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Biomonitoring System to Assist in Early Detection of Oxygen–deficient Sea Water using Shell Valve Movements of Pacific Oyster (*Crassostrea gigas*)

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To develop a biomonitoring system to assist in the early detection of oxygen–deficient water in aquaculture settings, we conducted laboratory and field experiments using shell valve movements (SVMs) of Pacific oysters (*Crassostrea gigas*). Typical patterns of SVM showed that closing was quick (closing time ~1 sec), whereas opening was slow (opening time ~10 min). Under normal conditions, the SVMs averaged 6 ± 3 times/h. In a laboratory test, however, SVMs responded immediately when the dissolved oxygen concentration decreased and increased to 30 ± 17 times/h. When the dissolved oxygen concentration was only 2.0 ppm, all experiments recorded no SVMs; instead, the shells remained closed. Abnormally high or low rates of SVM were a regular occurrence under hypoxic and anoxic conditions. In field experiments, oyster shells remained closed in hypoxic water, but the rate of SVMs rapidly increased before and after exposure to hypoxic water, just as in the laboratory experiment. Thus, the observation of rapid SVMs or no SVM might serve as a biomarker of abnormal conditions, such as oxygen–deficient water.

Key words: Biomonitoring system, Shell valve movement (SVM), Pacific oyster (*Crassostrea gigas*), oxygen–deficient water

INTRODUCTION

Excess nutrients and terrestrial run–off, often associated with eutrophication, lead to greater organic matter concentrations and higher primary production in aquatic systems. Such nutrient enrichment enhances hypoxia, defined as a dissolved oxygen (DO) concentration <2.5 ppm, and anoxia, DO concentration <0.025 ppm, in the benthic layer (Yanagi, 1989). Moreover, when these conditions are coupled with adverse meteorological and hydrodynamic conditions, the frequency and severity of hypoxia and anoxia increase (Karim *et al*., 2003). Hypoxic and anoxic water masses often migrate toward the surface due to the effect of physical factors such as advection and wind mixing (Fujiwara and Yamada, 2002; Grantham *et al*., 2004; Yanagi, 2004; Takahashi *et al*., 2009). These events cause reduced biodiversity locally and alter the community structure and ecology of a water body. Consequently, oxygen–deficient conditions have caused substantial economic losses to fisheries and the aquaculture industry (Imabayashi, 1983; Westernhagen *et al*., 1986; Lim and Hong, 1994; Karlson *et al*., 2002; Karim *et al*., 2003; Kim *et al*., 2006). In Korea, hypoxic water has been reported from several semi–enclosed bays located in the southern and western part of the Korean peninsula every summer (Lim *et al*., 2006). The hypoxic water also causes serious economic and social problems due to the inability of cultured mollusks (oysters, mussels, and Manila clams) and finfish (olive flounder, rockfish, and sea bream) to thrive.

Mooring systems with chemical/physical sensors in semi–enclosed areas are utilized to monitor abnormal water quality conditions such as hypoxia, red tides, and low–salinity water (Nam *et al*., 2005; Greenwood *et al*., 2010). In Korea, moored real–time monitoring systems under the control of the National Institute of Fisheries Science of Korea operate at 27 sites to protect the aquaculture environment (http://www.nifs.go.kr/risa/subpage/index.jsp). However, these systems require the specialized knowledge to operate and maintain. In addition, there has been no on–site monitoring method of biological responses of aquatic organism. An ideal early warning biomonitoring system would be one that is simple to install and easy to operate and that is highly sensitive to environmental factors with biological responses (Oh *et al*., 2013). Many organisms have been reported to react to various chemical substances (e.g., fishes, crustaceans, microalgae, and bacteria; Baldwin and Kramer, 1994; Sluyts *et al*., 1996; van der Schalie *et al*., 2001 Untersteiner *et al*., 2005; Allan *et al*., 2006; Gerhardt *et al*., 2006; Moroishi *et al*., 2009; Oh *et al*., 2013). In 2002, a biomonitoring system using zooplankton (i.e., *Daphnia* sp.) and Japanese rice fish was introduced to Korea's National Water Quality Auto–measurement System in order to manage and monitor water quality in rivers (Jung *et al*., 2009). However, that biomonitoring system was designed to function in freshwater by using freshwater organism.

Bivalves have been used as bio-indicators for determining the health index of regional environments because of the accumulation of trace contaminants in

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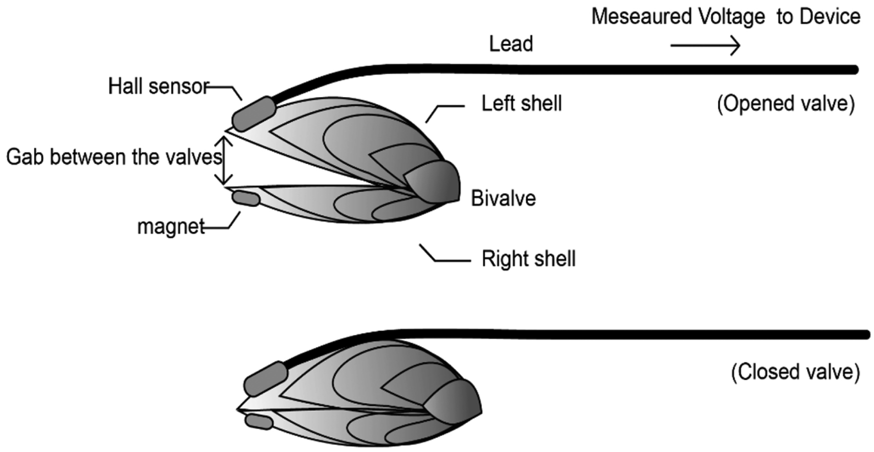

Fig. 1. Schematic diagram of a shell valve movement measuring system of a Pacific oyster (*Crassostrea gigas*).

their bodies and their sedentary behavior patterns (Kramer and Botterweg, 1991; Jeng *et al*., 2000; Moroishi *et al*., 2009). The shell valve movement (SVM) of bivalves is affected by physiological factors including respiration, filter feeding, heart rate, and circadian rhythm (i.e., diurnal and tidal cycles) and physical factors such as predator avoidance and external stimuli (Rao, 1954; Langton, 1977; Ameyaw–Akumfi and Naylor, 1987). The measurement of SVMs has been used to monitor the physiological condition of shellfish in aquaculture systems (Higgins, 1980; Fujii and Toda, 1991). Recently, SVMs have also been utilized as a monitoring tool for detecting abnormal conditions such as harmful algae and heavy metals (Markich *et al*., 2000; Nagai *et al*., 2006; Moroishi *et al*., 2009; Tran *et al*., 2010). Go *et al*. (2009) have tested changes in the SVMs of Akoya pearl oysters (*Pinctada fucata*) after exposure to hypoxic water and hydrogen sulfide and have found that a monitoring system using SVMs can detect abnormal conditions in coastal areas. To our knowledge, there is no study to evaluate the effect on behavior of bivalves exposed to hypoxia condition in the field experiment.

In this study, laboratory experiments were first conducted to identify the effects of oxygen–deficient seawater on the SVMs of Pacific oyster (*Crassostrea gigas*). Then, to assess the feasibility of using this organism as a sentinel for the early detection of oxygen–deficient seawater in the field, a biomonitoring system based on SVMs of Pacific oyster was tested in northern Gamak Bay, Korea. Based on our findings, we discuss the availability of using the SVMs of Pacific oysters for the early detection of oxygen–deficient seawater.

MATERIALS AND METHODS

Measurement of shell valve movements

The test organism is farmed widely in the southern and western coastal regions of Korea and is among the most highly produced (~200,000 ton/year) of all Korean shellfish in aquaculture (MOF, 2017). A method developed by Nagai *et al*. (2006) was used to measure SVMs. Other methods were considered unsuitable because the heavy weight and complexity of the sensors might have made it difficult to measure the SVMs in a natural environment (Fuji, 1979; Higgins, 1980; Jenner *et al*., 1989). The method developed by Nagai *et al*. (2006) is based on a magneto–electric device, the Hall element sensor (weight: ~2 g; sensitivity: 15–1000 mV; adjustable measurement speed: 0.5–2 sec; A1369EUA–24–T, Allegro MicroSystems LLC., Auburn Hills, MI, USA), which is a transducer that varies its output voltage (mV) in response to a magnetic field (Ramsden, 2006). Changes of the output voltages between the Hall element sensor on the left valve and the magnet on the right valve were continuously recorded in a data logger (OT–SVML–001, Oceantech Co., Busan). A Hall element was attached to the edge of the left valve of an oyster using CorAffix (Coral Gum 104.74, Tunze Co., Penzberg, Germany), which is a bio–friendly adhesive for coral. A magnet was attached to the edge of the right valve directly opposite the sensor on the other valve using the same adhesive (Fig. 1).

Shell valve movements during light and dark periods

We investigated changes in SVMs during light and dark periods to identify the circadian rhythm of Pacific oysters in response to a diurnal cycle. Eight 2–year–old Pacific oysters (shell length: 100 ± 30 mm; shell width: 30 ± 10 mm; shell height: 30 ± 10 mm; wet weight: 100 ± 30 g) that had been cultured in Gamak Bay were placed in an aquarium with 30 L of filtered seawater (pore size 1.0 μm), while controlling the temperature, salinity, pH, and light intensity at 15 ± 1°C, 32 ± 1, 8.1, and 130 μmol photons/m^2/sec (12:12 h L:D cycle), respectively. The DO concentration was maintained at 8.0 ppm so as to avoid the direct exposure of oysters to air bubbles. Before the experiments commenced, all oysters fasted for 3 days to exclude the influence of feeding and excretion. Changes in SVMs during day and night were measured under white light–emitting diodes attached to the roof of the aquarium that could be masked with blackout paper to achieve darkness. Light intensity underwater during the day was adjusted to about 130 μmol photons/m^2/s, which corresponds to the light intensity at the maximum depth of suspended oyster cultures in Gamak Bay. During experimental periods, every effort was made to protect the oysters from external stimuli. Output voltages changed in response to SVMs and were recorded at intervals of 0.5 sec for 2 days.

Laboratory test of shell valve movements in response to variation of dissolved oxygen

To track the SVM variations in response to DO concentration decreasing from 8.0 to 1.0 ppm, eight 2–year–old Pacific oysters cultured in Geoje–Hansan Bay were placed in an aquarium with 30 L of filtered seawater (pore size 1.0 μm), and we monitored their response to exposure to nitrogen gas (N_2). To measure SVMs under anoxic conditions, a DO concentration below 1 ppm was maintained for 4 h. N_2 gas was carefully aerated at the edge of the aquarium to minimize its direct influence on experimental organisms. The DO concentration was controlled by using an N_2 regulator with a DO detector (Oxygen Optode 4500, AADI, Bergen, Norway). The temperature and salinity were set as in the circadian rhythm experiment, and light intensity was maintained at less than 130 μmol photons/m^2/sec. As in previous experiments, every effort was made to protect the oysters from external stimuli. Output voltages changed in response to SVMs and were recorded at intervals of 0.5 sec.

Field test of shell valve movements in response to variation in dissolved oxygen in Gamak Bay

For three days (25–28 August 2016), Pacific oysters attached to Hall element sensors were installed at about 8 m depth in the oyster farms of Gamak Bay, Korea (GB station; Fig. 2). The oysters were periodically exposed to hypoxic seawater, which formed near the bottom layer, as the water moved in and out with the tides (maximum depth: ~15 m; Fig. 3). To measure the SVMs, eight oysters of the same age that had been raised in the Gamak Bay region were placed in separate chambers. Moreover, we also installed a conductivity–temperature–depth meter with a DO sensor to measure temperature,

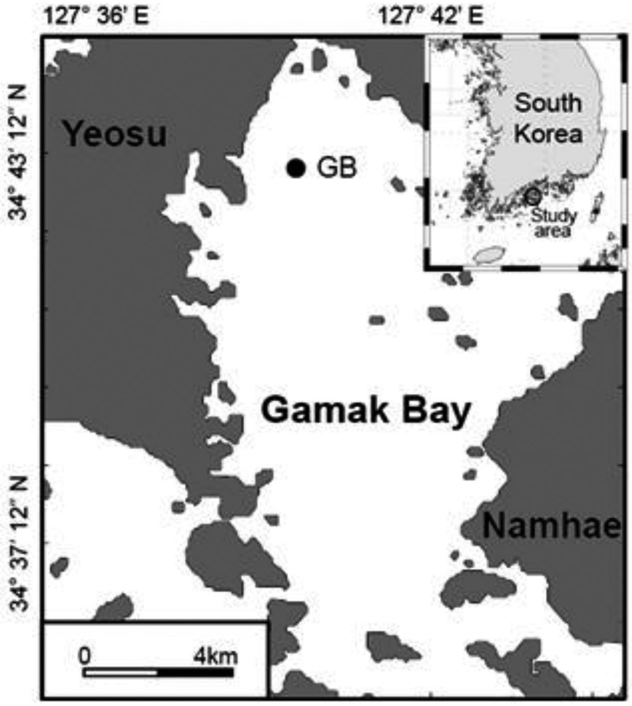

Fig. 2. Monitoring station for measured shell valve movements (SVMs) of Pacific oyster (*Crassostrea gigas*) according to variation of dissolved oxygen (DO) in Gamak bay, Korea. GB means abbreviation of Gamak Bay.

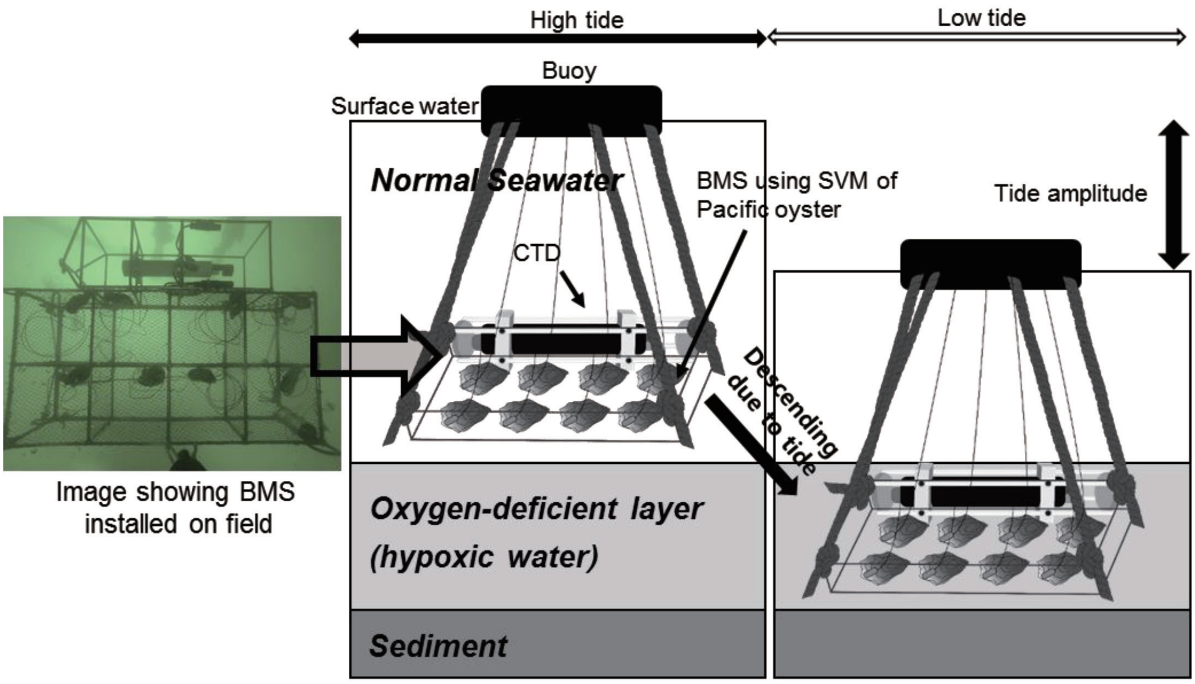

Fig. 3. Mooring design of biomonitoring system (BMS) based on shell valve movements (SVMs) of Pacific oyster (*Crassostrea gigas*) to the detecting of oxygen–deficient water. In case of high tide, the system was located in normal seawater condition. On the other hand, in case of low tide, the system was located in oxygen–deficient layer (hypoxic water) because of the changing tidal movement.

salinity, and DO concentration at various depths. Tidal movement data, including measurements of the height of the water column every minute, were provided by the Korea Hydrographic and Oceanographic Agency (http://www.khoa.go.kr/swtc/main.do). Previous reports have indicated that Akoya pearl oysters and Manila clams evidenced convulsive SVMs despite a low cell density of *Heterocapsa circularisquama*, a biotoxin–producing dinoflagellate that can kill bivalves (Nagai *et al*., 2006; Basti *et al*., 2011), although *H. circularisquama* has not been reported in the coastal area of Korea. SVMs of Pacific oysters were also reported to change as a function of the cell density of another toxic dinoflagellate (Haberkorn *et al*., 2011). Thus, each day we used microscopy to observe harmful dinoflagellates sampled from the surface (top 1 m), middle, and benthic layers (1 m above the bottom) during the experiment.

RESULTS AND DISCUSSION

Shell valve movements during light and dark periods

Figure 4 shows the SVMs of Pacific oysters during light and dark periods (12:12 h L:D cycle). Typical patterns of SVM indicated that closing was quick (closing time ~1 sec), whereas opening occurred slowly (opening time ~10 min). Under normal conditions, without any outside stimuli, SVMs occurred an average of 6 ± 3 times/h. Suzuki *et al*. (2011) have reported that SVMs of the pen shell (*Atrina lischkeana*) under aerobic conditions occur 2–5 times/h at intervals of about 13 sec after opening for long periods. In SVMs of Akoya pearl oysters, the opening speed was similar to the closing speed, and SVMs occurred 0–2 times/h (Nagai, 2006; Nagai *et al*., 2006). Manila clams (*Ruditapes philippinarum*) commonly maintain an opening of 4–5 mm between their two valves, but they occasionally close their valves at a frequency of about 2–3 times/h (Kozuki *et al*., 2015). Thus, the SVMs of Pacific oysters differ from those of bivalves such as Manila clams and Akoya pearl oysters, perhaps because the adductor muscular tissue and hinge ligament, which are the organs that cause the SVM, differ among these species (Oh *et al*., 2013).

The patterns of SVMs in Pacific oysters were not significantly different between light and dark periods (Fig. 4). Moreover, the rates of SVMs were 6.48 ± 2.32 and 4.91 ± 3.36 times/h during the light and dark periods, respectively, which were not significantly different. Although some researchers have reported that circadian rhythms exist in bivalves (García–March *et al*., 2008; Tran *et al*., 2011), our results indicated that there was no circadian rhythm in the SVMs of the Pacific oyster, and this potentially confounding factor could be ignored in our laboratory experiments. Therefore, laboratory experiments were conducted only during the light period.

Laboratory test of shell valve movements in response to variations of DO concentrations

After injecting N_2 gas into the test aquarium for 2 h, the DO concentration decreased rapidly. The water became at first hypoxic and then anoxic after 3 h. The SVMs responded immediately to the decrease of the DO concentration; they increased to 30 ± 17 times/h within a short time. When the DO concentration reached less than 2.0 ppm after 1 h, however, the fact that all experiments recorded no SVMs indicated that all the shells were closed. The rapid opening and closing of shells were a regular occurrence under hypoxic and anoxic conditions (Fig. 5). Previous studies have shown that the SVMs of Akoya pearl oysters also increase as DO

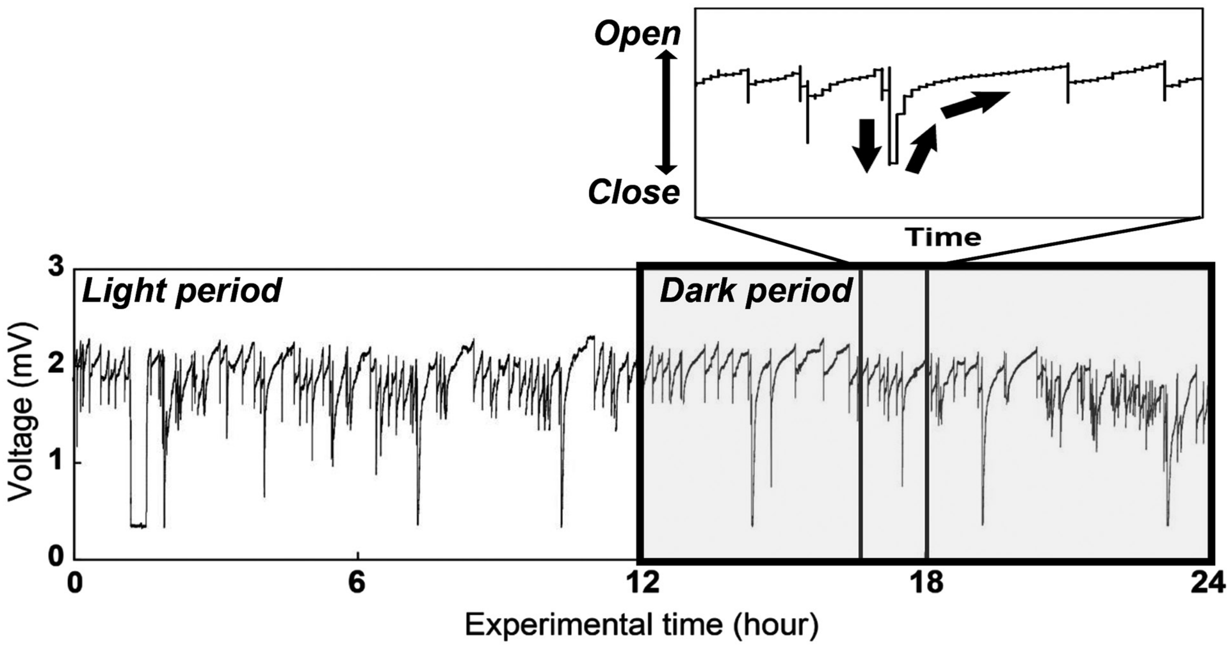

Fig. 4. Shell valve movements (SVMs) of Pacific oyster (*Crassostrea gigas*) during light period and dark period (L:D cycle of12:12 h). Also, the figure of right top showed typical patterns of SVMs.

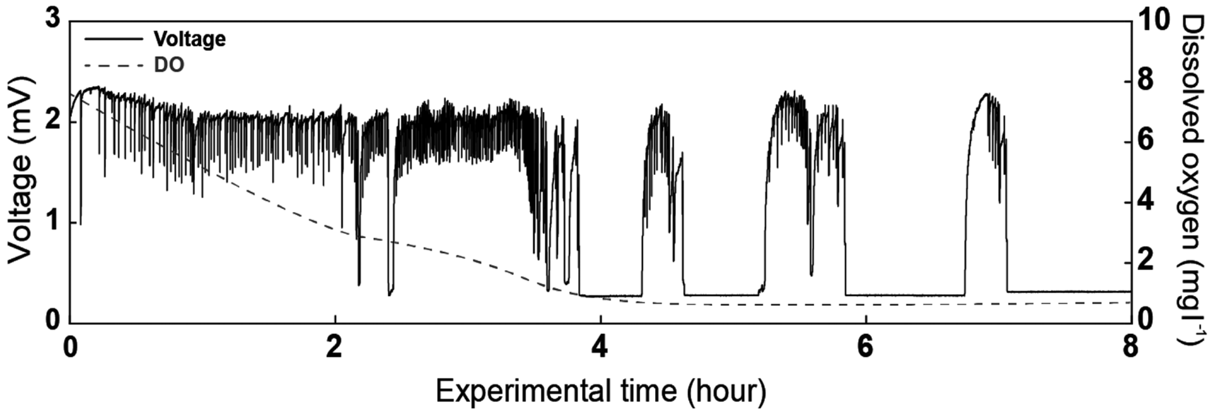

Fig. 5. Shell valve movements (SVMs) of Pacific oyster (*Crassostrea gigas*) under various dissolved oxygen (DO).

concentrations decrease (Go *et al.*, 2009).

The Pacific oyster's tolerance of low DO concentrations is dependent on the mechanism of water pumping/ventilation, which makes more oxygen available to the gills and improves its distribution to the tissues. In this way pumping/ventilation helps to maintain the rate of oxygen consumption (Tran *et al.*, 2000). Tolerance mechanisms may be able to operate above a DO threshold (Le Moullac *et al.*, 2007), such as 2 ppm, the DO concentration of hypoxic water (Diaz and Rosenberg, 1995). MacDonald *et al.* (2009) have argued that the ventilation volume of some bivalves is controlled by an exhalant and inhalant in the siphon, but the siphons of Pacific oysters are not fully developed. Moreover, the increases and decreases of the ventilation volume are closely connected with SVMs (Riisgård *et al.*, 2014). If the DO concentration decreases, the SVMs of Pacific oysters may increase in order to increase the ventilation volume. The closed condition of Pacific oyster shells when the DO concentration is less than 2 ppm likely represents a marked decline in metabolic activity due to a decreasing rate of oxygen consumption (Yamamoto and Handa, 2011). The Pacific oyster has a relatively low oxygen demand compared with Akoya pearl oysters and Japanese purple mussels (*Septifer virgatus*) and is therefore regarded as a relatively oxygen-independent species. Nevertheless, it may be useful as a biosensor for detecting hypoxic and anoxic conditions.

Shell valve movements in response to variation in DO concentrations in Gamak Bay

Gamak Bay is one of the largest areas in the seas surrounding the Republic of Korea for the aquaculture of shellfish, including oysters and mussels. There are more than 200 shellfish farms scattered along Gamak Bay (Hwang *et al.*, 2010). Although the bay has been designated by the Korean Ministry of Land Transport and Maritime Affairs as an environmental conservation area because of its ecological importance as a habitat for fishes and shellfish (Lee and Cho, 2002; Kim, 2003), the bay's physical stratification in summer, topographical features such as basins, and enhanced anthropogenic loading of pollutants from land have resulted in the depletion of bottom DO concentrations in the northern part of Gamak Bay since the 1980s (Hwang *et al.*, 2010; Seo *et al.*, 2012).

Based on the promising results of the laboratory studies of SVMs, a biomonitoring system for the early detection of oxygen–deficient water was tested in the field. Hypoxic water was observed in the northernmost part of Gamak Bay from May to September 2016 (MOF, 2016). Figure 6 shows the changes of temperature, salinity, tidal level, DO concentration, and SVMs around Pacific oyster farms when hypoxic water formed in the benthic layer during 25–27 August. The phytoplankton community was dominated by centric diatoms (i.e., *Skeletonema* spp. and *Chaetoceros* spp.), and harmful dinoflagellates (i.e., *Alexandrium* spp. and *H. circularisquama*) were not observed during any sampling period. There were typically two high and low tides each day, although the diurnal inequality of the tides was not clear because of the change from neap tide to spring tide. The mean high–water interval lasted for 12 h 49 min. The maximum and minimum water levels were 168 and 106 cm, respectively. Water temperature changed from 26.8 to 28.9°C with tidal level during the field experiment. Salinity was fairly constant during the study period, ranging from 32.6 to 32.8. From 25 to 26 August, the DO concentration changed from 0.00 to 7.53 ppm with the tidal level and water temperature. The biological and physical sensors therefore moved in and out of the hypoxic zone in the benthic layer, where the temperature was relatively low and the DO concentration extremely low (<1 ppm). On 27 August, the DO was in the normal range at 6.0 ppm because the thermocline was weak.

The changes of the SVMs matched the tidal level changes. The oyster shells closed as the water surrounding the oysters became hypoxic, and the SVMs resumed again when the oysters were no longer within the hypoxic zone. The rate of SVM rapidly increased to about 16 times/h before and after exposure to hypoxic water and recovered to 6–7 times/h when the DO concentrations reached ~5 ppm (Fig. 7). The results of both the field and laboratory experiments indicated that abnormally high or low SVMs of Pacific oysters can serve as a biomarker of oxygen-deficient water.

Many researchers have suggested that marine

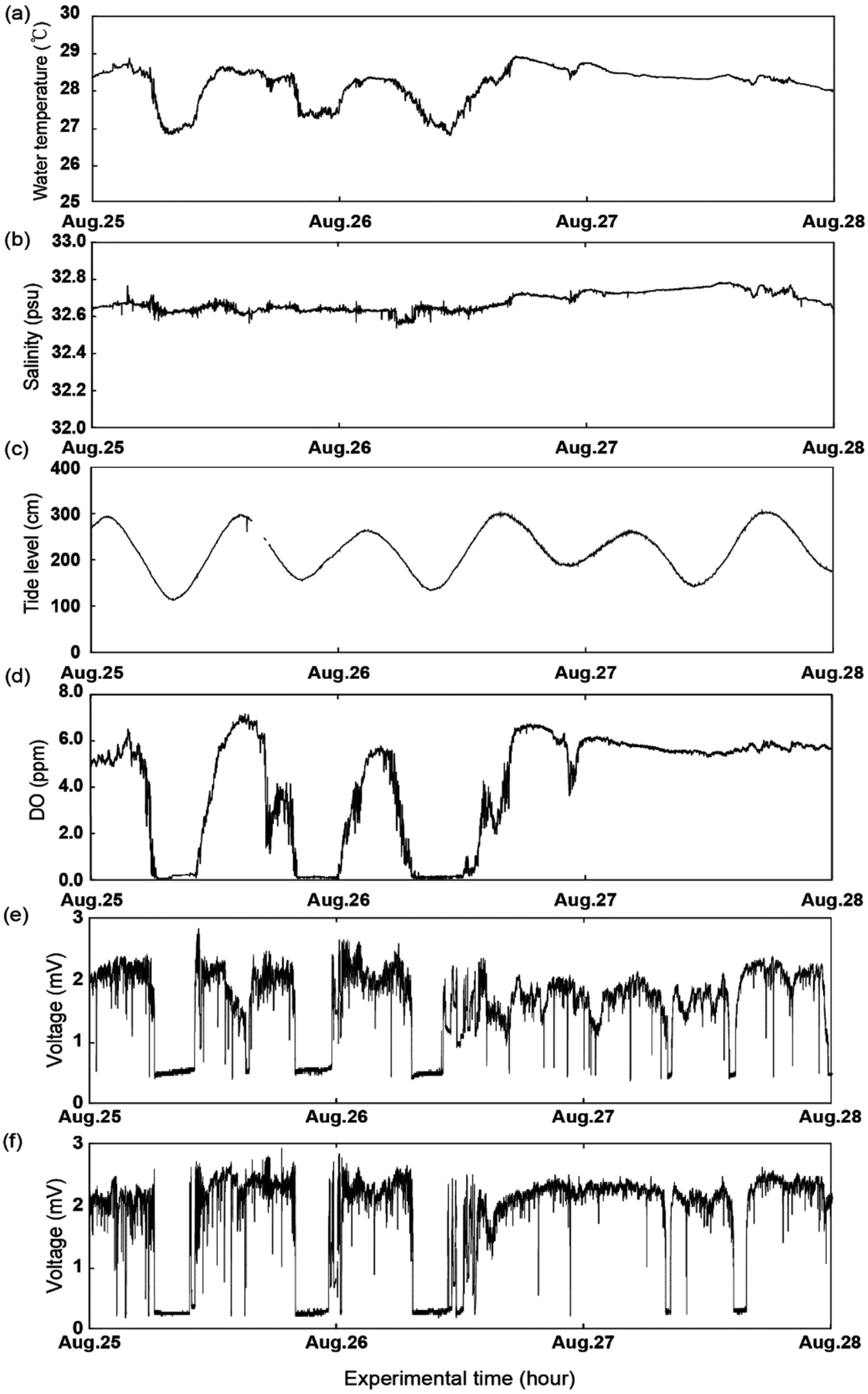

Fig. 6. Temporal variations of temperature (a), salinity (b), tide level (c), dissolved oxygen (d), and shell valve movements (SVMs) of Pacific oyster (*Crassostrea gigas*) (e, f) in Gamak Bay, Korea from 25 to 29 August.

bivalves are more suitable than fishes for monitoring chemical pollution of coastal waters (Goldberg *et al*., 1978; Jenner *et al*., 1989; Kramer *et al*., 1989; Farrington *et al*., 2016), because standardizing and quantifying responses of fish species for biomonitoring systems is difficult due to their relatively low sensitivity and long reaction time to toxic substances (Morse *et al*., 2007). It is also difficult to ascertain the typical behaviors of microalgae and bacteria that would allow them to be used as test organisms to detect the presence of toxic substances (Morse *et al*., 2007). However, bivalves are resistant to a wide range of contaminants and may thrive even in highly polluted environments (Goldberg *et al*., 1978). In Korea, bivalves such as clams, mussels, and oysters are widely distributed around coastal areas, and the production of bivalve accounts for more than 80% of the total coastal aquaculture volume. Therefore, we suggest that installation of a biomonitoring system using bivalves around aquaculture farms should help to reduce damage from oxygen–deficient water through simple

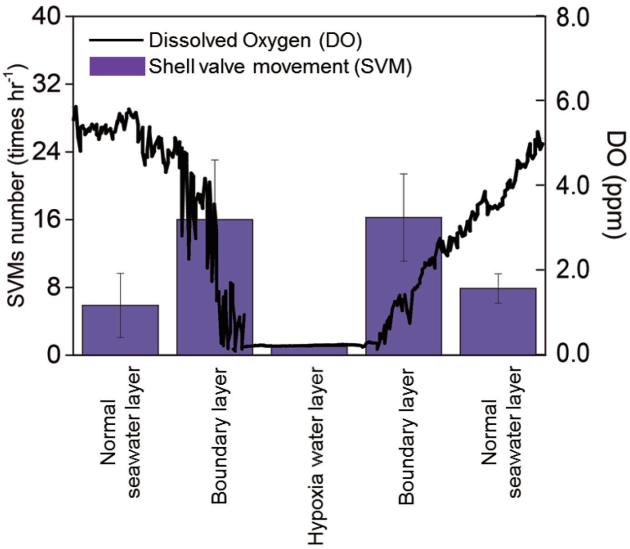

Fig. 7. Changing SVMs of Pacific oysters (*Crassostrea gigas*) and concentrations of dissolved oxygen in the normal seawater layer, boundary layer and hypoxic layer in Gamak Bay, Korea. Boundary layer means zone between normal seawater and hypoxic layer.

actions such as making shorter growing ropes for floating rafts.

CONCLUSIONS

We observed SVMs of Pacific oyster averaged 6 ± 3 times/h under normal conditions. However, SVMs responded immediately and dramatically when the dissolved oxygen concentration decreased and increased to 30 ± 17 times/h during laboratory experiments. All experiments recorded no SVMs in the 2.0 ppm dissolved oxygen concentration group. In field experiments, oyster shells remained closed in hypoxic water. However, the rate of SVMs rapidly increased before and after exposure to hypoxic water. Therefore, we concluded that the observation of rapid SVMs or no SVM in Pacific oyster might serve as signals of bioindicator to detect oxygen–deficient water quality condition. We suggest that online biomonitoring system using SVM of bivalves around aquaculture farms should help to reduce damage from oxygen–deficient water. Additional research on the effects of a variety of environmental factors such as the increase of water temperature, low salinity and the occurrence of red tides on SVMs of bivalves are needed to further outstanding of how onsite and online biological monitoring support to reduce environmental impact and minimize financial damage on aquaculture field.

AUTHOR CONTRIBUTIONS

Seok Jin OH was analyzed the data, designed the study and prepared the manuscript. Suyeon MOON was performed the experiments. Yang Ho YOON and Dae Hyun KIM assisted the experiments. IK Joon KANG participated in the design of the study and played the role of the corresponding author. Yohei SHIMASAKI contributed to revise the manuscript. All authors have read and agreed to the published version of the manuscript.

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