Acta Oceanol. Sin., 2018, Vol. 37, No. 12, P. 55–62
DOI: 10.1007/s13131-018-1223-3
http://www.hyxb.org.cn
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An ocean acidification-simulated system and its application in coral physiological studies

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Received 15 June 2017; accepted 15 March 2018

Abstract

Due to the elevated atmospheric carbon dioxide, ocean acidification (OA) has recently emerged as a research theme in marine biology due to an expected deleterious effect of altered seawater chemistry on calcification. A system simulating future OA scenario is crucial for OA-related studies. Here, we designed an OA-simulated system (OASys) with three solenoid-controlled CO_2 gas channels. The OASys can adjust the pH of the seawater by bubbling CO_2 gas into seawaters via feedback systems. The OASys is very simple in structure with an integrated design and is new-user friendly with the instruction. Moreover, the OASys can monitor and record real-time pH values and can maintain pH levels within 0.02 pH unit. In a 15-d experiment, the OASys was applied to simulate OA in which the expected target pH values were 8.00, 7.80 and 7.60 to study the calcifying response of *Galaxea fascicularis*. The results showed daily mean seawater pH values held at pH 8.00±0.01, 7.80±0.01 and 7.61±0.01 over 15 d. Correspondingly, the coral calcification of *G. fascicularis* gradually decreased with reduced pH.

Key words: ocean acidification, OASys, coral, *Galaxea fascicularis*

Citation: Zheng Xinqing, Wang Chenying, Hadi Tri Aryono, Ye Youyin, Pan Ke. 2018. An ocean acidification-simulated system and its application in coral physiological studies. Acta Oceanologica Sinica, 37(12): 55–62, doi: 10.1007/s13131-018-1223-3

1 Introduction

Ocean acidification (OA) is the progressive decline in seawater pH due to oceanic uptake of anthropogenic CO_2 (Kleypas et al., 1999). The projected 0.3–0.4 pH drop by 2100 will result in a 50% decrease in the carbonate ion concentration and a 3–fold increase in hydrogen ions. This modifies the calcium carbonate ($CaCO_3$) saturation state of surface waters (Orr et al., 2005). OA is attracting increasing attention especially because it poses a threat to calcium-based marine organisms. OA has recently emerged as a research theme in marine biology. The response of calcifying marine organisms (i.e., hermatypic corals, coralline algae, mollusks) to OA in terms of their physiological, molecular and community response is a research hotspot (Gattuso et al., 1999; Buddemeier et al., 2008; Abbasi and Abbasi, 2011).

One important research topic is the effects of OA or varied carbonate chemistry of seawater to calcifying marine organisms under controlled laboratory conditions (Hoegh-Guldberg et al., 2007; Anthony et al., 2008; Jokiel et al., 2008; Hofmann et al., 2010; Edmunds, 2011; Comeau et al., 2013a, 2014a; Huang et al., 2014). Studies examining the effects of ocean acidification on marine organisms are usually done via perturbation experiments: organisms are exposed to acidified seawater and the changes in growth or calcification are monitored over hours to weeks (Doney et al., 2009). Recently, scientists have studied the molecular response of calcifying organisms to OA (Hillhouse and Grammatopoulos, 2006; Carreiro-Silva et al., 2014). These studies reveal the physiological plasticity of calcifying organisms under OA, which allows them to endure or compensate for the varied carbonate chemistry of seawater (Hillhouse and Grammatopoulos, 2006; Fabry et al., 2008; Hofmann et al., 2008, 2010).

One of key elements in OA-related physiological studies is that the systems simulate future OA scenarios. Due to technical limitations, however, most of OA experiments in the past were conducted via the addition of HCl, $NaHCO_3$ or NaOH (Marubini and Atkinson, 1999; Langdon et al., 2000; Langdon and Atkinson, 2005; Marubini et al., 2008; Andersson et al., 2009). For example, more than 70% of OA studies on hermatypic corals publicized before 2011 used diluted HCl, $NaHCO_3$ or NaOH to change the pH and carbonate chemistry of seawater (Erez et al., 2011). Strictly speaking, there is a different scenario of OA. For example, the addition of HCl simultaneously reduces the pH, HCO_3^- and CO_3^{2-}, but OA is caused by oceanic uptake of anthropogenic CO_2 that lowers pH and CO_3^{2-}, but increases HCO_3^-. Therefore, an OA model was created by bubbling CO_2 into experimental seawaters in OA-related studies in recent years (Anthony et al., 2008; Edmunds, 2011; Comeau et al., 2014a).

In this study, an OA-simulated system (OASys) was designed with three solenoid-controlled CO_2 gas channels. The OASys adjusts the pH of the seawater by bubbling CO_2 gas into seawater via feedback systems. The pH controller is adjusted using a

Foundation item: The China-ASEAN Maritime Cooperation Fund Project "China-ASEAN Countries Collaboration on Marine Endangered Species Researches" and "China-ASEAN Marine Protected Areas Ecosystem Management Network", Regional Demonstration of Marine Economy Innovative Development Project under contract No. 16PZY002SF18; the China-Indonesia Maritime Cooperation Fund Project "China-Indonesia Bitung Ecological Station Establishment"; the National Natural Science Foundation of China under contract No. 41506123.

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solenoid valve that open when the pH increases 0.02 unit above the desired level. The OASys prototype monitors and records pH in real-time via table computer. To test the effectiveness of the OASys in coral physiological studies, a 15-d experiment was carried out to study the calcification effect of OA to a scleractinian coral *Galaxea fascicularis*. The OASys was applied to simulate the scenarios of OA in which the expected target pH is 8.00, 7.80 and 7.60.

2 Materials and procedures

2.1 *System schematic and structure*

A schematic of the simulation is shown in Fig. 1. It is composed of a pH intelligent feedback systems (IFS) and solenoid-controlled CO_2 gas regulation system (SCGRA) regulated by IFS. IFS features a pH controller that is connected with a pH probe immersed in experimental seawater, solenoid valve, indicator light and both power adaptors. They supply 24 V power for the solenoid valve and 220 V power for the pH controller, respectively. The SCGRA contains a gas tube, CO_2 gas bottle, pressure valve, control valve and gas diffuser. The OASys adjusted the pH of the seawater by bubbling CO_2 gas into seawater via the solenoid valve. The pH controller is adjusted using a solenoid valve that opened when the pH increases 0.02 unit above the desired level. Pure CO_2 is injected from the compressed CO_2 tanks via this solenoid valve.

2.2 *Description of OASys prototype with three gas pathway*

According to OASys schematic, an OASys prototype with three solenoid-controlled CO_2 gas channels was designed and created to simulate OA. The structural diagram and resulting OASys prototype are shown in Fig. 2 and Fig. 3. A custom solenoid valve made from stainless steel was used to avoid possible metal contamination seawater via the gas tube (i.e., most of solenoidvalves is made of Cu that is lethal to marine organisms). This prolongs the life of the solenoid valve. Three paralleled pH controllers with RS–485 were connected with a table computer with USB interface that control and record the pH of the seawater in the tank. We programmed the procedure to transmit data from three paralleled pH controllers to table computer. A monitor and control generated system (MCGS) for the OASys prototype was programmed. This is a controlling software, that shows the real-time variation of pH in the software interface, which collects data when the OASys prototype is running (Fig. 3). The data can be exported via USB interface after the experiments end.

2.3 *Stability test of OASys prototype*

Three glass tanks with fresh seawater were prepared to test the stability of OASys prototype. Seawater was made by mixing the deionized water and reef crystal (Aquarium System, French) to a salinity of 33. Three targeted pH (8.00, 7.80 and 7.60) were set in the OASys prototype. The MCGS software saves data every 5 min. The OASys prototype continuously ran for 6 d. The pH probe of the prototype was calibrated before the test begun. The pH buffer 4.01, 7.00 and 9.18 were used, and were held precisely to ±0.02 units. pH values of seawater in three tanks were measured for 2 d (F5, Mettler Toledo) to compare the difference between the values measured by F5 and monitored by the OASys prototype. The pH probe of the OASys prototype was calibrated 4 d after the OASys prototype run.

2.4 *The application of OASys in coral physiological studies*

To test the effectiveness of the OASys in coral physiological studies, a 15-d experiment was carried out to study the calcification effect of OA on a scleractinia coral *G. fascicularis*. Experiments were performed at the Laboratory of Coral Conservation, Xiamen, where colonies of *G. fascicularis* were sampled from Houhai coastal waters (18°16′41.77″N, 109°44′0.01″E), Sanya City, Hainan and cultivated in a recycling aquaria system based on the Berlin System featuring ample live rock.

The parent colonies of each coral were divided into at least fifty small fragments (3–4 cm in length) of similar size using pliers. All of the nubbins were scaled using the buoyant weight method prior to the experiment (Davies, 1989), and choose

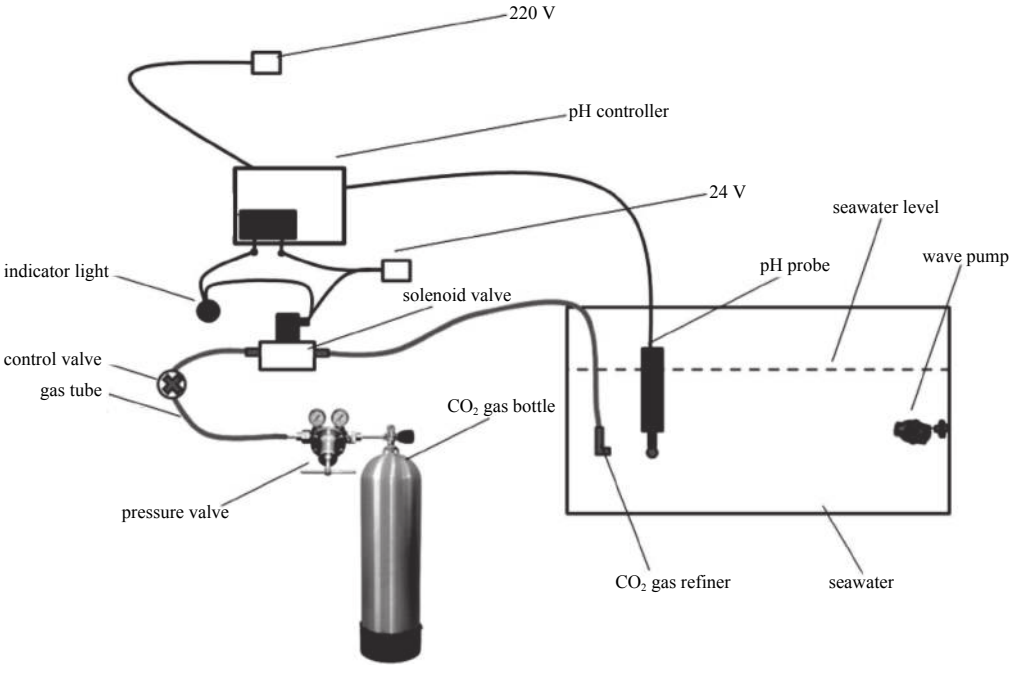

Fig. 1. The schematic of the experimental system designed to simulate ocean acidification.

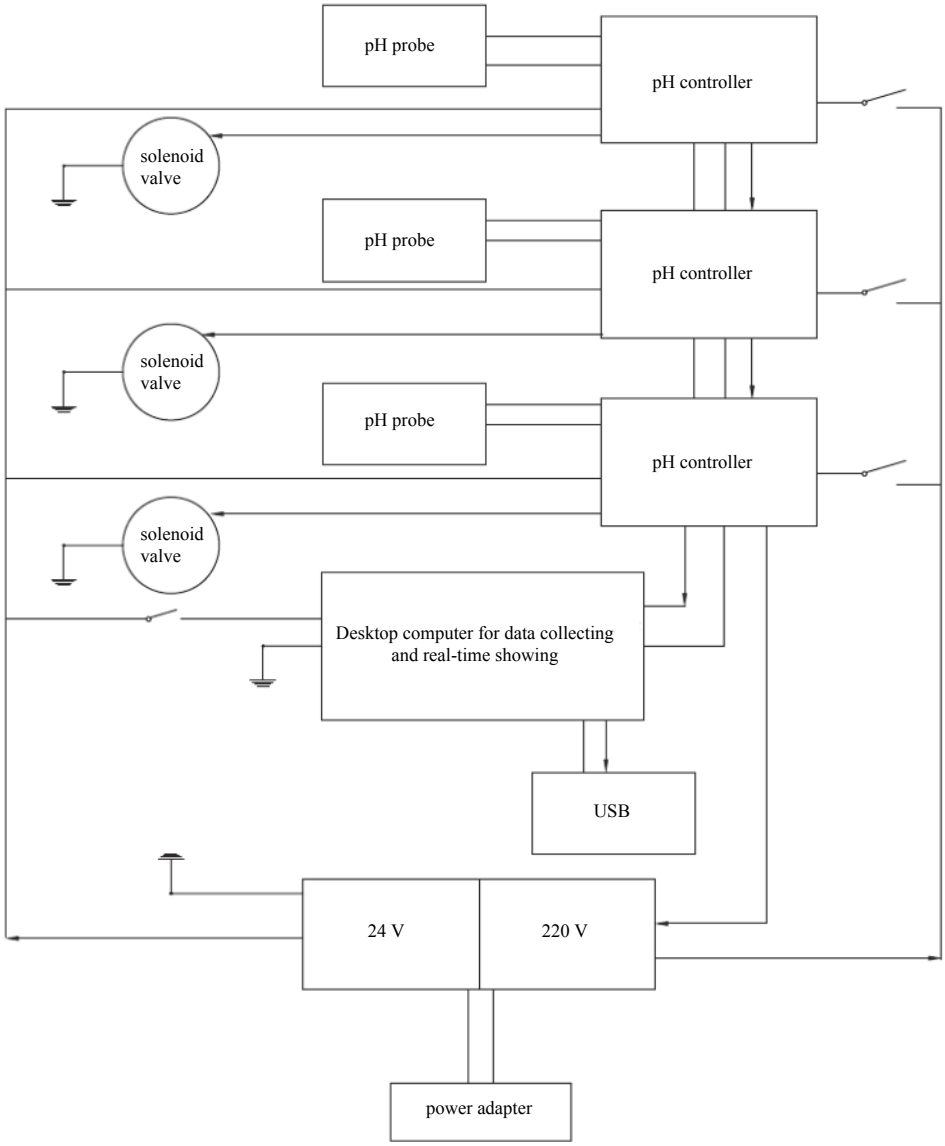

Fig. 2. The structure diagram of OASys prototype with three gas pathways.

thirty-six nubbins with similar growth rates to minimize coral growth difference.

Treatment was created in three 50 L glass tanks ($L\times W\times H$: 30 cm×30 cm×50 cm) indoors with artificial illuminating. The tanks were divided into two parts by black acrylic plate with a comb separator. The back part was equipped with a water compensator and the pump (Rio 400, 250 L/h). The water compensator can supply the evaporated freshwater, and the water pump introduces the seawater into the front part of tanks and causes water flow. Seawater was made by mixing deionized water and reef crystal with a salinity of 33. Three OA treatments (8.0, 7.8 and 7.6) were created by bubbling CO_2 diffuser into the tanks.

When the OASys prototype starts, seawater will be acidified to our set values by bubbling CO_2 diffuser into the tanks. After the pH values of tanks stabilized for 24 h, *G. fascicularis* fragments were placed in the tanks. The MCGS software saves data every 5 min. There are 12 randomly chosen *G. fascicularis* fragments in each tank (Fig. 4). The experiments last for 15 d. The calcification rates of two corals were determined as differences in buoyant weight (underwater) between the time interval (Davies, 1989).

Seawater chemistry in the tanks was sampled in 200 mL volumes with a two-drop saturated $HgCl_2$ solution daily. Total alkalinity (TA) used potentiometric titration according to standard operation procedure SOP 3 b (Dickson et al., 2007). TA certified reference materials (CRMs) provided by A. G. Dickson (SIO, UCSD) were used for calibration and accuracy assessment. The titrant (0.1 mol/L HCl) was standardized using the CRM with ±0.1% precision.

The calcification rates of hermatypic coral *G. fascicularis* among three pH treatments were compared by HSD test of One-Way ANOVA. The significance level was $p<0.05$.

3 Results

3.1 *Stability test of OASys prototype*

Figure 5 shows the real-time pH values of the seawater that was continuously monitored by OASys prototype. The pH values of the seawater for three test tanks were very stable with average values of 7.59±0.01, 7.81±0.01 and 8.01±0.01, respectively. It is highly consistent with our target values (7.60, 7.80 and 8.00, re-

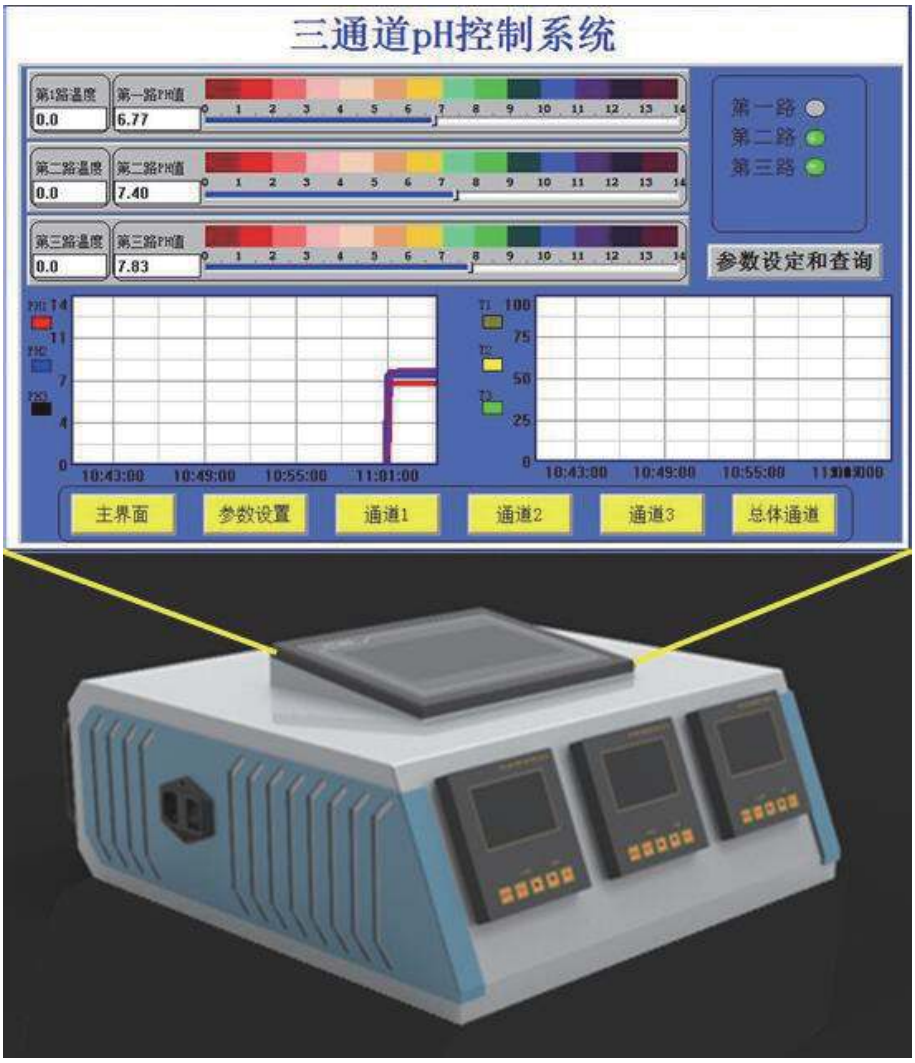

Fig. 3. OASys prototype and its MCGS software.

Fig. 4. OASys prototype applied in physiological studies of scleractinia coral *Galaxea fascicularis*. Three pH values (8.00, 7.80 and 7.60, respectively) were set in OASys prototype.

spectively) in OASys prototype. The sharp slope in the figure shown with the rectangle is due to CO_2 gas continuously placed into the tanks during OASys prototype calibration. Thus, the CO_2 gas pathway must be turned off during the calibration.

To test the stability of OASys prototype, pH values of seawater in three tanks were measured with an F5 portable pH-meter on March 6, March 9 and March 11 to compare the difference between the values measured by pH meter and monitored by OASys prototype. The results showed that there is a positive linear relationship between the real-time pH values measured by

OASys prototype and those by pH-meter in fresh seawater (y=0.996x–0.047, r^2=0.997, n=9, p<0.001, Fig. 6a). Moreover, there was no difference between pH values of seawater measured by the F5 pH-meter before and after the calibration (March 9 vs. March 11, Paired Samples t-test, t=–0.383, df=2, p=0.739, Fig. 6b). This suggests that the pH values monitored by OASys prototype are reliable and can be used in OA-related physiological studies if the calibration is carried out every 3 d.

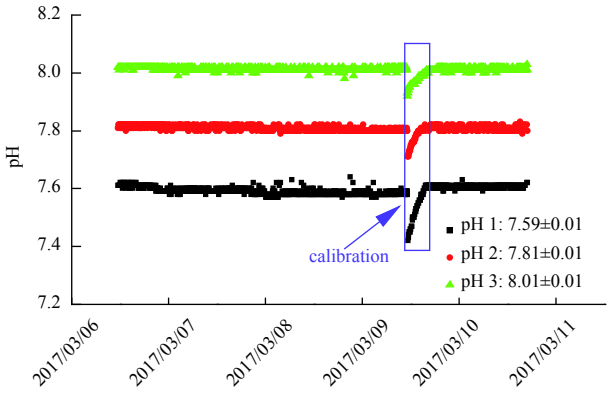

Fig. 5. The real-time pH values of the seawater continuously monitored by OASys prototype over a 6-d experimental period. Three pH values (8.00, 7.80 and 7.60, respectively) were set in OASys prototype. The sharp slope in figure shown with the arrow is because the CO_2 gas pathway was held when the OASys prototype was calibrating.

3.2 *The application of OASys in coral physiological studies*

The OASys prototype was applied to study the calcified effect of OA on reef corals. As shown in Fig. 7a, pH values of the seawater for the three tanks were very stable during 15 d experiments and were maintained at pH 7.60±0.02 for Tank 1, 7.81±0.01 for Tank 2 and 8.01±0.01 for Tank 3, respectively, which are highly consistent with the set target points (7.6 for Tank 1, 7.8 for Tank 2 and 8.0 for Tank 3). Extremely significant differences were found for pH among three tanks (p<0.001).

The OASys prototype also recorded the variation of water temperature during 15–d experiments from 21.80°C to 23.95°C for Tank 1, from 21.71°C to 23.94°C for Tank 2, from 21.46°C to 23.69°C for Tank 3. A similar variation trend of the temperature for the three tanks showed the obvious effects of ambient environment due to a lack of a chiller.

Although the mean calcification of *G. fascicularis* was higher

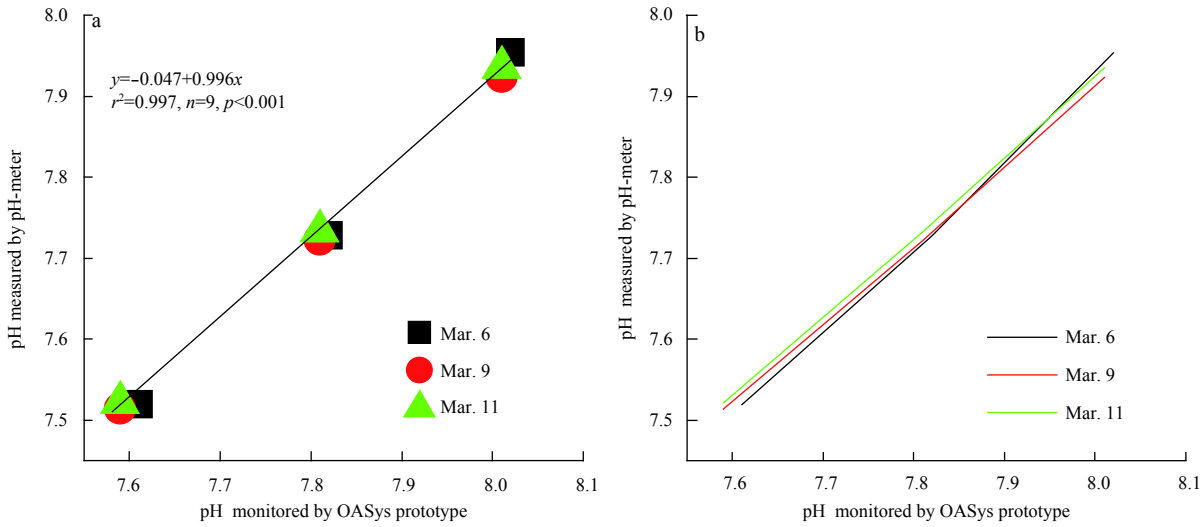

Fig. 6. Comparison of real-time pH values measured by OASys prototype and by pH-meter in fresh seawater. The pH-meter was calibrated before measuring the pH in the seawater. Paired Samples t-test showed no difference between pH values of seawater measured by F5 pH-meter before and after the calibration (March 9 vs. March 11).

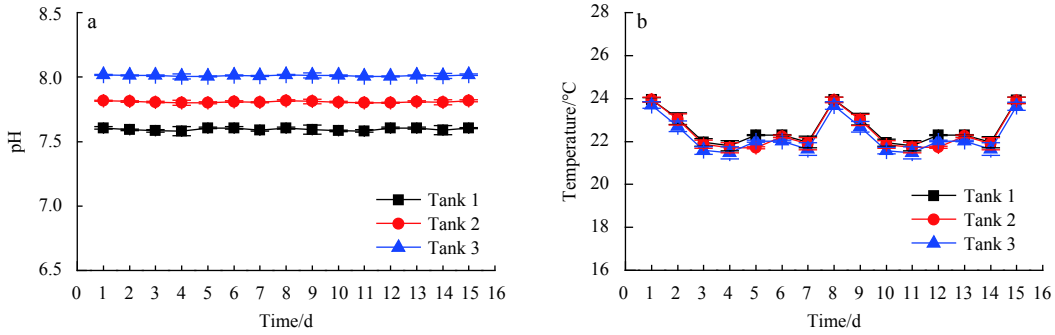

Fig. 7. The pH (a) and temperature (b) in three tanks acidified via bubbling CO_2 gas that was regulated by an OASys prototype over a 16-d experimental period. Three pH values (8.0, 7.8 and 7.6, respectively) were set in the OASys prototype. The pH values and temperature were recorded every day (144 data every day) and were averaged.

at pH 8.0 than that at pH 7.8, no significantly statistical difference was found (Fig. 8, p=0.174). With pH reduced down to 7.6, the mean calcification of *G. fascicularis* at pH 7.6 was (0.082±0.050)%/d, which is far lower than that at pH 8.0, indicating *G. fascicularis* is strongly affected by reduced pH and elevated CO_2.

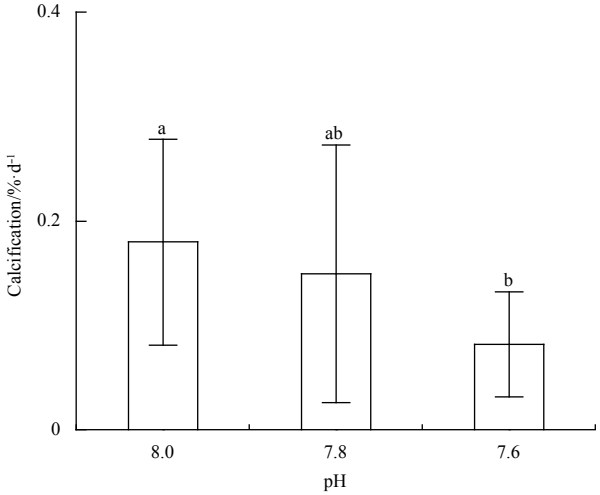

Fig. 8. The calcification of *Galaxea fascicularis* in acidified seawater (mean±SE) over the 16-d experimental period. The difference letter over the bar showed the difference among OA treatments. The significance level is p<0.05.

4 Discussion

4.1 *Comparison of OASys and other OA-simulated systems*

Previous reports have described common OA-simulated systems in OA-related physiological studies via pCO_2 generation system (PGS) (Comeau et al., 2013b, 2014b; Dufault et al., 2013; Edmunds et al., 2013; Rivest and Hofmann, 2014), in which the pCO_2 treatments were established by bubbling CO_2–enriched air into the tanks. The CO_2–enriched air was created with a solenoid-controlled gas regulation system that received pure CO_2 from a gas cylinder and ambient air from an air pump. Mass-flow controllers and a solenoid valve were used to blend the air and CO_2 in a mixing chamber from which gas was drawn to measure pCO_2 with a gas analyzer (Fig. 9). The chemistry of the seawater regulated by PGS varied only slightly (Fangue et al., 2010), which is very suitable for OA studies. However, as shown in Fig. 9, PGS consist of many pieces of equipments and electronic components, including CO_2 gas cylinders, air pump/compressor, solenoid valves, mass-flow controllers, the chamber for blending air and CO_2, CO_2 gas analyzer, the small devices making the dry air without CO_2 gas and particles, etc. It is very complex in structure, and researchers have to spend a lot of time learning how to assemble PGS due to the lack of mature products on the markets that can be used directly. It is bad for the development of OA physiological studies. Moreover, the PGS makes tremendous noise when running air compressors, which possibly interrupts the physiological status of the experimental organisms, especially for fish (Codarin et al., 2009; Slabbekoorn et al., 2010).

The OASys is very simple with an integrated design (Figs 3 and 4), and the researchers do not need to learn how to assemble it but can easily use it via the instruction. Most importantly, the OASys is very accurate and can maintain pH levels within 0.02 pH (Figs 5 and 7a). Moreover, the OASys can continuously record the real-time pH variation, and it records the variation of pH at any time over a long-term experimental period. If the OASys encounters problems, we can identify and diagnose the issue. In fact, OA-simulated systems based on the same principles as the OASys have been used in previous studies, and called a pH-stat systems (Iguchi et al., 2012; Carreiro-Silva et al., 2014), but real-time pH variation in experimental seawater was not logged or publicized in these papers (Leclercq et al., 2002; Iguchi et al., 2012; Carreiro-Silva et al., 2014), therefore we cannot compare the advantages and disadvantages between the OASys and the pH-stat systems. However, OASys was experi-

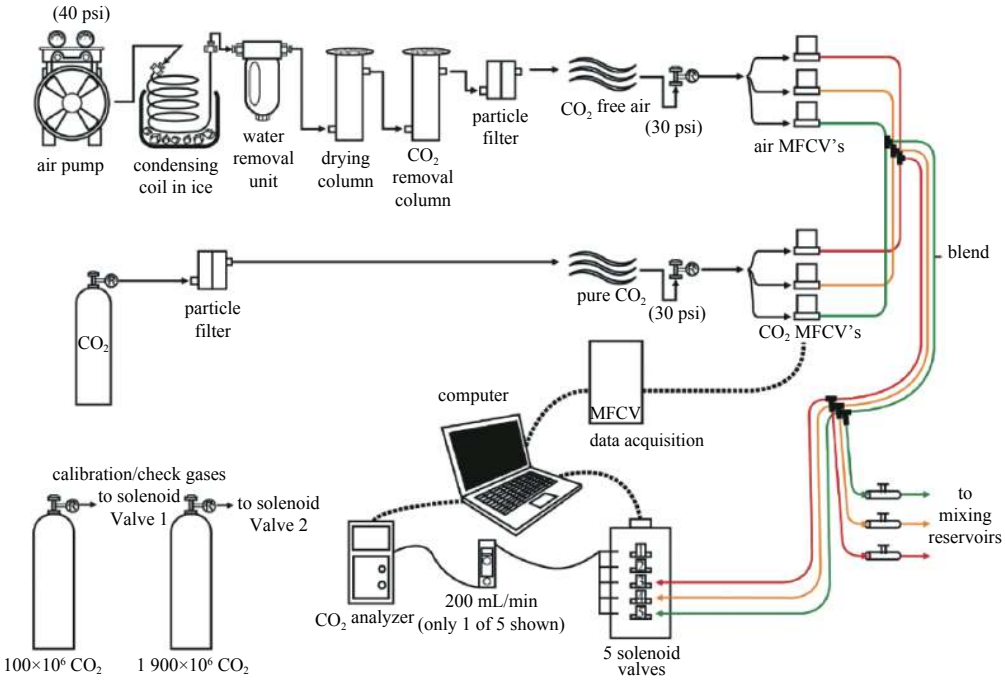

Fig. 9. Diagram of the pCO_2 generation system (originated from Fangue et al. (2010)).

mentally confirmed to be accurate and stable (Figs 5, 6 and 7a). The pH probes of the OASys need be calibrated every 5 d to guarantee the accuracies and stabilities (Fig. 6).

Barry et al. (2008) designed a gas controlled aquarium (GCA) system used for laboratory studies of ocean acidification on marine animals (Barry et al., 2008). Membrane contactors connected to the recirculation pumps and gas sources control gas concentrations in experimental tanks (Barry et al., 2008). Thus, this has a similar operating principle as PGS. Control of CO_2 is slightly less accurate with variability of about 10% (Barry et al., 2008), which is far lower than the OASys. McGraw et al. (2010) also developed an automated 12-tank culture system using a spectrophotometric pH measurement system with a feedback system that can maintain pH levels between 7.51 and 8.00 within 0.02 pH units at 15.1°C (McGraw et al., 2010). The system is close to the OASys in accuracy, but it is meant for small indoor experiments, i.e., 150-mL culture tank reported by McGraw et al. (2010). Thus, it is very useful for a range of small marine organisms, including phytoplankton or invertebrate larvae (McGraw et al., 2010), but it is not suitable for meso- or macrocosm with water bodies from 100 L to several tons. OA studies conducted in mesocosm or macrocosm have attracted more attention from scientists (Jokiel et al., 2008; Andersson et al., 2009) because they build an environment similar to the field for animals and the conclusions or findings originating from these experiments are more convincing. Obviously, McGraw's systems are not fit for these studies. The OASys is more stable for larger body of water because of larger pH or CO_2 buffering capacity when bubbling CO_2 gas into seawater. Therefore, it is an effective tool to simulate OA environments in a mesocosm or macrocosm.

4.2 *Effects of OA to the calcification of hermatypic coral Galaxea fascicularis*

Galaxea fascicularis is a common hard coral with thick colonies in Indo-Pacific region (Zheng et al., 2013), which has been previously used in OA-related studies, but focused on heterotrophy after long-time exposure to elevated CO_2 (Smith et al., 2016) and coral competition mechanism for space, i.e., by mesenterial filaments (Evensen et al., 2018), instead of calcification. In this case, we concentrated on the variation of *G. fascicularis* calcification rates under OA and showed that the reef coral showed a decreased trend in mean calcification with reduced pH (Fig. 8), in line with most previous studies (Langdon and Atkinson, 2005; Anthony et al., 2008; Jokiel et al., 2008; Albright and Langdon, 2011; Chauvin et al., 2011; Ries, 2011; Dufault et al., 2013; Comeau et al., 2014b; Huang et al., 2014; Zheng et al., 2018), although only slight differences were found between at pH 8.1 and at pH 7.8.

This may be due to the diffusion limitation of net H^+ transport through the boundary layer caused by increasing $[H^+]$ in the water column (Jokiel, 2011). Because coral calcification is directly affected by the chemical composition in the calcification fuild (CF) rather than ambient seawater, the evidence of skeletal $\delta^{11}B$ in other hermatypic corals that reflects the pH in the calcification fluid (pH_{CF}) (McCulloch et al., 2012a, b; Tanaka et al., 2015), suggests that *G. fascicularis* gradually decreased the calcification with elevated CO_2 is related to the possibilities that the reef-building coral is insufficient to significantly up-regulate the pH_{CF}. Consequently, the Ω_{CF} increases via exchange of H^+ with Ca^{2+} and decrease seawater pH.

McCulloch et al. (2012a) stated that up-regulation of pH is not ubiquitous among calcifying organisms; those lacking this ability are likely to undergo severe declines in calcification as CO_2 levels increase. Tanaka et al. (2015) estimated that the aragonite saturation state of the Ω_{CF} for *Acropora digitifera* is elevated by a factor of 5–10 relative to ambient seawater in seawater pH of 8.1, 7.8 and 7.4 (Tanaka et al., 2015). Thus, reduced pH by bubbling CO_2 into seawater from 8.25±0.02 to 7.56±0.04 has no effects on its calcification (Takahashi and Kurihara, 2013). Strong tolerance to OA were revealed in typical scleractinian corals *Stylophora pistillata* and *Porites* spp. (McCulloch et al., 2012a), some species of cold water corals (Rodolfo-Metalpa et al., 2010; McCulloch et al., 2012b) using boron isotope systematics.

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