Falcon) were attached to the outflow lines, and all other collections were made at maximum pump speed for a short time period (<2.5% volume collected), where after sample collection, the pump was turned off for an appropriate interval to compensate for the faster pump speed used during sampling. Samples were analyzed for phytoplankton biomass as Chl *a* using nonacidification (Welschmeyer 1994) in vitro fluorometric analyses after filtration onto Whatman GF/F filters (0.7 μm nominal pore size). Samples were extracted at sea in 90% acetone for ca. 24 h in the dark at –20°C, and the fluorescence subsequently measured with a Turner Designs 10AU fluorometer calibrated with pure Chl *a* standards (Turner Designs). Unfiltered samples for dissolved inorganic nutrient analyses were collected in precleaned polypropylene tubes, and analyzed at sea for nitrate plus nitrite (NO_3^- + NO_2^-, hereafter referred to as nitrate), ortho-phosphate (PO_4^{3-}), and silicic acid ($Si[OH_4]$) with a Lachat QuikChem 8000 Flow Injection Analysis system using standard colorimetric techniques (Smith and Bogren 2001; Knepel and Bogren 2001; Wolters 2002, respectively). In vivo cellular fluorescence capacity was determined as the ratio of variable (F_V) to maximum (F_M) fluorescence, an analog of the quantum yield of PSII, measured after dark acclimation (0.5 h) using the 3-[3,4-dichlorophenyl]-1, 1-dimethylurea (DCMU) technique (Parkhill et al. 2001).

Size-fractioned Chl *a* biomass (0.7 μm, GF/F; 20.0 μm, Osmonics) was collected on the initial and final days of the incubation, along with sub-samples for epifluorescent microscopy cell counts and taxon identifications. Slides for epifluorescent analysis were prepared on-board according to Lessard and Murrell (1996), where 100-200 mL sample aliquots, fixed in a final concentration of 1% glutaraldehyde, were filtered onto 0.2 μm and 0.8 μm black polycarbonate filters (Poretics, 25-mm dia.). Samples were filtered in a dark fume hood, and dual stained with the fluorescent DNA stain 4_ 6-diamidino-2-phenylindole dihydrochloride (DAPI; 5 μg mL^{-1}) for 5 min, and the nuclei and cytoplasm stain 3,6-acridinediamine, monohydrochloride (proflavine; 2 μg mL^{-1}) for 30 s. Filters were placed on labeled glass slides with small drops of immersion oil (Resolve®) and covered with a glass cover slip. Slides were sealed with paraffin wax and stored frozen until microscope analysis ashore, where cells were counted using a Zeiss Axiovert 35 inverted epifluorescence microscope.

Assessment

Two shipboard Ecostat experiments were designed to evaluate the continuous culture system in two physically and chemically disparate environments: the effect of nitrate amendments in nitrate-limited coastal waters during early fall, and the effect of iron amendments in offshore HNLC waters at the onset of a muted spring bloom. The purpose of the coastal experiment was to establish the functionality of our system by testing whether cultures responded to nutrient amendments in a predicable and consistent way and to determine whether dynamic steady state conditions could be achieved. The objective of the iron experiment was to demonstrate the application of this method by determining the effect of continuous, low-level iron enrichment on phytoplankton biomass, and evaluate the trajectory of the phytoplankton community.

Coastal nitrate experiment—Surface seawater was collected near the mouth of the Strait of Juan de Fuca in the Pacific Northwest on 12 September 2004 (48°16.72′ N; 124° 44.33′ W). Incubator temperature was held constant at 13°C and light reduced to ~500 μmol photon m^{-2} s^{-1} (mid-day), by covering the incubator with one layer of neutral density screening. Feed-water flow to the culture vessels was not turned on for the first 24 h to enable cultures to adjust to the media and light conditions. Dilution rates (D) were set at 0.30 d^{-1} and the experiment ran for 7 d. This low dilution rate was chosen to minimize the loss (flushing) of slower growing cells from the system to allow better assessment of the relationship between nitrate supply and biomass generated. To ensure that PO_4^{3-} and $Si(OH)_4$ were in excess, concentrations of these nutrients in the reservoir feed-water (for all treatments and controls) were increased to 1.5 μM and 21.8 μM, respectively. Ambient nitrate concentrations of 4.7 μM served as the base 'control' concentration, and nitrate was added to the other two treatments to achieve initial concentrations of 9.7 μM and 14.7 μM. Iron data for these specific sampling time points are not available, but other data showed dissolved iron concentrations well in excess of 0.5 nM so these phytoplankton were not expected to be iron limited.

Total (>0.7 μm) Chl *a* biomass of the natural phytoplankton community increased as a function of increasing NO_3^- concentration, with 14.7 μM producing the greatest biomass (Fig. 2A). Chlorophyll *a* peaked by day 4 then leveled off, with slightly diminishing values through day 7 and excellent replication between duplicates. The good precision was due in

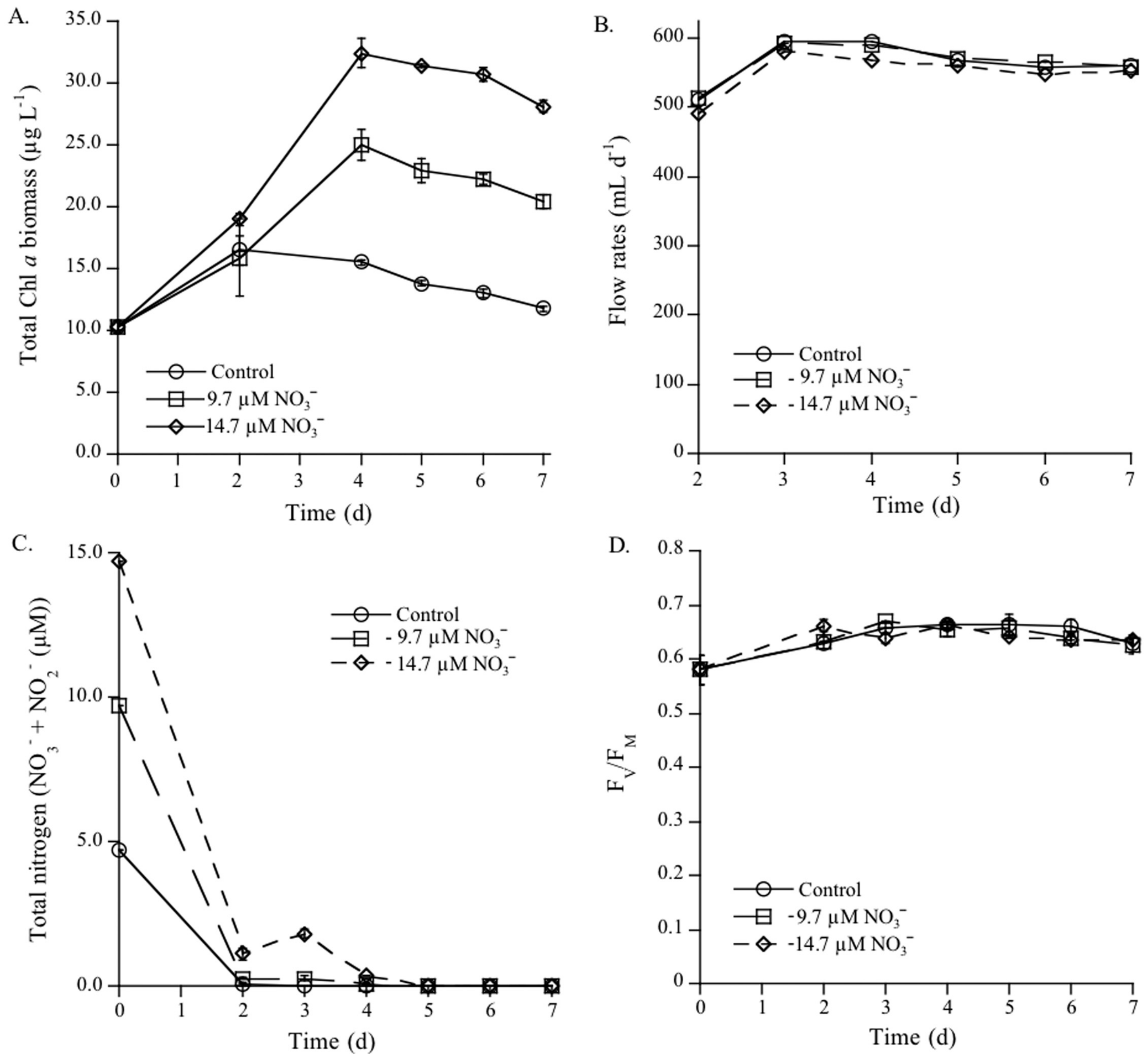

Fig. 2. (A) Mean total (> 0.7 μm) Chl *a* biomass of coastal phytoplankton grown in NO_3^- amended continuous cultures over 7 d. Error bars represent the range of duplicate (*n* = 2) treatments. (B) Mean flow rates (± range) measured via outflow from the control and treatment bottles throughout the experiment. (C) Mean total nitrogen (NO_3^- + NO_2^-) concentrations (± range) measured in the outflow medium of the cultures and (D) Mean (± range) in vivo cellular fluorescence capacity determined as the ratio of variable (F_V) to maximum (F_M) fluorescence.

large part to very constant and uniform flow rates among treatments and controls after an initial adjustment of pump speed (Fig. 2B). Nitrate concentration in the outflow media of the control was depleted to below detection limits (0.05 μM) by day 2, and remained undetectable for the rest of the experiment, whereas NO_3^- in the other treatments was not exhausted until days 4 and 5 (Fig. 2C). At low growth rates (i.e., <0.5 d^{-1}), the limiting nutrient is typically below detection in the culture vessels, and usually indicates achieved steady state (Calcott 1981), as was the case here with nitrate, whereas ortho-phosphate remained ≥ 0.25 μM in the control and treatments, well above levels that might limit growth (data not shown). Silicate depletion was more significant, particularly in the NO_3^- amended treatments where values

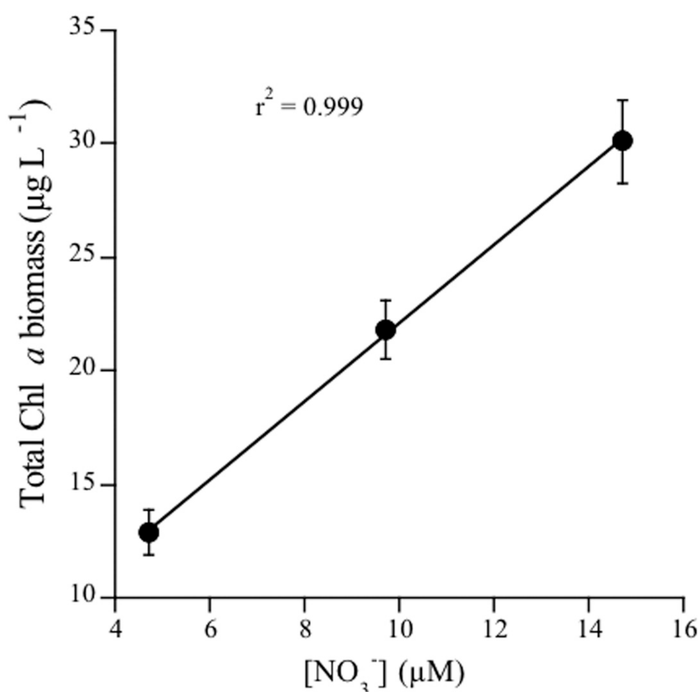

Fig. 3. Least squares linear regression of Chl *a* biomass values (±SD) averaged over the steady state (days 5-7) as a function of nitrate concentration.

decreased to ≥ 2.0 µM at the end of the experiment, however Si was not limiting throughout the bulk of the experiment (data not shown). The photo-physiological index of cellular fluorescence capacity (CFC) also indicated steady state was achieved in our cultures, as F_V/F_M remained fairly uniform over the final days of the experiment (Fig. 2D). However, while photosynthetic efficiency is often a good indicator of steady state and nutrient stress, some studies have shown that the DCMU method can be insensitive to nutrient-limited diatom cultures in balanced growth (Cullen et al. 1992; Parkhill et al. 2001), which may explain the lack of difference between nitrate-amended cultures. Chlorophyll *a* biomass varied by ≤ 12% from day 5 to day 7, and when averaged over this time period, there was a positive linear relationship between Chl *a* biomass and nitrate enrichment (Fig. 3). The phytoplankton community composition was very similar among treatments; i.e., nitrate amendments had little impact on phytoplankton community structure. The bulk (> 98%) of the phytoplankton assemblage in each treatment comprised *Chattonella* sp., *Pseudo-nitzschia* sp., *Chaetoceros* sp., and small autotrophic flagellates (< 5.0 µm) though total cell abundances were higher in the nitrate-amended treatments (Table 1).

Despite these indicators of balanced growth, Chl *a* biomass decreased slightly (≤ 12%) during the latter stage of the experiment. Similar biomass oscillations have been observed in other continuous and semi-continuous culture experiments using natural communities having a light/dark photocycle, and have been attributed to factors influencing cell division timing (Frish and Gotham 1979; Rhee 1980; Rhee et al. 1981; MacIntyre and Cullen 2005). For example, our deck-board continuous cultures experienced variations in light intensity due to diel periodicity and changing weather conditions that may have affected circadian rhythms of the cells and manifested as small changes in biomass. Additionally, phytoplankton can "overshoot" balanced growth-determined biomass in the early stages of continuous cultures by using internal nutrient reserves bolstered by luxury uptake that occurred prior to initiation of the experiment (Rhee 1980), and may explain the slight decrease in biomass between days 4 and 7. Grazing pressure adds a further complication for interpreting results with natural communities. Although large grazers (≥ 200 µm) were removed upon sample collection, micro-zooplankters (mainly heterotrophic dinoflagellates) were present, and protozoans remain active even after transport through the pumping system used for water collection (E. Lessard unpubl. data). Although heterotrophic dinoflagellates comprised a very small (< 1.0%) component of the total population in each treatment, their grazing activity may still have contributed to the small decline in Chl *a* during the latter stage of the experiment (i.e., effects on cell population size without affecting cell physiology).

These findings show that constant flow rates were achieved in each bottle, with biomass reflecting the concentration of amended nitrate. The combination of reasonably uniform biomass, photo-physiology, and complete nitrate drawdown during the latter stage of the experiment indicates that the system had attained a dynamic equilibrium in bulk community parameters, though we cannot assess whether community composition had stabilized (see below).

Table 1. Abundances of the dominant phytoplankton assemblage in the control and nitrate amended treatments in the coastal community continuous culture experiment. Error ranges are shown in parentheses.

Species	Control (4.7 µM NO_3^-)	9.7 µM NO_3^-	14.7 µM NO_3^-
Chattonella sp.	3106.2	4152.6	6894.1
		(346.1)	(467.4)
Pseudo-nitzschia sp.	6962.2	7613.1	9698.4
		(230.7)	(1343.8)
Autotrophic flagellates	1124.7	1787.9	2234.7
		(519.1)	(628.1)
Chaetoceros sp.	3223.3	3687.5	6446.1
		(519.1)	(1259.9)
Total abundances:	**1.44 x 10^4**	**1.72 x 10^4**	**2.53 x 10^4**

Open ocean iron experiment—Surface seawater was collected in the northeastern, subarctic Pacific at Ocean Station PAPA on 24 May 2006 (50°07.13′ N; 144°56.14′ W). Incubator temperature was held constant at 7.0°C and light reduced to ~400

μmol photon m^{-2} s^{-1} (persistent foggy/overcast conditions), without additional screening placed over the incubator. The culture vessels were wrapped with UV-absorbing film (GAM Products) to eliminate light < 400 nm to limit the photochemical cycling of trace metals under the full spectrum surface irradiance, an interesting but potentially complicating issue for assessing performance of the continuous culture system in response to iron addition. Feed-water flow to the culture vessels was not turned on for the first 48 h to enable cultures to adjust to the media and light conditions. Dilution rates (D) were set at 0.5 d^{-1} based on average whole community growth rates typical of the subarctic Pacific Station PAPA region in the spring (Boyd and Harrison 1999; Harrison 2002), and the experiment ran for 10 d. No macronutrient amendments were made over the ambient concentrations of 12.4 μM $NO_3^- + NO_2^-$, 1.2 μM PO_4^{3-}, and 21.8 μM $SiOH_4$, which served as the control, and the iron treatment was enriched with 1 nM Fe (ferric nitrate, AA standard Sigma). Based on previous Fe manipulation studies in this area, the nanoplankton were expected to be Fe-limited (Martin and Fitzwater 1988; Coale 1991; Boyd et al. 2004).

Biomass (as Chl *a*) remained low in both the control and Fe treatment until day 6 when biomass began to rise steadily (Fig. 4A). This increase continued for the remainder of the experiment, but never resulted in the complete drawdown of macro-nutrients (NO_3^-: ≥ 9.6 μM; PO_4^{3-}: 0.7 μM; $SiOH_4$:19.8 μM). While Chl *a* of both size fractions, principally the larger cells, increased relative to initial values, the addition of Fe did not significantly ($P < 0.05$) enhance growth of the small (0.7-20.0 μm) or large (> 20.0 μm) phytoplankton Chl *a* biomass relative to controls (Fig. 4B), suggesting that light, rather than iron, was the primary limiting factor. Water collection at Ocean Station PAPA occurred immediately before the spring bloom and shallowing of the mixed layer, and other deck-board batch culture experiments run from this time period showed the same increasing growth in the controls as in the iron treatments. Dissolved iron concentrations in the deep mixed layer still were relatively high (~180 pM; Roy and Wells unpubl. data), indicating that iron would not be a primary limiting factor, particularly with continuous media replenishment. Cellular fluorescence capacity (F_V/F_M) doubled from a low initial value of 0.18 to 0.37 in the control and 0.36 in the Fe addition over the first 2 d. Phytoplankton are co-limited by light and iron prior to the spring bloom (Maldonado et al. 1999), and these results here suggest that light was a primary limiting factor in-situ at this time. Indeed, the spring bloom occurred roughly 1 week after this experiment was initiated. Dissolved iron concentrations then decreased in the mixed layer to ~90 (Roy and Wells unpubl. data) and both deck-board batch and continuous culture experiments clearly showed iron limitation (manuscripts in preparation). While dissolved iron concentrations in the culture bottles and feed water media were not measured at the end of this experiment, it would have been highly informative with respect to drawdown by the community as

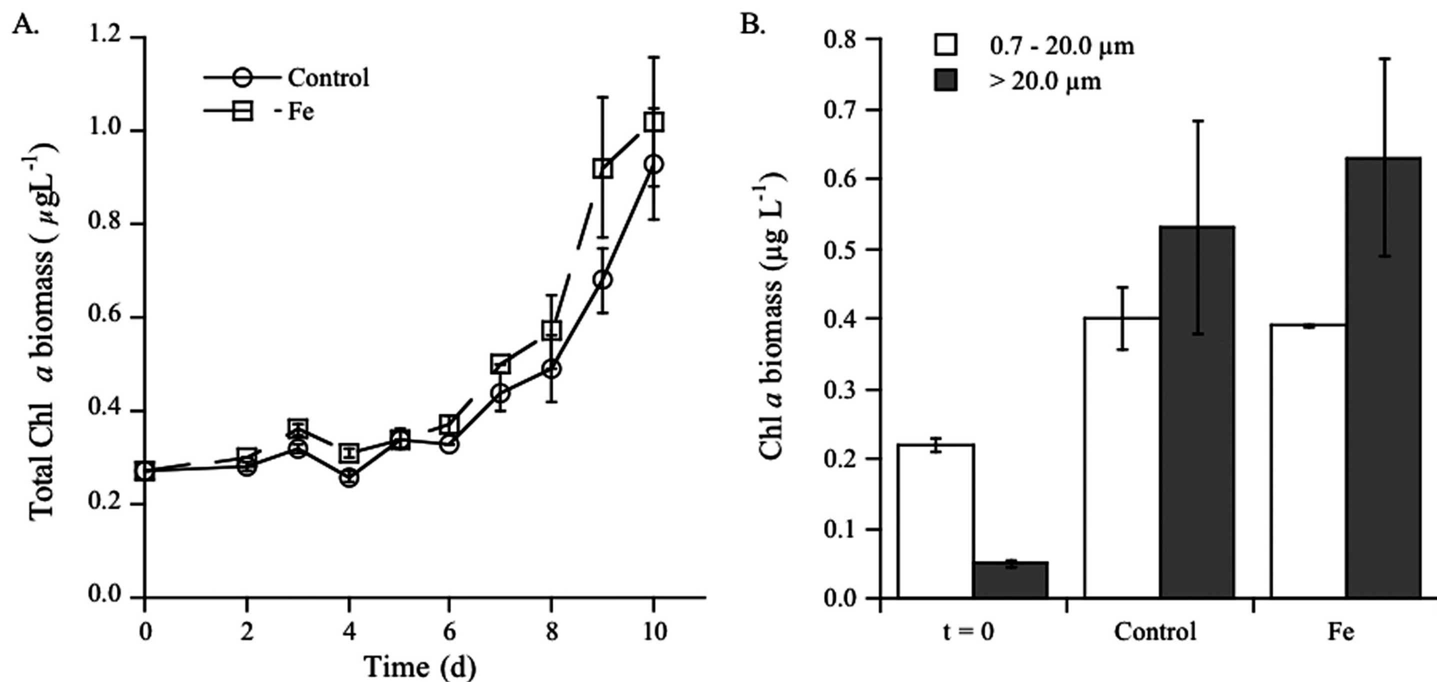

Fig. 4. (A) Mean total (> 0.7 μm) Chl *a* biomass of oceanic phytoplankton grown in unamended (control) and iron-amended (1 nM) continuous cultures over 10 d. Error bars represent the range of duplicate (n = 2) treatments. (B) Mean size-fractioned Chl *a* biomass of small (> 0.7–20.0 μm) and large (> 20.0 μm) cells (± range) of control and Fe treatment at t = 0 and t = final (day 10) of the continuous culture experiment. Values for the small size fraction were calculated by subtracting > 20.0 μm biomass from the total.

well as address issues of Fe contamination and should be determined in future continuous culture experiments.

Even if iron was not the primary factor limiting the community level biomass, iron nevertheless substantially affected community composition. The initial community comprised mainly smaller cells, such as cyanobacteria and picoplankton < 4.0 µm (data not shown), autotrophic flagellates (~5 µm; > 90% *Phaeocystis*), with a small diatom seed population typical of HNLC areas (Fig. 5). However, there was a 6-fold increase in *Pseudo-nitzschia* abundance (30 to 190 cells mL^{-1}) in the iron treatment relative to the control, while no equivalent increase was observed by *Chaetoceros* or *Tropidoneis* species. These data clearly show that not all diatoms responded equally to continuous low-level iron amendments. The *Pseudo-nitzschia* population was mainly small cells (19.5 ± 3.6 µm × 1.2 ± 0.28 µm [n = 50]), tentatively identified as *P. subcurvata*, and therefore would have accounted for only a minor portion of the total Chl *a* biomass relative to the large diatoms, *Chaetoceros* and *Tropodoneis* spp. As a consequence, the sharp increase in *Pseudo-nitzschia* cell abundance was not apparent in the Chl *a* biomass data.

Continuous culture experiments using natural assemblages, and therefore having multiple trophic levels, adds the complexity of grazing effects (Frost and Franzen 1992). In HNLC areas, grazing rates can nearly match net community growth rates, asserting strict control over biomass levels achieved by the phytoplankton population (Chavez et al. 1991). Cyanobacterial abundance decreased steadily in the control and iron treatment, reaching a ~98% loss by the end of the experiment (data not shown). These changes likely resulted from grazing pressure rather than a direct response to iron addition. Protozoan grazing rates were not quantified here, but a small population of heterotrophic dinoflagellates (~35 cells mL^{-1}) was present in the community. Therefore, grazing on nanoplankton also may have influenced the community response, though grazer abundances did not change in either the control or Fe treatment by the end of the experiment. It is important then to remember that Ecostat's create a relationship between dilution rate and net growth rate (including grazing and viral lysis) rather than intrinsic or specific growth rates, and so might not be appropriate for testing some aspects of phytoplankton ecophysiology.

Continuous culture systems offer an excellent platform to study shifts in natural community structure, and evolution of the assemblage in response to the applied treatments. Similar biomass among treatments may shield entirely different community structures, as in our case, where small increases in iron availability strongly affected the relative abundances of diatoms. While these changes can be detected in batch cultures when the shifts are large, it is difficult to distinguish more subtle community change in batch cultures because noncompetitive or nonviable cells remain in the vessels. Theoretically, a continuous system with a natural assemblage should result in a rapid and complete selection for one species whose growth

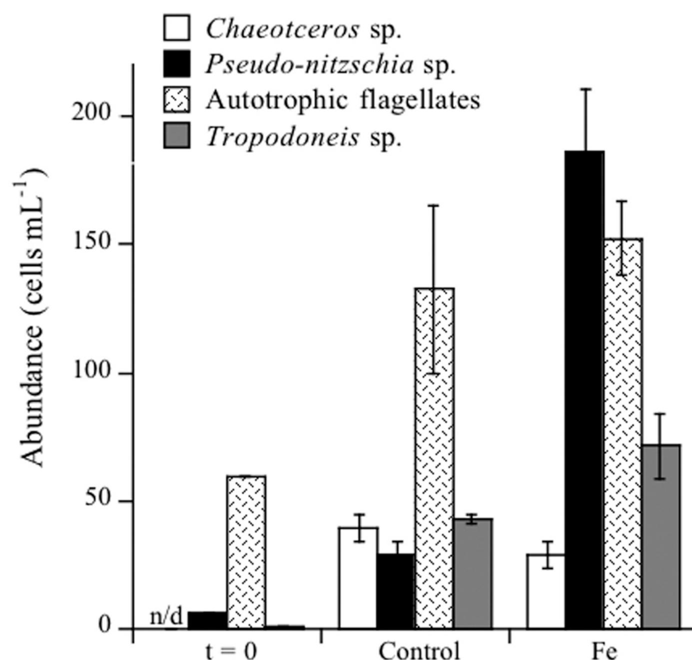

Fig. 5. Mean (± range) abundances of dominant eukaryotic phytoplankton measured via epifluorescent microscopic cell counts at t = 0 and t = final (day 10) in the control and iron addition continuous cultures; n/d indicates "not detected."

rate is most favored by the conditions, competitively excluding all others from the system (Mickelson et al. 1979; Rhee 1980; Turpin 1981). However, while steady state can be achieved using natural mixed communities, changes in species composition and diversity can be surprisingly slow (e.g., Dunstan and Menzel 1971; Thomas et al. 1980), often requiring 25 to > 40 d for ~99% dominance of one species (competitive exclusion) to occur (Tilman 1977). Our coastal nitrate experiment showed only ~65% dominance of two species after 7 d; the Raphidophyte (*Chattonella* spp.) and a pennate diatom (*Pseudo-nitzschia* spp.). Thus while total chlorophyll approached steady state at the community level, competitive species exclusion had not yet been achieved. Co-existence observed in previous cyclostat experiments has been attributed to differential timing in cell cycles due to changes in light/dark periods (Eppley et al. 1971; Frisch and Gotham 1979; see below discussion). A mixed community also remained after 10 d in our oceanic experiment, however Fe addition still was exerting selective pressure on the diatom community (Fig. 5).

Discussion

Traditional batch cultures have been widely used for assessing biological responses to environmental perturbations and have resulted in valuable insights about phytoplankton ecology. In addition they are quick, straightforward, and are amenable to trace metal clean techniques. However, interpreting results from batch culture experiments requires the recognition that the constantly changing (decreasing) nutrient

conditions will directly affect growth and interspecies competition, resulting in shifting pressures on the phytoplankton assemblage. To minimize these complicating effects, secondary macro-, and sometimes micro-nutrients, are added far in excess of what would naturally be present, straining the ability to extrapolate findings to natural conditions (Hutchins et al. 2003). For example, species that have a fast, adaptive response will tend to thrive and thus dominate numerically in these bottle communities. Species that might otherwise be replaced or have substantially reduced populations under natural-scale nutrient fluctuations. Additionally, loss variables in closed systems are reduced or altogether absent, particularly for nano- and microphytoplankton, so the short-lived exponential growth of a natural mixed community often yields very high cell concentrations relative to natural conditions (Rhee 1980). This effect is compounded by the retention of non-competitive or senescent phytoplankton in culture, which may have been the prevailing physiological state of the cells collected (MacIntyre and Cullen 2005). In these regards, batch cultures tend to bias species outcome in ways that are avoided in continuous cultures with sustained, low-level nutrient manipulations.

More recently, insight to iron limitation effects on phytoplankton growth and community response has been gained through large-scale in situ manipulation experiments (fertilizations) in the open ocean (see a comparative review of the first nine Fe enrichment experiments by de Baar et al. 2005). However, these experiments typically require large or multiple inoculations of iron, and despite the inclusion of loss terms (advective mixing, sinking, grazing), the resulting biomass can still be artificially high (Hutchins et al. 2003). There is a need for alternate experimental methods to assess the dynamic equilibrium between low-level continuous nutrient inputs, phytoplankton growth, and the resulting community structure.

Previous continuous culture experiments have been largely restricted to the study of bacterial and phytoplankton monocultures in the laboratory, with few conducted outdoors or with natural community assemblages (deNoyelles and O'Brien 1974; Malone et al. 1975; Harrison and Davis 1979; Turpin and Harrison 1979). Continuous culture systems adapted for use at sea have only recently been applied in field-going studies (Hutchins et al. 2003; Hare et al. 2005, 2007a, 2007b), yet these systems offer advantages over traditional batch or semi-continuous culturing methods in that the physiological response to more ecologically relevant nutrients levels can be determined at both the species and community level (Jannasch 1974; Thomas et al. 1980). These attributes make continuous cultures particularly well suited to study the competitive interactions for iron acquisition near or at ambient iron concentrations. In contrast, the changing environmental conditions in batch cultures complicate the interpretation of iron effects on the community.

Continuous cultures in particular offer the possibility to better study some of the subtleties of trace metal availability now recognized to be very important; e.g., determining chemical speciation effects on separate members of the phytoplankton community, and the effects of low-level, continuous iron supply, more analogous to natural processes in HNLC regions (Hare et al. 2005, 2007a). It is estimated that most large natural aerosol deposition events may increase surface dissolved iron concentrations by only ~500 pM (Sedwick et al. 2005), meaning that the majority of deposition events may result in iron inputs << 100 pM. There is a strong need therefore to assess the impacts of picomolar-level iron inputs on phytoplankton communities and carbon cycling. Moreover, the effects from single or repeated perturbations of iron input, such as occurs in nature, can be mimicked by adjusting dilution rates or feed-water solutions (Hare et al. 2007a). The effects of changing pCO_2 and temperature on community structure and productivity also have been studied using deck-board continuous cultures to predict the response of phytoplankton to longer-term global scale changes (Hare et al. 2007b). In addition, nutrient effects on phytoplankton species trajectories can be separated from possible allelopathic effects in mixed assemblages because the flow-through system will minimize the latter impact, contrary to batch cultures (Rhee 1980).

It is important to realize that while many natural conditions can be simulated using continuous cultures, these systems are not meant to, nor are they capable of, replicating a natural habitat (Jannasch 1974; Rhee 1980; MacIntyre and Cullen 2005). For instance, the loss or "removal" of organisms in nature are due to individual selective forces—predation, cell death, sinking or competition—whereas in continuous cultures, there will be indiscriminate removal of cells growing slower than the dilution rate (Jannasch 1974; Thomas et al. 1980). The dilution rate and its time scale of implementation therefore are very important parameters to consider when using continuous cultures. Too low a dilution rate and poor-growing cells are sustained in culture, perhaps leading to unnaturally enhanced nutrient recycling effects. Too high a dilution rate risks wash out of cells that otherwise might adapt to the growth conditions given sufficient time. Our chosen dilution rates reflected our understanding of the average net community growth rates based on the literature, but steady state can be achieved across a range of dilution rates (Herbert et al. 1956). For example, Harrison and Davis (1979) studied a mixed community in outdoor continuous cultures and found different species dominated depending on dilution rate, and it is expected that higher or lower dilution rates would have significantly affected the community composition findings in our study.

Despite their advantages, continuous cultures rarely have been used in field-based studies, in large part because a compact, robust architecture for such systems has not been available (Hutchins et al. 2003). Additionally, two of the main challenges to successful continuous culture operations are to verify continuous flow rates and ensure complete homogeneity within culture vessels. The initial approach of Hutchins et al.

(2003) relied on hourly mixing of culture vessels by hand to maintain homogeneity, and flow rates could not be measured accurately over the time course of sample collection in their gravity overflow design. Since then, they have introduced an air-driven mechanical mixing system and other improvements to their gravity overflow design, including a recirculating incubator with an aquarium chiller/heater temperature control system (Hare et al. 2007b). Hutchins and colleagues also experimented with multi-channel pumps but found they could not maintain consistent flow rates, so they returned to using the more cumbersome approach of multiple single channel pumps (Hutchins pers. comm.). The design presented here differs by using forced outflow, positive pressure displacement in sealed culture vessels rather than gravity outflow that would occur in small pulses with the ships motion. It may be that these small changes in culture volume vary the pump backpressure enough to prevent the highly consistent flow rates measured here. Our system also employs an electric motor to generate continuous rocking of the reclined culture vessels, which we believe is an improvement over the innovative compressed air-driven design that rotates (120°) a Plexiglas rack of upright bottles on a timed cycle (Hare et al. 2007b). The reclined bottle design also allows more uniform light conditions in the vessels that may better enable studies of photochemical metal cycling. All system components in contact with media are fabricated of plastics amenable to cleaning for ultra-low trace metal studies, and the stand-alone, self-contained unit is robust enough to be transported by air or ground freight for operation on both large and small research vessels. This device provides an excellent platform to study the effects of low level, continuous or pulsed nutrient perturbation on the trajectory of phytoplankton communities, and the resultant impacts on the biogeochemical cycling of carbon and trace metals.

References

Bazin, M. J. 1981. Theory of continuous culture, p. 27-49. *In* P. H. Calcott [ed.], Continuous cultures of cells. Vol. I. CRC Press.

Boyd, P. W., and others. 2004. The decline and fate of an iron-induced subarctic phytoplankton bloom. Nature 428:549-553.

——— and P. J. Harrison. 1999. Phytoplankton dynamics in the NE subarctic Pacific. Deep-Sea Res. II 46:2405-2432.

Bruland, K. W., E. L. Rue, G. J. Smith, and G. R. DiTullio. 2005. Iron, macronutrients and diatom blooms in the Peru upwelling regime: brown and blue waters of Peru. Mar. Chem. 93:81-103.

Calcott, P. H. 1981. The construction and operation of continuous cultures, p. 13-25. *In* P. H. Calcott [ed.], Continuous cultures of cells. Vol. I. CRC Press.

Chavez, F. P., K. R. Buck, K. H. Coale, J. H. Martin, G. R. DiTullio, N. A. Welschmeyer, A. C. Jacobson, and R. T. Barber. 1991. Growth rates, grazing, sinking, and iron limitation of equatorial Pacific phytoplankton. Limnol. Oceanogr. 36:1816-1833.

Coale, K. H. 1991. Effects or iron, manganese, copper and zinc enrichments on productivity and biomass in the subarctic Pacific. Limnol. Oceanogr. 36:1851-1864.

Cullen, J. J., X. Yang, and H. L. MacIntyre. 1992. Nutrient limitation of marine photosynthesis. In P. G. Falkowski and A. Woodhead [eds.], Preliminary productivity and biogeochemical cycles in the sea. Plenum.

de Baar, H. J. W., and others. 2005. Synthesis of iron fertilization experiments: from the iron age in the age of enlightenment. J. Geophys. Res. 110: [doi: 10.1029/2004JC002601].

deNoyelles, F., and W. J. O'Brien. 1974. The in situ chemostat – a self-contained continuous culturing and water sampling system. Limnol. Oceanogr. 19:326-331.

Dunstan, W. M., and D. W. Menzel. 1971. Continuous cultures of natural populations of phytoplankton in dilute, treated sewage effluent. Limnol. Oceanogr. 16:623-632.

Eppley, R. W., J. N. Rogers, J. J. McCarthy, and A. Sournia. 1971. Light/dark periodicity in nitrogen assimilation of the marine phytoplankters *Skeletonema costatum* and *Coccolithus huxleyi* in N-limited chemostat culture. J. Phycol. 7:150-161.

Frisch, H. L., and I. J. Gotham. 1979. A simple model for periodic cyclostat growth of algae. J. Math. Biol. 7:149-169.

Frost, B. W., and N. C. Franzen. 1992. Grazing and iron limitation in the control of phytoplankton stock and nutrient concentration: a chemostat analogue of the Pacific equatorial upwelling zone. Mar. Ecol. Prog. Ser. 83:291-303.

Hare, C. E., G. R. DiTullio, C. G. Trick, S. W. Wilhelm, K. W. Bruland, E. L. Rue, and D. A. Hutchins. 2005. Phytoplankton community structure changes following simulated upwelled iron inputs in the Peru upwelling region. Aquat. Microb. Ecol. 38:269-282.

———, G. R. DiTullio, S. F. Riseman, A. C. Crossley, L. C. Popels, P. N. Sedwick, and D. A. Hutchins. 2007a. Effects of changing continuous iron input rates on a Southern Ocean algal assemblage. Deep-Sea Res. I 54:732-746.

———, and others. 2007b. Consequences of increased temperature and CO_2 for algal community structure and biogeochemistry in the Bering Sea. Mar. Ecol. Prog. Ser. 352:9-16.

Harrison, P. J., and C. O. Davis. 1979. The use of outdoor phytoplankton continuous cultures to analyze factors influencing species selection. J. Exp. Mar. Biol. Ecol. 41:9-23.

———. 2002. Station Papa time series: Insights into ecosystem dynamics. J. Oceanogr. 58:259-264.

Herbert, D., R. Elsworth, and R. C. Telling. 1956. The continuous culture of bacteria; a theoretical and experimental study. J. Gen. Microbiol. 14:601-622.

Hutchins, D. A., F. Pustizzi, C. E. Hare, and G. R. DiTullio. 2003. A shipboard natural community continuous culture system for ecologically relevant low-level nutrient enrichment experiments. Limnol. Oceanogr.: Methods. 1:82-91.

Jannasch, H. W. 1974. Comment: Steady state and the chemostat ecology. Limnol. Oceanogr. 19:716-720.

Knepel, K., and K. Bogren. 2002. Determination of orthophosphorate by flow injection analysis: QuickChem Method 31-115-01-1-H., p. 14. Methods Manual. Lachat Instruments.

Lessard, E. J., and M. C. Murrell. 1996. Distribution, abundance and size composition of the heterotrophic dinoflagellates and ciliates in the Sargasso Sea near Bermuda. Deep-Sea Res. 43:1045-1065.

MacIntyre, H. L., and J. J. Cullen. 2005. Using cultures to investigate the physiological ecology of microalgae, p. 287-326. In R. Anderson [ed.], Culturing techniques. Academic.

Maldonado, M. T., P. W. Boyd, P. J. Harrison, and N. M. Price. 1999. Co-limitation of phytoplankton growth by light and Fe during winter in the NE subarctic Pacific Ocean. Deep Sea Res. II 46:2475-2485.

Malone, T. C., C. Garside, K. C. Haines, and O. A. Roels. 1975. Nitrate uptake and growth of *Chaetoceros* sp. in large outdoor continuous cultures. Limnol. Oceanogr. 20:9-19.

Martin, J. H., and S. E. Fitzwater. 1988. Iron deficiency limits phytoplankton growth in the northeast Pacific subarctic. Nature 331:341-343.

Mickelson, M. J., H. Maske, and R. C. Dugdale. 1979. Nutrient-determined dominance in multispecies chemostat cultures of diatoms. Limnol. Oceanogr. 24:298-315.

Monod, J. 1950. La technique de culture continue, theorie et applications. Ann. Inst. Pasteur. 79:390-410.

Novick, A., and L. Szilard. 1950. Description of the chemostat. Science 112:715-716.

Parkhill, J. -P., G. Maillet, and J. J. Cullen. 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. 37:517-529.

Price, N. M., G. I. Harrison, J. G. Hering, R. J. Hudson, P. M. V. Nirel, B. Palenik, and F. M. M. Morel. 1988/89. Preparation and chemistry of the artificial algal culture medium Aquil. Biol. Oceanogr. 6:443-461.

Rhee, G. -Y. 1980. Continuous culture in phytoplankton ecology, p.151-203. In M. R. Droop and H. W. Jannasch [eds.], Advances in aquatic microbiology Vol.2. Academic.

———, I. J. Gotham, and S. W. Chisholm. 1981. Use of cyclostat cultures to study phytoplankton ecology, p.159-187. In P. H. Calcott [ed.], Continuous cultures of cells. Vol. II. CRC Press.

Riser, S. C., and K. S. Johnson. 2008. Net production of oxygen in the subtropical ocean. Nature 451:[doi:10.1038/nature06441].

Roy, E. G., M. L. Wells, and D. W. King. 2008. Persistence of iron(II) in surface waters of the western subarctic Pacific. Limnol. Oceanogr. 53:89-98.

Sedwick, P. N., and others. 2005. Iron in the Sargasso Sea (Bermuda Atlantic Time-series Study region) during summer: Eolian imprint, spatiotemporal variability and ecological implications. Glob. Biogeochem. Cycles 19: [doi:10.1029/2004GB002445].

Smith, P., and K. Bogren. 2001. Determination of nitrate and/or nitrite in brackish or seawater by flow injection analysis colorimetry: QuickChem Method 31-107-04-1-E., p. 12, Methods manual, Lachat Instruments.

Thomas, W.H., M. Pollock, and D. L. R. Seibert. 1980. Effects of simulated upwelling and oligotrophy on chemostat-grown natural marine phytoplankton assemblages. J. Exp. Mar. Biol. Ecol. 45:25-36.

Tilman, D. 1977. Resource competition between planktonic algae: an experimental and theoretical approach. Ecol. 58:338-348.

Turpin, D. H., and P. J. Harrison. 1979. Limiting nutrient patchiness and its role in phytoplankton ecology. J. Exp. Biol. Ecol. 39:151-166.

Turpin, D. H. 1981. Physiological mechanisms in phytoplankton resource competition, p. 316-368. *In* C. D. Sandgren [ed.], Growth and reproductive strategies of freshwater phytoplankton. Cambridge Univ.

Veldkamp, H. 1976. Continuous culture in microbial physiology and ecology, p. (7)1-68. In J. G. Cook [ed.], Patterns of progress. Meadowfield Press.

Welschmeyer, N. A. 1994. Fluormetric analysis of chlorophyll *a* in the presence of chlorophyll *b* and pheopigments. Limnol. Oceanogr. 39:1985-1992.

Wolters, M. 2002. Determination of silicate in brackish or seawater by flow injection analysis. QuickChem Method 31-114-27-1-D., p. 12, Methods Manual. Lachat Instruments.

Submitted 17 May 2008
Revised 12 November 2008
Accepted 3 December 2008