(Guillard & Ryther, 1962)Shake it easy: a gently mixed continuous culture system for dinoflagellates

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Abstract

An important requirement of continuous cultures is a homogeneous distribution of resources and microorganisms, often achieved by rigorous mixing. Many dinoflagellate species are known to be vulnerable to turbulence. Here, we present a newly developed continuous culture system based on gentle mixing in which the two dinoflagellate species *Scrippsiella trochoidea* and *Alexandrium tamarense*, having different turbulence sensitivities, grew well under steady state conditions. We also show that the continuous culture system can be applied at low nutrient conditions and low population densities.

Body text

Continuous cultures allow testing a wide range of environmental factors under well-defined growth conditions, and have therefore contributed greatly to our understanding of microbial physiology and ecology (Bull, 2010; Fredrickson, 1977; Huisman *et al.*, 2002; Monod, 1950; Novick & Szilard, 1950). In conventional batch cultures, population growth will deplete one or more resources. Consequently, growth rates and resource concentrations can substantially change during the course of an experiment. In a continuous culture system, the population growth rate is controlled by the dilution rate (D). With a fixed dilution rate and a sufficiently low initial population density, resource conditions may support transient growth rates larger than the dilution rate ($\mu > D$). As a consequence, population densities will increase until a resource becomes growth limiting, causing the net population growth rate to decrease until it equals dilution rate, and a so-called steady state is

reached ($\mu = D$). Once in steady state, growth rate, resource conditions and population densities remain constant. Thus, net population growth is fixed by the dilution rate, which in turn controls the extent to which resources become limiting.

The application of continuous cultures has a long history (Monod, 1950; Novick & Szilard, 1950), and has been applied for a variety of organisms including bacteria, fungi, as well as phytoplankton (Bull, 2010). Continuous cultures have been used to study the impact of growth and resource limitation on phytoplankton physiology, for instance on their biochemical composition (Droop, 1974; Goldman *et al.*, 1979). Continuous cultures have further been applied to study ecological processes such as competition for various resources (Passarge *et al.*, 2006; Tilman, 1982; Van de Waal *et al.*, 2011), evolutionary processes like mutation and selection (Novick & Szilard, 1950; Rosenzweig *et al.*, 1994), and natural community dynamics (Harrison & Davis, 1979; Hutchins *et al.*, 2003; Sommer, 1985).

An important requirement of continuous cultures is a homogeneous distribution of resources and cells, needed for a representative dilution of the system. This is typically achieved by rigorous mixing via aeration and/or stirring. Clearly, this mixing should not negatively affect the species of interest. Here, we describe a continuous culture system based on gentle mixing, and test its applicability for two dinoflagellate species, *Alexandrium tamarense* and *Scrippsiella trochoidea*. Many dinoflagellate species are known to be vulnerable to turbulence, and often show decreased or even arrested growth rates (Berdalet *et al.*, 2007). Both species tested here have been shown to differ in their sensitivity towards turbulence, with *A. tamarense* being insensitive to moderately sensitive (Sullivan & Swift, 2003; White, 1976) and *S. trochoidea* being highly sensitive (Berdalet & Estrada, 1993), especially to high shaking levels.

We grew *S. trochoidea* GeoB267 (culture collection of the University of Bremen) and *A. tamarense* Alex5 (Tillmann *et al.*, 2009) at 15°C in 0.2 µm filtered North Sea water (salinity 34 ‰) containing 18 µM NO_3^-, 0.8 µM NH_4^+, and 0.3 µM PO_4^{3-}. The seawater was enriched with vitamins, trace metals and 36 µM PO_4^{3-} according to the recipe of f/2 medium (Guillard and Ryther, 1962), with additional 10 nM H_2SeO_3 and 6.3 nM $NiCl_2$ according to the recipe of K medium (Keller *et al.*, 1987). In the first series of experiments, cultures were grown under high nutrient conditions, by adding 100 µM NO_3^- to the medium, which yielded an initial concentration of 118 µM NO_3^-. A second series of experiments were conducted under low nutrient conditions. In that case, the medium did not contain additional NO_3^-, which allowed us to test whether the continuous cultures are also applicable at low population densities. Culture medium was pre-aerated with moistened air containing 380 µatm CO_2 (Fig. 1). Cultures were grown in custom-made glass tubes (diameter 95 mm; length 370 mm) closed by Duran GLS80 caps at both ends, yielding a working volume of 2100 ± 50 mL. The glass tubes were placed on a three-dimensional orbital shaker (TL10; Edmund

Bühler GmbH, Hechingen, Germany), set at an angle of 9° with a shaking speed of 16 rpm, to allow homogenous mixing (i.e. rocking) by moving a 55 mm diameter polyoximethylen ball and a 50-100 mL headspace back and forth (Fig. 1). Light was provided from above by day light tubes (18W/965 Biolux; OSRAM GmbH, München, Germany) at a light:dark cycle of 16:8 h and average incident irradiance of 200 ± 25 µmol photons m^{-2} s^{-1}. Medium was continuously supplied using a peristaltic pump with a dilution rate D = 0.14 d^{-1} for the high nutrient incubation with *S. trochoidea*, and D = 0.11 d^{-1} for high nutrient incubation with *A. tamarense*. To further lower population densities in the low nutrient incubations, dilution rates were increased to D = 0.2 d^{-1}. Prior to the experiments, cells were acclimated to the respective culture medium and experimental conditions for at least seven generations.

Samples for population density, pH and dissolved inorganic nitrogen (DIN) were taken every second or third day, except for the *A. tamarense* high nutrient incubations for which no DIN samples were taken. Population densities were assessed as cell number and biovolume by means of automated cell counts, applying triplicate counts of 2 x 1 mL culture suspension with a Multisizer III Coulter Counter (Beckman-Coulter, Fullerton, USA). Automatic cell counts were regularly confirmed by microscopic cell counts with an inverted light microscope (Axiovert 40C), using a settling chamber containing 0.2-10 mL culture suspension fixed with Lugol's solution (2% final concentration). Because cell volume changed during the transient phase, average growth rate was based on biovolume according to: $\mu = D + (\ln(N_2) - \ln(N_1)) / (t_2 - t_1)$, where N_1 and N_2 represent the average total biovolumes at times t_1 and t_2, respectively (Bull, 2010). The calculated growth rate was corrected for the dilution rate by adding D. pH was measured with a pH electrode (Schott Instruments, Mainz, Germany), applying a two-point calibration on the NBS scale prior to each measurement. For DIN analyses (i.e. NO_3^-, NO_2^-, and NH_4^+), 15 mL of culture suspension was filtered over a 0.45 µm membrane filter and duplicates were measured colorimetrically using an Evolution III continuous flow analyzer (Alliance Instruments, Salzburg, Austria) according to Grasshoff *et al.* (1999).

Both dinoflagellate species grew well in the continuous culture system and showed a gradual increase in population density reaching steady state after 21-29 days in the high nutrient incubations (Figs. 2A,B), and after 14-17 days in the low nutrient incubations (Figs. 3A,B). The average growth rates achieved during the transient phase in the high nutrient incubations were 0.46 (0.31-0.64) d^{-1} for *S. trochoidea* and 0.33 (0.30-0.36) d^{-1} for *A. tamarense*, which were somewhat lower in the low nutrient incubations with 0.30 (0.22-0.36) d^{-1} for *S. trochoidea* and 0.27 (0.25-0.29) d^{-1} for *A. tamarense*. The average growth rates during steady state were equal to D in all experiments. Relatively stable cell numbers were reached at steady state, with 11.5 ± 0.2 × 10^6 cells L^{-1} and 1.3 ± 0.1 × 10^6 cells L^{-1} in the high nutrient incubations, and 0.76 ± 0.03 × 10^6 cells L^{-1} and 0.10 ± 0.02 ×

10^6 cells L^{-1} in the low nutrient incubations, for *S. trochoidea* and *A. tamarense*, respectively (Figs. 2B and 3B). The associated biovolumes at steady state were 40.1 ± 1.5 mm^3 L^{-1} and 17.2 ± 2.9 mm^3 L^{-1} in the high nutrient incubations and 4.2 ± 0.3 mm^3 L^{-1} and 1.3 ± 0.1 mm^3 L^{-1} in the low nutrient incubations, for *S. trochoidea* and *A. tamarense*, respectively. In both *A. tamarense* incubations, we observed a slight decrease in population density prior to reaching steady state. This is presumably the result of initial cell growth towards higher population densities than can be sustained under the given experimental conditions. Consequently, cell growth and population densities decrease to a level where growth equals *D*, and steady state is reached.

Regular microscopic inspection throughout the experiment revealed no visual changes in cell morphology or motility of the tested dinoflagellate species, suggesting that there were no direct negative effects of the applied mixing. Furthermore, test batch experiments with the highly sensitive *S. trochoidea* under the same experimental conditions without shaking, with shaking but without the ball, or with shaking and with the ball, showed comparable growth rates as observed in our high nutrient incubation, yielding average growth rates of 0.51 (0.31-0.61) d^{-1}, 0.48 (0.25-0.65) d^{-1}, and 0.46 (0.33-0.63) d^{-1}, respectively. Thus, growth of *S. trochoidea* does not seem to be affected by the induced mixing, which presumably also applies to *A. tamarense*. The growth rates attained during the transient phase of the high nutrient incubations are also in line with values reported earlier in batch experiments with the same *A. tamarense* strain, and another strain of *S. trochoidea* (Tillmann & Hansen, 2009). This further confirms that growth of both species remained unaffected by the applied mixing conditions. The lower growth rates during the transient phase in the low nutrient incubations are presumably caused by the low initial DIN concentrations, which limited growth even at the start of the experiment. It remains to be tested whether other dinoflagellates will also be unaffected by the applied mixing strategy. However, many dinoflagellates show a comparable or lower sensitivity towards turbulence as *S. trochoidea* (Berdalet *et al.*, 2007, and references therein). It is thus conceivable that the presented continuous culture system is applicable to many more dinoflagellates species.

The increase in biomass was associated with a decrease in DIN and an increase in pH (Fig. 2C and 3C,D). In the high nutrient incubation with *S. trochoidea*, DIN decreased from about 86 μM measured at the start of the experiments to 10.2 ± 3.6 μM at steady state. At the same time, pH increased from about 8.1 to 9.33 ± 0.05 (Fig. 2C). In high nutrient incubation with *A. tamarense*, pH increased from about 8.2 at the start to 8.68 ± 0.02 at steady state (Fig. 2D). In the low nutrient incubations, changes in pH were substantially smaller, increasing from about 8.1 at the start of the experiment to 8.43 ± 0.04 at steady state for *S. trochoidea*, while it remained stable around 8.20 ± 0.02 for *A. tamarense*. Also in terms of DIN, changes were much lower in the low nutrient incubation compared to the high nutrient incubation. More specifically, in the *S. trochoidea* culture,

DIN decreased from about 3.3 μM measured at the start down to 0.73 ± 0.63 μM at steady state (Fig. 3C). In the low nutrient incubations with *A. tamarense*, DIN was about 1.0 μM at the start, decreased upon cell growth, but increased afterwards reaching 1.08 ± 0.37 μM at steady state (Fig. 3D). The increase in DIN at the end of the low nutrient incubations may be associated to a minor decrease in population densities (observed for *A. tamarense*), or by bacterial mineralization of organic nitrogen, which may occur in non-axenic cultures. Under such low nutrient concentrations, fluctuations caused by minor shifts in nutrient uptake by the tested dinoflagellate species, or by bacterial mineralization of organic nitrogen, are relatively strong. Future experiments should proceed for a longer period in order to better assess the residual nutrient concentrations at steady state.

In the high nutrient incubations, increasing population densities not only caused a strong decrease in DIN (only available for *S. trochoidea*, Fig. 2C), but also a substantial increase in pH (Fig. 2C,D). Although initial NO_3^- concentrations were lowered compared to full f/2 medium (118 μM compared to 883 μM), the concentrations were sufficiently high to support substantial biomass build-up. The high population densities also caused a pH drift towards values potentially affecting growth of dinoflagellates, as has been demonstrated for the tested strain of *A. tamarense*, and for another strain of *S. trochoidea* (Tillmann & Hansen, 2009). Consequently, growth at steady state is presumable also controlled by the shift in carbonate chemistry. With the low nutrient incubations, we show that a lowering of the initial NO_3^- concentration can prevent such a strong drift in carbonate chemistry and ensures that cultures become limited by DIN only, maintaining low and relatively stable population densities (Fig. 3).

Our findings presented here demonstrate that both *S. trochoidea* and *A. tamarense* are able to grow well towards stable population densities at steady state in the continuous culture system based on gentle mixing. Population densities and accompanied changes in carbonate chemistry can be modulated by changing the supply of nutrients, as well as by adjusting other chemical parameters such as pH or the CO_2 concentration used for aeration of the medium, which may proof valuable in testing the consequences of ocean acidification. We believe this gently mixed continuous culture system to be very suitable for eco-physiological studies with dinoflagellates and possibly other turbulence sensitive phytoplankton species as well.

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Figure legends

Figure 1. Schematic overview of the continuous culture system. Culture medium is pre-aerated with humidified air containing additional CO_2. The culture medium is pumped at a fixed rate (i.e. dilution rate) into the culture vessel. Mixing in the culture vessels is achieved by gentle rocking, where ball and headspace move in opposite direction, covering the entire length of the vessel. The culture medium containing cell material runs out of the culture vessel by overpressure, and is transported to a waste container. The air outlet allows for stabilization of overpressure.

Figure 2. Dynamic changes in population densities, given in cell number and biovolume, and pH in the high nutrient incubations of (A,B) *S. trochoidea,* and (C,D) of *A. tamarense*. For *S. trochoidea*, dissolved inorganic nitrogen (DIN) is also shown (C). Values for population densities indicate mean ± SD (n=3).

245 Figure 3. Dynamic changes in population densities, given in cell number and biovolume, and
246 dissolved inorganic nitrogen (DIN), and pH in the low nutrient incubations (A,B) of *S. trochoidea,*
247 and (C,D) of *A. tamarense*. Values for population densities indicate mean ± SD (*n*=3).
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