Ontogenetic and temperature-dependent changes in tolerance to hypoxia and hydrogen sulfide during the early life stages of the Manila clam *Ruditapes philippinarum*

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ABSTRACT

Wind-induced upwelling of hypoxic waters containing hydrogen sulfide (H_2S) sometimes causes mass mortalities of aquatic organisms inhabiting coastal areas, including the hypoxia-tolerant Manila clam *Ruditapes philippinarum*. We examined the tolerance of Manila clam to H_2S under controlled laboratory conditions. Larvae and juveniles obtained by artificial fertilization or from a wild population were exposed to normoxic or to hypoxic water with or without un-ionized H_2S (concentrations, 0.2–52.2 mg/L). Twenty-four-hour exposure experiments revealed ontogenetic changes in the clam's tolerance to H_2S exposure: tolerance was enhanced from the larval stages to juveniles just after settlement but was attenuated as juveniles grew. Tolerance of larvae and juveniles to H_2S exposure weakened as the water temperature rose from 20 to 28 °C. Prolonged 48-h exposure to H_2S attenuated the tolerance of juveniles to H_2S. Temporary suspension of H_2S exposure by 24-h reoxygenation improved the ability of juveniles to withstand repeated H_2S exposure.

1. Introduction

Bottom hypoxia (dissolved oxygen, DO ≤ 2 mL/L) usually occurs in eutrophic coastal regions worldwide. Excessive nutrient loading enhances primary production, resulting in increased deposition of organic matter on the seafloor (Diaz and Rosenberg, 2008). Oxygen demand in the microbial decomposition of bottom organic materials often exceeds the supply of oxygen from the surface layers, especially in summer, when there is thermal or density stratification of the water column. These conditions frequently lead to the development of hypoxia in bottom waters. A number of studies have investigated the adverse effects of hypoxia on marine benthic organisms, including effects on growth, metabolism, reproduction, and development, as well as mass mortalities of benthic populations or communities (Kodama and Horiguchi, 2011; Kodama et al., 2010, 2012, 2014, 2018; Thomas and Rahman, 2009; Thomas et al., 2007; Wu, 2002, 2009).

Hydrogen sulfide (H_2S) is produced under anoxic conditions through the reduction of sulfate. H_2S is highly toxic to aquatic organisms through respiratory disorder caused by inhibition of the respiratory enzyme cytochrome *c* oxidase (Smith et al., 1977; Vismann, 1996). Wind-induced upwelling of hypoxic waters containing H_2S (referred to as blue tides; Furukawa, 2015) sometimes causes mass mortalities of aquatic organisms inhabiting shallow and tidal flat areas. For example, despite relatively high tolerance of the Manila clam *Ruditapes philippinarum* to severely hypoxic conditions (Kakino, 1982; Kamohara et al., 2012), mass mortalities of this clam sometimes occur after these catastrophic upwellings: 4750 t of Manila clam was killed by the upwelling of hypoxic water containing H_2S in northern Tokyo Bay in 2010 (Iimura et al., 2010), and 5000 t was similarly killed on the Rokujo tidal flat in Mikawa Bay in 2008 (Aoki et al., 2014). However, in other instances of upwelling of hypoxic water with H_2S, no Manila clam mortalities were observed: for example, no clam kills were observed in two out of five blue-tide events from 2004 to 2011 (Aoki et al., 2014). These field observations imply the presence of a threshold H_2S level for mass mortalities of this clam.

A number of studies have examined the adverse effects of sulfide species under hypoxic conditions on benthic organisms, including the Manila clam, in coastal regions (reviewed by Bagarinao, 1993; Diaz and

Rosenberg, 1995; Marumo and Yokota, 2012), but toxicity has been assessed mostly on the basis of the total sulfide (TS) concentration. TS consists of dissolved sulfides (un-ionized H_2S, bisulfide ion [HS^-], and sulfide ion [S^{2-}]) and acid-volatile sulfides (such as mackinawite/pyrrhotite [FeS], greigite [Fe_3S_4], and amorphous monosulfides of other metals) (American Public Health Association, 2005). However, sulfide species other than un-ionized H_2S do not show critical toxicity to aquatic organisms (Smith et al., 1977; Vismann, 1996). To better understand the toxicity of H_2S to organisms, the concentration of only H_2S—excluding other sulfide species—needs to be examined. Recent developments in amperometric microsensing technology now enable the direct measurement of un-ionized H_2S concentrations in aquatic environments (Jeroschewski et al., 1996). Here, we used an H_2S microsensor to examine the levels of H_2S that were lethal to the Manila clam during its early life stages under controlled laboratory conditions.

The Manila clam is a suitable model species for examining the effects of exposure to hypoxia and H_2S for a number of reasons. It is a dominant species of benthic infauna in eutrophic coastal regions, as well as a major target of the local commercial fisheries. The clam is pelagic during the larval stage, and it is distributed over a wide spatial range in coastal systems where hypoxia frequently occurs (Kasuya, 2005). The Manila clam starts its benthic life after metamorphosing to juveniles, which are sometimes subjected to the abovementioned up-wellings of hypoxic water containing H_2S. The clam may therefore encounter hypoxic water containing H_2S throughout its life history. It has a relatively high tolerance to hypoxic exposure during the larval stages, the juvenile stages, and thereafter; larvae at the D-shaped stage, the umbo stage, and full-grown stage, as well as settled juveniles and adults, have been to survive up to at least 2 days of the start of hypoxic exposure (Kakino, 1982; Kamohara et al., 2012). Therefore, the combined effects of short-term exposure to hypoxia and H_2S need to be investigated by laboratory experiment. Assessment of the tolerance of Manila clams to hypoxia and H_2S would make it possible to establish environmental criteria to conserve local ecosystems and help fisheries managers to regulate the efforts of both commercial and recreational fisheries to maintain sustainable population sizes. Here, we examined the tolerance of Manila clams to hypoxia and H_2S at the larval and juvenile stages, because the recruitment success of populations of benthic organisms is generally influenced by mortality during the early life stages (Jennings et al., 2001).

2. Materials and methods

2.1. Samples for laboratory experiment

2.1.1. Progeny of broodstock

To reveal the tolerance of Manila clams at the early life stages under various conditions of water temperature and H_2S concentration, we conducted controlled laboratory experiments using larvae and juveniles obtained by artificial fertilization. We purchased from a local shellfish distributor (Marue Suisan Co. Ltd., Tahara, Aichi) 20 kg of adult Manila clams that had been caught in the southern part of Mikawa Bay (off the coast of the city of Tahara; Fig. 1), Japan, on 17 May 2016. These clams were transferred to the Aichi Fisheries Research Institute (Fig. 1) to serve as broodstock to obtain progeny for use in the laboratory experiments. We artificially fertilized the broodstock following the procedure of Kamohara et al. (2013), whereby adult clams were immersed in turn in two aquaria filled with filtered seawater, one at 13 °C and the other at 26 °C, at a time interval of 20 min. This procedure was repeated until the clams started spawning.

Approximately 70 million individual trochophore larvae were hatched from the artificially fertilized eggs, and these larvae were kept in two 0.5-t tanks filled with approximately 400 L of filtered seawater at 22 °C. We fed 1.5×10^4 cells/mL of the haptophycean alga *Pavlova lutheri* to the larvae in each tank every day. Before the start of the exposure experiment for each developmental stage, we randomly took a
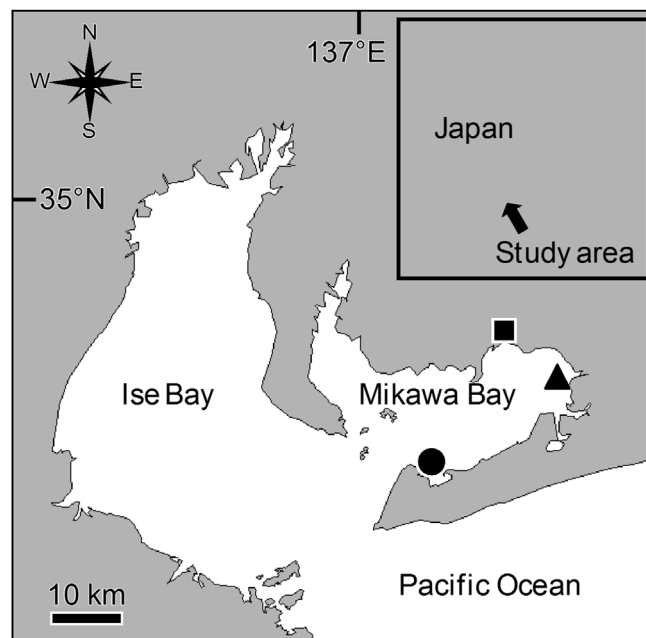

Fig. 1. Locations of sampling area for mature individuals (off the coast of the city of Tahara; solid circle) and juveniles (Rokujo tidal flat; solid triangle) of the Manila clam *Ruditapes philippinarum*. Artificial fertilization and the laboratory experiments were conducted at the Aichi Fisheries Research Institute (solid square).

Table 1
Means and standard deviations of shell length of the Manila clam *Ruditapes philippinarum* used in the exposure experiments. Numbers of individuals in each experimental container are also shown. Samples were obtained by artificial fertilization (AF) of broodstock or collected from a wild population (WP) on the Rokujo tidal flat, Mikawa Bay, Japan. Continuous 24-h or 48-h exposure to H_2S and hypoxia was conducted. Intermittent exposure was also performed: clams were exposed to hypoxia plus H_2S for 24 h, followed by a 24-h rest period under normoxic conditions, and then a repeat 24-h exposure to hypoxia and H_2S.

Sample	Exposure (h)	Stage	Shell length (mm)	No. individuals
AF	24	D-shape	0.10 ± 0.01	119 ± 17
AF	24	Umbo	0.13 ± 0.01	160 ± 33
AF	24	Umbo to full-grown	0.17 ± 0.02	63 ± 15
AF	24	Full-grown	0.20 ± 0.01	57 ± 11
AF	24	Juvenile	0.73 ± 0.04	10
AF	24	Juvenile	2.53 ± 0.23	10
AF	24	Juvenile	5.32 ± 0.33	10
AF	48	Juvenile	0.84 ± 0.03	10
AF	Intermittent	Juvenile	8.93 ± 0.31	10
WP	24	Juvenile	6.94 ± 0.73	10
WP	24	Juvenile	11.88 ± 0.87	10
WP	48	Juvenile	11.33 ± 0.33	10

combined total of 30 clams from the two 0.5-t tanks and measured their shell length (SL) (Table 1).

To raise juvenile clams, all of the remaining full-grown larvae were transferred to, and kept in, a polyethylene rectangular container (size, 100 cm × 80 cm × 20 cm) under flow-through conditions using filtered seawater. The water temperature fluctuated between 20 °C and 28 °C, reflecting the variation in the water temperature of Mikawa Bay. Fine sand (< 435 μm in diameter) was laid on the bottom of the container so that juveniles could inhabit the sand substrate. Juvenile clams were fed 1.5×10^4 cells/mL of *P. lutheri* every day.

2.1.2. Juveniles from wild population

To investigate the tolerance of juvenile clams grown in the field, we used wild juveniles (SL, 4.6–14.3 mm, approximately 1 year old) collected from the Rokujo tidal flat in the northeastern part of Mikawa Bay