



Approaches and involved principles to control pH/pCO₂ stability in algal cultures

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Abstract

Experimental cultures of both microalgae and macroalgae are commonly carried out by phycologists or environmental biologists to look into morphological, physiological, and molecular responses to aquatic environmental changes. However, the species of inorganic carbon in algal cultures is often altered by algal photosynthetic CO₂ removal and/or bicarbonate utilization. The pH changes associated with altered carbonate chemistry in cultures impact physiological processes in microalgae and macroalgae even at their exponential growth phases, since extra energy is required to sustain intracellular acid–base homeostasis. Usually, pH increases during light period due to inorganic carbon uptake and utilization for photosynthesis and decreases during dark period because of respiratory CO₂ release. Therefore, to obtain relevant data aimed for physiological and/or molecular responses of algae to changed levels of environmental factors, stability of pH/pCO₂ in the cultures should be considered and controlled to rule out impacts of carbonate chemistry and pH changes. In this work, principles involved in changing pH processes in algal cultures are mechanistically analyzed and several approaches to control pH and pCO₂ are introduced. In order to sustain stability of pH/pCO₂, the principles underline the following key points: (1) maintaining the rate of photosynthetic C removal less than or equal to the rate of CO₂ dissolution into the cultures which are aerated; or (2) sustaining dilute cultures with very low cell density without aeration, so that photosynthetic C removal is small enough not to cause significant pH/pCO₂ changes; or (3) stabilizing the changes in micro-environments surrounding the cells or thallus. To maintain pH drift < 1% in growing typical unicellular microalgae, the recommended cell concentration ranges from 50 × 10³ to 200 × 10³ mL⁻¹ with aeration (air replacement rate of ca 500–1000 mL L⁻¹ min⁻¹) in semi-continuous cultures of < 1 L, and it ranges from 100 to 5000 cells mL⁻¹ for diatoms and from 100 to 100 × 10³ cells mL⁻¹ for coccolithophores in dilute cultures without aeration, respectively. For macroalgae, maintaining the thalli in flowing through- system or in semi-continuous cultures (continuously control algal biomass density) is recommended.

Keywords Algae · CO₂ · Culture methods · pH · Phytoplankton

Introduction

Algal cultures can be run in different ways, such as batch, fed-batch, continuous (including turbidostat and chemostat), and semi-continuous growing methods. Frequently used batch and fed-batch cultures are easy to operate, but

physical and chemical conditions in the cultures change drastically with growth time and increasing biomass density. For instance, light exposures to each cell or individuals decrease with increasing density of biomass (Richmond et al. 2003), and pH along with dissolved O₂ levels rise in the period of light with reduced pCO₂ and/or bicarbonate ions, while the changes of these factors are reversed in darkness (Gao et al. 1991; Moheimani and Borowitzka 2011) (Fig. 1). On the other hand, continuous cultures are more ideal compared to the batch cultures in terms of stability in chemical and physical conditions. However, the pH and pCO₂ levels are also dependent on biomass densities which are maintained stable by continuous dilution, since rate of photosynthetic CO₂ removal with certain amounts of algal cells can usually exceed that of dissolution of CO₂ from air

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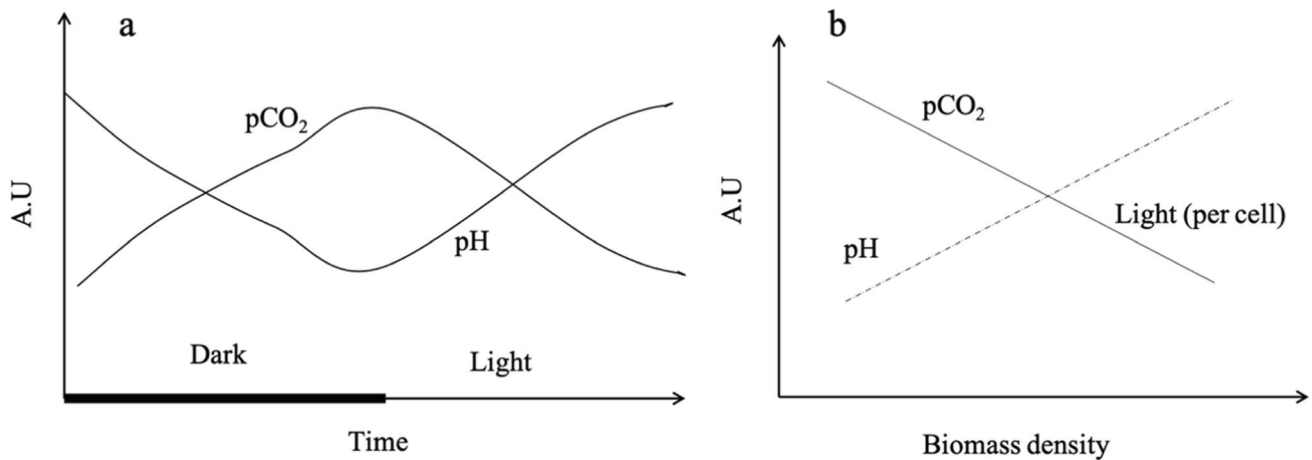


Fig. 1 The changes in pH and pCO₂ during a diel cycle, with pH declining and pCO₂ rising during dark and pH rise with pCO₂ decrease during daytime (a). Amplitude of changes in pH and pCO₂

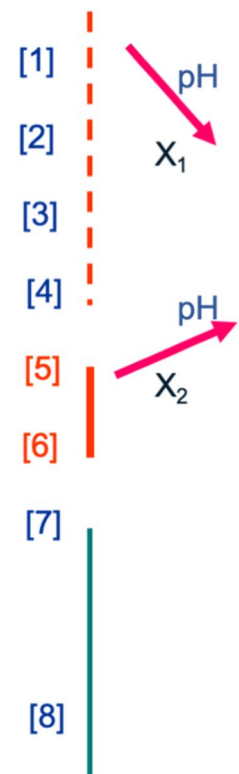
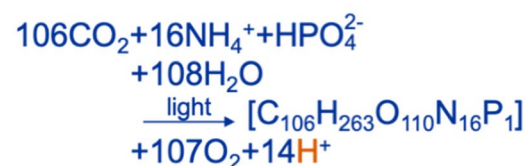
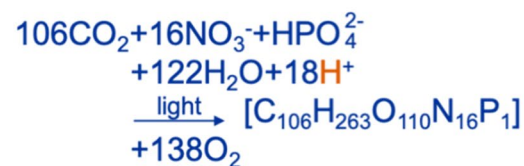
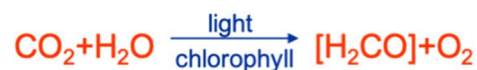
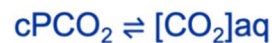
as well as light received per cell change with increased biomass density (b). A.U.: arbitrary unit

into the medium (Fig. 2). In addition, assimilation of inorganic nitrogen and phosphate is accompanied by the uptake or release of H⁺ and OH⁻ altering the total alkalinity, with a slight decrease and increase of H⁺ along with uptake of nitrate and ammonium ions, respectively (Stum and Morgan 1996) (Fig. 2). Consequently, assimilation of the inorganic nitrogen has a negligible effect on pH stability except in very

dense cultures (Goldman et al. 1982) in view of Redfield ratios and the counteractive impacts during utilization of nitrate and ammonium ions on pH (Fig. 2). The amplitude of pH changes is usually larger in batch cultures with high cell density (Fig. 1b).

Since changed levels of physical drivers can also affect chemical conditions by influencing biological processes

Fig. 2 Dissolution of CO₂ into culture medium (X₁) and subsequent carbonate equilibrium (equations next to broken vertical red line), photosynthetic carbon removal (X₂, equations next to solid red vertical line), and assimilation of inorganic nitrogen which has little impacts on pH (equations next to solid green vertical line). K₁ and K₂ represent the coefficients for the first and second dissociations of carbonic acid, respectively, and CA is carbonic anhydrase. Downward or upward arrows indicate directions of pH drop or rise, respectively



(Flynn et al. 2012; Hurd 2015), they have to be considered in algal cultures. Light levels and quality (proportions of differential wavelengths for different light sources) can change due to different levels of cells or algal tissues or number of individuals. Higher levels of algal biomass can result in self-shading, therefore, leading to reduced levels of photosynthetically active radiation (PAR) and/or altered exposures of changed light spectrum to the cells due to differences in primary and secondary absorption (re-absorption of the attenuated light by other cells). On the other hand, water motion dynamics in aerated, mixed or shaken cultures can affect the thickness of diffusion boundary layers (DBL) surrounding cells or individuals; therefore, different levels of water motion or current can result in different levels of pH, $p\text{CO}_2$, and $p\text{O}_2$ near the cell or thallus surface compared to the bulk medium (Hurd 2015; McNicholl et al. 2019). Consequently, inadequate mixing can lead to significant localized variations in all physical and chemical parameters during cultures of algae (Borowitzka 2016). Biologically pumped efflux and influx of ions across the cell membrane is affected by the DBL (Hofmann et al. 2018), the thickness of which influences the transport of nutrients or gases (Märkl 1977) and therefore can affect concentrations of ions, O_2 , and/or CO_2 at the cell surface (Fig. 3), leading to differences in metabolic rates. Usually, faster water current result in thinner DBL (Noisette et al. 2018), and large cells or thalli have thicker DBL; therefore, faster water current speeds can enhance photosynthetic rates of some macroalgae (Gao 1992), mainly due to reduced barrier of the DBL. On the other hand, large cells may experience more acidic (lowered pH) stresses induced by respiratory CO_2 release

during night period due to a thicker DBL (Flynn et al. 2012) when grown in static (without water motion) water, where levels of pH and O_2 are expected to be higher in light (Fig. 3) and to be lowered in darkness.

As aforementioned, biological and physical conditions should be taken into account when trying to manipulate and control pH/ $p\text{CO}_2$ stability in the cultures. In the context of ocean acidification simulation tests, different ways for manipulation of pH and associated seawater carbonate chemistry parameters are explained in general (Gattuso et al. 2010) and for algal research (Gao et al. 2021). In this work, approaches to control pH stability are analyzed according to the involved biological and chemical processes along with the basic principles.

Methodology in different approaches

Controlling seawater pH with aeration in the absence and presence of carbonic anhydrase

When CO_2 dissolves in water, as shown in the equations in Fig. 2, it combines with water to form carbonic acid, which dissociates to form bicarbonate and hydrogen ions, and the bicarbonate ions can further dissociate to form carbonate ions. The protons produced in the first dissociation may reverse to convert carbonate to bicarbonate as shown in the following equation, leading to reduced carbonate concentration due to increased concentration of H^+ . In aerated cultures, air containing certain levels of CO_2 is consistently bubbled into water, $p\text{CO}_2$, and pH in

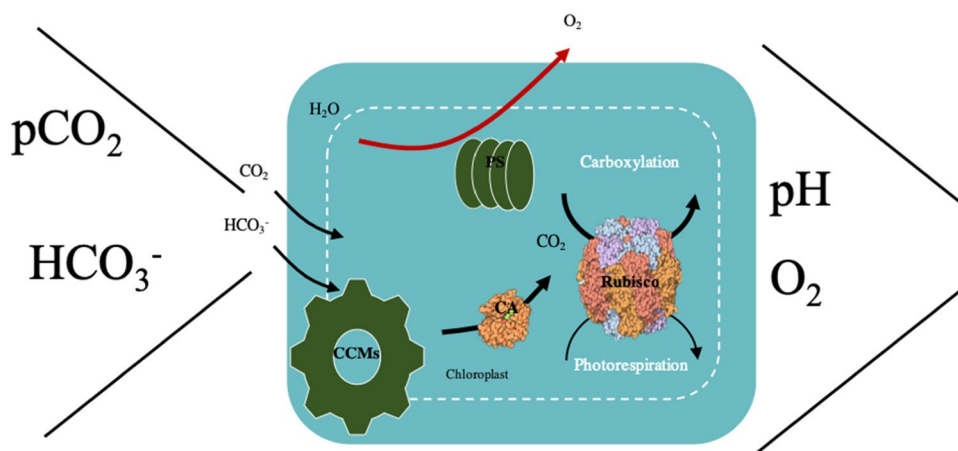


Fig. 3 Conceptual illustration for the thickness (width between the opposingly sloped lines aside the cell) of the diffusion boundary layers (DBL) surrounding the algal cells under light. Note, larger cells or thicker macroalgal thalli are supposed to have a thicker DBL, in which CO_2 and O_2 gradients (as well as pH and bicarbonate) are inversely different toward the cell surface. The width between the

downward and upward sloping lines changes toward the cell surface at each side, indicating that the levels of CO_2 and bicarbonate decline (right side) and that of O_2 and pH increase (left side) due to photosynthetic activities. CCMs within the cell indicate CO_2 concentrating mechanisms. Note that Rubisco is disproportionately illustrated within the chloroplast (circled by broken lines)

the culture are supposed to be constant because the aeration continuously supplies CO_2 which is supposed to be in equilibrium between the air and the culture media. However, since CO_2 dissolution and diffusion (approximately 8 thousand times slower than in air) in water is very slow (Raven and Beardall 2003), the rate of photosynthetic carbon fixation can easily exceed the dissolution rate of CO_2 into the culture system, and therefore, bicarbonate in seawater is utilized by algae via CO_2 concentrating mechanisms (CCMs) with carbonic anhydrase (CA) involved to convert bicarbonate to CO_2 for photosynthesis (Beardall and Raven 2020), leading to pH rise (Figs. 1–3) (Gao et al. 1991; Qiu and Gao 2002). On the other hand, when pH in milieu is high with little pCO_2 available, algae tend to utilize bicarbonate, which further increases the pH of cultures (Keymer et al. 2014).

There is a way to accelerate the dissolution of CO_2 into water, so that the equilibrium in CO_2 partial pressure between the air and water can be reached. Carbonic anhydrase (CA) in water or within cells catalyses the interconversion of bicarbonate and CO_2 ($\text{HCO}_3^- \xrightleftharpoons{\text{CA}} \text{CO}_2 + \text{OH}^-$). By adding CA (soluble) in the culture media, continuous aeration with air of ambient or enriched CO_2 can rapidly lower the pH, and eventually make it stabilized due to the balanced rates of CO_2 dissolution and photosynthetic CO_2 removal (Fig. 4). The function of CA can be visualized in pH changes by stopping or starting the bubbling (Fig. 4) (Gao et al. 1991).

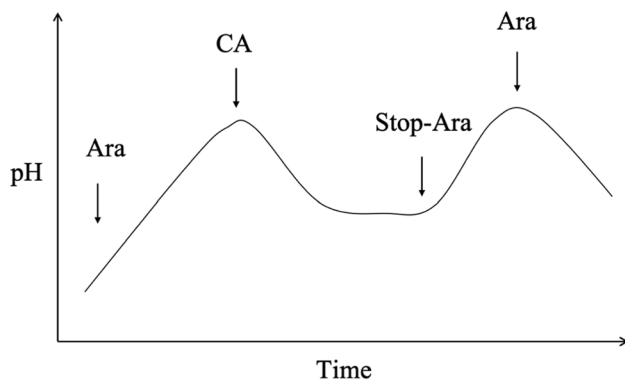


Fig. 4 Changes of pH in algal cultures under light with (Ara) or without (Stop-Ara) aeration before and after the addition of carbonic anhydrase (CA), which accelerates the dissolution of CO_2 into the culture. Addition of CA increases dissolution of CO_2 into seawater, resulting in equilibrium between photosynthetic carbon removal (X_2) and CO_2 dissolution rate (X_1) and stable pH before stopping the aeration (Stop-Ara). Note, even in the presence of CA, pH rises due to lack of CO_2 supply after the aeration is stopped. The arrows indicate the time point when aeration started or stopped or when CA was added (redrawn on the basis of Gao et al. 1991)

Manipulating biomass density to achieve stable carbonate chemistry

Since increasing biomass or cell density in cultures can increase CO_2 removal rate (X_2), which can be greater than the rate (X_1) of CO_2 dissolution into the culture, then utilization of bicarbonate ions by algae results in decreased DIC with increased pH (Fig. 1b; Fig. 2). Usually, O_2 level can be maintained constant if the cultures are aerated. Flow rates of air for bubbling are frequently controlled within a range of 100–500 mL min^{-1} in culture systems of less than 1 L, which is empirically known to be able to maximize the dissolution rate of CO_2 with the tested biomass densities (Table 1). Larger culture vessels of more seawater and/or of higher biomass density require higher aeration rates and need longer time to achieve the equilibrium of CO_2 between the air and medium. It took about 25 h for 30 L pelagic seawater ($< 1 \mu\text{g Chl } a$) to reach carbonate chemistry equilibrium (stable pH) by aerating at a rate of 500 mL min^{-1} with CO_2 levels of 1000 ppmv (Gao et al. 2012a). When $X_2 < X_1$, it takes less than 1 h for the culture of < 1 L seawater to reach stable pH.

Irrespective of the culture methods and various approaches to agitate micro-environments near algal cell surface (Tamiya 1957), increasing biomass density can easily result in faster rate of photosynthetic carbon removal over that of the CO_2 dissolution, therefore, leading to pH rise and unbalanced partial pressures of CO_2 between water and air. In semi-continuous cultures, time interval or span between continual dilutions and the cell density prior to dilution affect the stability of pH in the culture. The cell concentrations and dilution spans in the semi-continuous cultures recommended in Table 1 indicate that lower cell concentrations can usually allow for relatively longer intervals for consecutive dilutions. For the species of slower growth rate, dilution frequency can also be elongated, such as in the cyanobacterium *Trichodesmium* sp. Growing diatoms in bubbled cultures with consecutive dilutions every 24 h is frequently run, since this approach can provide enough cells for physiological and/or molecular measurements (Gao et al. 2012a). The initial or diluted cell concentrations (C_o) can be set at about 5×10^4 cells mL^{-1} , and that (C_i) prior to dilution can be near or less than 20×10^4 cells mL^{-1} (Table 1) (Fig. 5). In dilute cultures of very low cell concentrations (about 100–200 cells mL^{-1}) without aeration, dilution intervals can be elongated with sustained stability of pH, since photosynthetic carbon removal was sufficient not to bring about significant changes in DIC and pH. In this case, cultures should be shaken or rolled or circulated to diminish the effects of DBL, which was shown to be as thick as about 65 mm in a coralline alga in still seawater (Cornwall et al. 2013, 2014). Within the DBL, pH significantly differ from that of

Table 1 Cell concentration ranges, dilution intervals and aeration rates at various culture volumes (air replacement rates per unit volume of culture per time can be obtained by dividing the aeration rate with the volume) documented in some literatures or provided by the

authors for microalgal cultures with pH changes maintained less than 1%. Biomass densities are expressed as chl. a in cyanobacteria and as fresh weight in macroalgae. N indicates none, NS not shown, and flow-thr represents flow-through system, respectively

Algal species	Cell or biomass density (10^3 cells mL^{-1} or g L^{-1})		Dilution intervals	Aeration (mL min^{-1})	Culture volume (L)	References
	Initial or after dilution	Before dilution				
	C_0	C_t				
Diatoms						
<i>Phaeodactylum tricoratum</i>	50	200	24 h	300	0.1	Gao et al. (2012a)
	80	200	24 h	350	0.3	Wu et al. (2010)
	0.1	120	5 day	N		Li et al. (2017)
<i>Skeletonema costatum</i>	50	200	24 h	300	0.1	Gao et al. (2012a)
<i>Thalassiosira pseudonana</i>	50	200	24 h	300	0.1	Gao et al. (2012a)
	0.1	50	4 day	N		Li et al. (2019)
<i>T. weissflogii</i>	0.1	9	4 day	N		Li et al. (2019)
Prymnesiophytes						
<i>Gephyrocapsa oceanica</i>	0.1	80	6 day	N		Tong et al. (2018)
	15	60	24 h	N		Miao et al. (2018)
<i>Emiliana huxleyi</i>	50	120	3 day	N		Xu and Gao (2015)
	20	45	24 h	N		Jin et al. (2015)
	0.2	95	4 day	N		Zhang et al. (2020)
<i>Phaeocystis globosa</i>	90	110	Continuously	700	1.2	Chen and Gao (2011)
Cyanobacteria						
<i>Trichodesmium sp</i>	5	40	4 day	100	<1	Cai et al. (2017)
<i>Trichodesmium sp</i>	3	15 (μg)	3 day	400	NS	Hutchins et al. (2015)
<i>Trichodesmium sp</i>	3	10 (μg)	3 day	400	0.75	Yi et al. (2020)
Dinoflagellates						
<i>Prorocentrum micans</i>	0.5	0.8	24 h	300 (air)	0.8	Zheng et al. (2015)
<i>Alexandrium fundyense</i>	0.4	1.0	3 day	300	0.25	Hattenrath-lehmann et al. (2015)
<i>Karenia brevis</i>	6	30	7 day	4	<1	Bercel and Kranz (2019)
<i>Amphidinium carterae</i>	0.9	6	3 day	100 (air)	1.2	Bausch et al. (2019)
Macroalgae						
<i>Ulva prolifera</i>	1	2 (g)	24 h	300	0.5	Xu and Gao (2012)
<i>Gracilaria lemaneiformis</i>		14 (g)	Continuously	Flow-thr	0.44	Gao et al. (2012b)
<i>Corallina sesilis</i>		30 (g)	Continuously	Flow-thr	0.44	Gao et al. (2012b)
<i>Arthrocardia corymbosa</i>		NS	Continuously	Flow-thr	0.15	McGraw et al. (2010)
<i>Arthrocardia corymbosa</i>		NS	Continuously	Flow-thr	0.65	Cornwall et al. (2014)
<i>Ecklonia radiata</i>		NS	Continuously	Flow-thr	NS	Noisette and Hurd (2018)

milieu (Fig. 3), being higher in the light and lower in the dark compared to that beyond the DBL. The time intervals for these dilute cultures to have stable pH differ according to species with different growth rates or of different CCMs efficiencies. For example, calcification process in calcifying microalgae or macroalgae generates CO_2 and lowers pH, therefore, counteracting the alkalization associated with photosynthetic utilization of inorganic carbon (Gao and Zheng 2010; Gattuso et al. 2010). Consequently, the time span for consecutive dilutions can be longer for the

algal calcifiers (Table 1). Nevertheless, cell or biomass density should be maintained as low as possible to avoid pH drift and significant changes in carbonate chemistry. C_0 (cell concentration after dilution) and C_t (cell concentration before dilution) should fall within the range illustrated in Fig. 5 to maintain pH drift less than 1%, and the values for C_0 and C_t are recommended in Table 1. Since bubbling is known unfavorable for coccolithophores and dinoflagellates (due to dynamic disturbance for attachments of coccoliths and to flagellate movement) (Xing 2015; Zhou

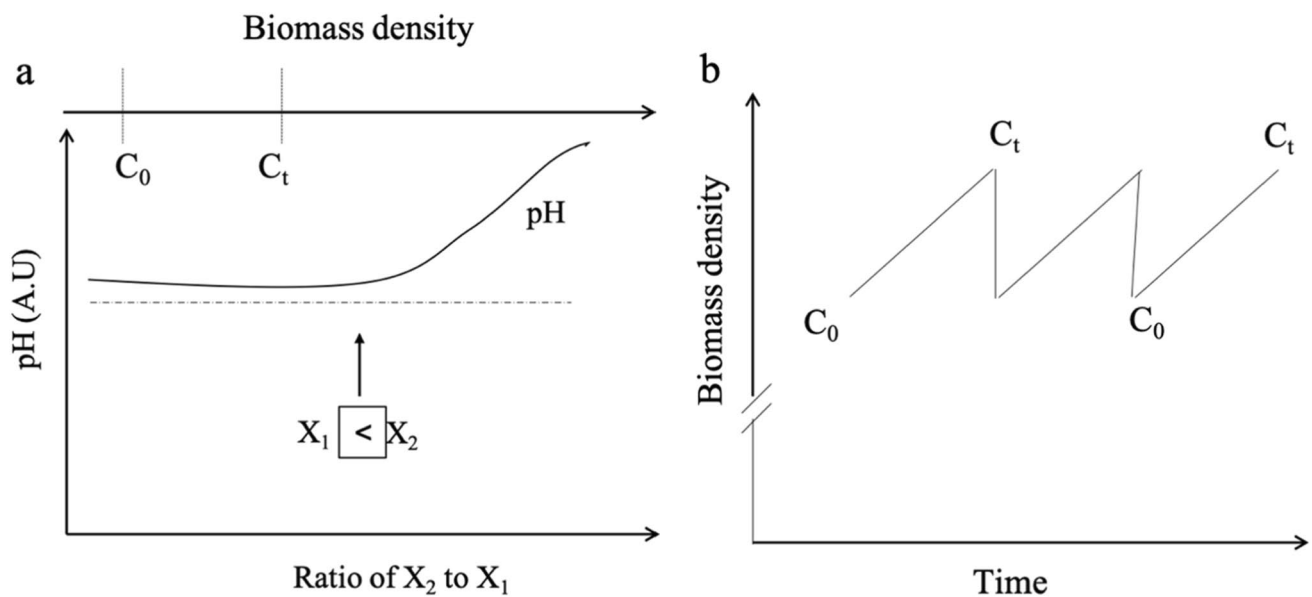


Fig. 5 The stability of pH during dilute (a) and semi-continuous (b) cultures when biomass densities were maintained within the range of C_o (cell concentration after dilution) and C_t (cell concentration before

dilution). Note, within the range of C_o to C_t , rate (X_2) of photosynthetic carbon fixation (CO_2 removal) is negligible or slower than the rate (X_1) of CO_2 dissolution into the cultures

2016), dilute cultures are often run with low cell concentration without aeration. For evolutionary experiments, it is logistically difficult to dilute the cultures frequently; therefore, dilute cultures of very low cell concentrations with relatively longer dilution interval have also been applied to diatoms (Li et al. 2017) (Table 1).

Growing algae in flowing through systems or in natural waters

Both macroalgae and microalgae can be grown in a flow-through system (McGraw et al. 2010; Gao et al. 2012b). For macroalgae, individuals can be fixed on something hard and placed in an assimilation tube, the size of which can differ according to the individual sizes or experimental purpose, and the diameters of the assimilation tubes inversely relate to the current speed within them (Gao and Zheng 2010; Gao et al. 2012b). For microalgae, especially those sensitive to aeration-induced disturbances, the cells can be collected in a dialysis bag or tube and then the bags or tubes can be mounted in the flowing-through system similar to that recommended for macroalgae. Note that larger bags may result in thicker DBL if the “pore size” of dialysis bags is not large enough. Since the dialysis or other polymeric membranes allow gases, nutrients, and small particles to penetrate, carbonate chemistry equilibrium between inner and outer sides can reach within short-time. The absolute time required for the equilibrium can be obtained by testing with the selected membranes and cell concentrations within the membrane bags or tubes. Note that current speed

of seawater is proportional to flowing rate in the assimilation tube, and different current velocities may affect physiological status and the growth of the algae, since thickness of the diffusion boundary layer (Fig. 3) surrounding the thalli, the cells, or the dialysis bags is negatively correlated with current speed (Cornwall et al. 2013). To select membranes of certain permeance, those used for microalgae cultivations can be referred (Bilad et al. 2014). It is worth mentioning that the dialysis or permeable membrane bags should be replaced with new ones regularly to avoid biofouling.

In parallel with the aforementioned flow-through system, one can also consider growing microalgae in the sea (Fig. 6) with permeable membranes or dialysis bags which are commercially available and low-cost (Bilad et al. 2014). However, the carbonate chemistry can also be altered when cell concentrations within the bags reach to high levels that results in $X_2 > X_1$ (Fig. 5a). If one needs to maintain the stability of carbonate chemistry or to expose the cells to similar changes of pH in situ, the cell densities within the permeable membrane or dialysis bags should be tested in advance under the in situ conditions, since intensity of water mixing affects rate of exchanges of molecules between the inside and the outside of the bags (Fig. 6).

Approaches to elevate pCO_2 and control pH in cultures

For massive microalgae cultures, there are a number of approaches to supply CO_2 in order to raise productivity per volume of water (Doucha and Lívanský 2006; Zheng

et al. 2018) or to sustain laboratory microalgae cultures (Pörs et al. 2010). Here, the need to enrich CO_2 is raised for the purpose to simulate high CO_2 environmental conditions, such as that in upwelling areas or under the scenario of future ocean acidification. Gas in researches to investigate ocean acidification effects, general approaches to obtain the desired CO_2/pH conditions can be referred to Gattuso et al. (2010). CO_2/pH in seawater flow-through culturing systems for macroalgae can also be manipulated (McGraw et al. 2010; Gao and Zheng 2010). To aerate the cultures with air of target pCO_2 levels, one can manually mix the air with pure CO_2 and then measure the CO_2 partial pressure or CO_2 concentration. Such an approach can be achieved continuously using a mass-flow meter or by monitoring the partial pressure of CO_2 with a CO_2 measuring device. Alternatively, large bags can be used to store the mixed air of target pCO_2 . The bags should be sealed and be equipped with an inlet and an outlet openings, so that air and desired amount of CO_2 can be pumped in, then they should be rolled to mix for the CO_2 uniform distribution within the inner space (Gao and Zheng 2010). Then, the air of target CO_2 can be pumped out into cultures. This approach is simple and costs less.

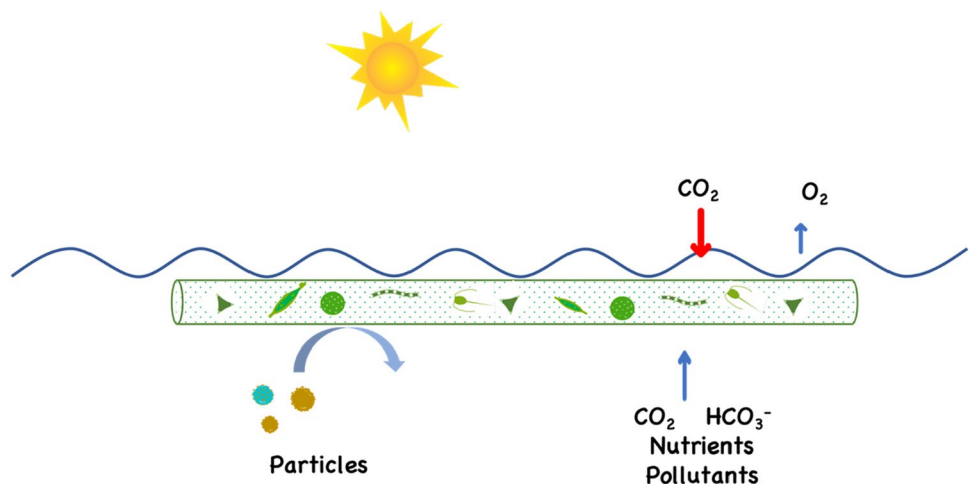
While pH-stats can be used to control pH by automatically and periodically sparging controlled dosage of pure CO_2 into the cultures (Hulatt and Thomas 2011; Han et al. 2013), the pH changes with zigzag-shaped pattern following the on and off of the injection, because dissolution of CO_2 into water and the following adjustment of the carbonate chemistry equilibrium take time (minutes to hours depending on the culture volume, aeration intensity and temperature). This approach is useful for photobioreactors or dense cultures of algae, but the fluctuating pH is not suitable for experiments that require stable pH or carbonate chemistry. Note, even increasing frequency of CO_2 injection can hardly avoid pH zigzag-shaped changes, since dissolution of CO_2 into water is a slow process. Alternatively, there are other CO_2 controlling devices (<http://ruihua.cn/Page/PList.aspx?>

[Cid=53&Page=1](#)), which automatically mix the air with pure CO_2 and supply the air of target pCO_2 levels at adjustable flow rates. Such devices can be used in laboratory and mesocosm tests (Jin et al. 2015) or during research cruises for deck incubations (Gao et al. 2012a). There are also automatic devices that can be used to simulate diel or diurnal pH fluctuations in coastal waters, so that physiological performances can be examined under fluctuating pH regimes (Li et al. 2016). Elevating pCO_2 by adding acid has also been used (Gattuso et al. 2010), but this approach alters total alkalinity of seawater. Therefore, it is not recommended for manipulating pH in algal cultures.

Conclusion and recommendations

There are a many methods or approaches for growing algae. To control pH in dense cultures of both microalgae and macroalgae, high CO_2 over 10 thousand ppmv or pure CO_2 is injected in the cultures (Moheimani and Borowitzka 2011; Hulatt and Thomas 2011). These approaches are useful for massive algal cultures, but can hardly be applied to studies aiming to examine physiological responses to changes in pH and carbonate chemistry, since dense cultures usually lead to large variation in pH as aforementioned. To choose an appropriate method for testing hypotheses or answering scientific questions under constant and/or stable pH levels, it is essential to understand principles involved in the method to be used in order to avoid artifacts or incorrect results (Borowitzka 2020). It has been documented that changes in pH or carbonate chemistry alter physiological performances of algae (see the reviews by Rost et al. 2008 and Gao et al. 2019 and references therein). Therefore, maintaining comparable levels of pH between experimental treatments and controls is essential for comparative studies and/or for reliable values to be compared with others. Bear in mind that (1)

Fig. 6 Illustration of using permeable membranes or dialysis bags to grow microalgae in the sea or lakes. This approach can be used to investigate responses of microalgal species or phytoplankton assemblages to natural environmental changes. Nutrients, pollutants (such as metal ions), CO_2 , and O_2 are allowed to exchange or diffuse along their concentration gradients



dissolution of CO₂ from air phase into water and its diffusion are very slow, usually takes minutes to hours depending on temperature, water volume, aeration rates, and algal biomass density; (2) photosynthetic carbon fixation can result pH rise by reducing CO₂ and/or DIC in the algae-growing medium, and the extent of pH changes depend on the balance of CO₂ dissolution into the cultures and photosynthetic CO₂ removal (Figs. 4 and 5). Therefore, the followings are recommended to sustain pH stability in cultures: (1) make sure to understand the principles for the chosen methods and report details of your experiments, including aeration rates (if the cultures are bubbled), biomass density or its range, dilution frequency if semi-continuous cultures are run; (2) always be aware of that biological processes can affect levels of pH and pCO₂ along with changes in carbonate chemistry, so you can minimize or make the changes identical for reasonable comparisons; and (3) become familiar with the merits and demerits of the chosen method, since almost all methods for aquatic physiology studies have shortcomings (see the chapters in Gao et al. 2021).

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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