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Pathological survey of Manila clam *Ruditapes philippinarum* and Pacific oyster *Crassostrea gigas* in Garorim Bay on the west coast of Korea

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ABSTRACT

Shellfish production on the west coast Korea has been declined for the past two decades, and the parasite infection is considered to be one of the causes responsible for the decline of Manila clam *Ruditapes philippinarum*. In this study, we first investigated the pathologic condition of Manila clams and the Pacific oysters *Crassostrea gigas* in Garorim Bay on the west coast, where Manila clams and the Pacific oysters are co-cultured. Histology and Ray's fluid thioglycollate medium assay (RFTM) revealed that most of Manila clams are infected by protozoan parasite *Perkinsus olseni* (98.0-100.0% prevalence). In contrast, none of the Pacific oysters diagnosed by the histology showed *P. olseni* in their tissue. The mean infection intensity of *P. olseni* in Manila clams was high, ranging from 0.796 (Ohiji-ri) to 2.058 (Dangsan-ri) × 10^6 cells/g wet tissue weight. In histology, metacercaria of *Parvatrema duboisi* was identified from the mantle tissue of Manila clam. Ancistrocoma-like ciliates (ALCs) were found from the digestive tubules of the oysters, with the prevalence ranging from 10.0 to 13.0 %. Rickettsia-like organisms (RLOs) were commonly identified from the epithelial cells of the digestive tubule of the Pacific oysters and Manila clams, with a prevalence ranging from 0.0 to 10.0% in Manila clams and 6.7 to 10.0% in the oysters. The mean condition index (CI) of Manila clams from Ohji-ri was significantly lower than CI of clams at Dangsan-ri tidal flat (ANOVA, $P < 0.05$). The mean digestive gland atrophy (DGA) of clams at Ohji-ri was significantly higher than the DGA of clams at Dangsan-ri (ANOVA, $P < 0.05$). The mean infection intensity of *P. olseni* in the host clams from Dangsan-ri was significantly higher than the clams in Ohji-ri (ANOVA, $P < 0.05$). The observed spatial variation in the CI and DGA was believed to be linked to a spatial change in the available food in the environment, although more investigations must be carried out to validate this hypothesis.

Key words: *Ruditapes philippinarum*, *Crassostrea gigas*, RFTM, *Perkinsus olseni*, *Parvatrema* sp. Ciliate parasite

INTRODUCTION

Native to the north-west Pacific Ocean, the Manila clam *Ruditapes philippinarum* and the Pacific oyster *Crassostrea gigas* occur commonly in mid to low tide areas in muddy sand tidal flats on the west coast of Korea. On the west coast, oysters and clams production varies year to year, and the clam production decreased at a fast rate. Several studies have reported that the decrease in shellfish production on the west coast is linked to the human-made and natural stresses, which often end up with mass mortality of the shellfish resources (Park *et al*., 2013; Mondol *et al*., 2015; Park *et al*., 2015). Several species of protozoan and metazoan parasites have been identified from the Pacific oysters and Manila clams in

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Korean waters, including protozoan *Perkinsus olseni* (Park *et al*., 2005, 2006), *Marteilia tapetis* (Kang *et al*., 2019), *Marteilioides chungmuensis* (Limpanont *et al*., 2013), *Urosporidium* sp. (Le *et al*., 2015), and metazoan trematode *Parvatrema duboisi* (Chang *et al*., 2020) and *Cercaria tapidis* (Ngo and Choi, 2004). Park *et al*. (2006) first reported the adverse effects of a high level of *P. olseni* infection on the growth and reproduction of Manila clams in Gomso Bay on the west coast. On the south coast, Manila clams are known to host several different species of protozoan parasites besides *P. olseni*, including the paramyxean parasite *M. tapetis* and *M. chungmuensis* (Kang *et al*., 2017, 2019; Limpanont *et al*., 2013). Contrary to the south, Manila clams infected by the paramyxean parasites are yet to be reported from the west coast.

Located on the northern Taean area, Garorim Bay includes well-developed muddy-sand tidal flats, where the Pacific oysters and Manila clams are co-cultured in an intensive mode. In this study, we surveyed overall health conditions and pathogenic organisms associated with the clams and oysters using histology. Accordingly, we report symbiotic microorganisms observed from the oysters and clams and spatial variation in the health condition of the oysters and clams in Garorim Bay.

MATERIALS AND METHODS

For the investigation, we visited three tidal flats in Garorim Bay, Ohji-ri, Jungwang-ri, and Dangsan-ri in April 2015 (Fig. 1). From each tidal flat, we collected 40 adult Manila clams size ranging from 27 to 50 mm in shell length (SL). We also obtained 30 adult oysters size ranging from 50 to 80 mm SL for the analysis. After measuring SL (i.e., the longest axis of the shell), the body was removed and measured the wet weight to mg using an electronic valence. The dry shell weight of Manila clam was recorded to mg to determine the condition index (CI). The shell cavity volume of the oysters was determined by subtracting shell volume from the total volume (Beninger and Lucas, 1984; Mondol *et al*., 2015). CI of Manila clams was established as a ratio of the wet tissue weight (g) to the shell dry weight (g). CI of the oysters was determined by dividing the wet tissue weight (g) by the shell cavity volume × 100 (Mondol *et al*., 2012).

To diagnose *Perkinsus olseni* infection, we adapted Ray's fluid thioglycollate medium (FTM) assay and subsequent 2 M NaOH digestion (Ray, 1966; Choi *et al*., 1989). For FTM assay, the whole body of individual clam (20 clams from each site) was cut into several pieces then added in a 15 ml conical tube filled with 10 mL FTM and antibiotics. The FTM tubes were incubated at room temperature for one week in the dark. The FTM incubated clam tissues were digested using 2 M NaOH, washed, and the number of hypnospores in the tube was counted using a hemocyte counter (Yang *et al*., 2012). Finally, the infection intensity was expressed as the number of *P. olseni* cells/g tissue wet weight.

Parasitic organisms in the oysters and clams were identified based on morphology using histology. For the assay, 30 oysters and 20 clams were selected from each tidal flats and analyzed. For histology, we sliced a 2-3 mm thick cross-section from the middle of the body and fixed in Davidson's solution over 24 h. After dehydrating the tissues in a series of ethanol using an automatic tissue processor, we embedded the tissues in paraffin, sectioned the tissues at 5 μm, then stained with Harris hematoxylin and counterstained with eosin Y. Under a compound microscope, we examined the mantle, gills, gonad, and the digestive system of oysters and clams to identify the symbiotic organisms (Park and Choi, 2004; Limpanont, 2010). The prevalence of each parasite type was calculated as the percentage of infected clams or oysters out of the whole clams or oysters examined. Nutrition condition of clams or oysters was also evaluated from the histology by categorizing the status of the digestive tubules. Based on the microscopic appearance of the digestive tubules and the lumens, the level of digestive gland atrophy (DGA) of an individual oyster or clam was graded from 0 (no atrophy) to 4 (highest) according to Ellis *et al*. (1998) and Kang *et al*. (2010).

Spatial variation in CI and *Perkinsus* infection intensity of clams from the three tidal flats in the bay were compared using a one-way analysis of variance

Table 1. Prevalence of parasites observed from *Ruditapes philippinarum* and *Crassostrea gigas* in Garorim Bay on the west coast of Korea. ALCs, Ancistrocoma-Like Ciliates, RLOs, Rickettsia-Like Organisms

Species	Site	N	Protozoa (%)			Metazoa (%)		Bacteria (%)
			P. olseni	*Urosporidium* sp.	ALCs	*P. duboisi*	*C. tapidis*	RLOs
R. philippinarum	Ohji-ri	20	90.0	5.0	0.0	15.0	0.0	0.0
	Jungwang-ri	20	80.0	0.0	0.0	10.0	0.0	5.0
	Dangsan-ri	20	95.0	0.0	0.0	20.0	10.0	10.0
C. gigas	Ohji-ri	30	0.0	0.0	10.0	0.0	3.3	10.0
	Jungwang-ri	30	0.0	0.0	13.3	0.0	0.0	6.7
	Dangsan-ri	30	0.0	0.0	13.3	0.0	0.0	6.7

(ANOVA) followed by Tukey's range test. Statistical analysis was performed using SAS statistical package (SAS Institution, USA), and the level of significance was set at $P < 0.05$.

RESULTS

Histology indicated that most of the oysters and clams collected from the three tidal flats in the Garorim Bay in April 2015 were reproductively in the early developing stage (N = 90 oysters and 60 clams). Table 1 summarizes the types of symbiotic organisms identified from *C. gigas* and *R. philippinarum*. In histology, the oysters and clams included rickettsia-like organisms (RLOs) in the epithelial cells of the digestive tubule, appearing as globular dark blue spots (Fig. 2F). Prevalence of the RLOs ranged 0.0 to 10.0% in Manila clams, and 6.7 to 10.0% in oysters, respectively.

In histology, trematode metacercaria of *Parvatreama duboisi* and sporocyst of *Cercaria tapidis* were identified. The metacercaria of *P. duboisi* (Fig. 1C) distributed mostly in the mantle epithelial tissue of Manila clams with the prevalence ranging from 10.0 to 20.0 % (Table 1). Sporocyst stage of trematode, *C. tapidis* (Fig. 2E) was detected from the gonad of Manila clam with a prevalence of 10.0% and oyster with a prevalence of 3.3%, respectively. *Urosporidium* sp. is a single-celled parasite infecting metazoan parasite trematode. The histology revealed that *P. duboisi* metacercaria was infested by *Urosporidium* sp., exhibiting a hyper-parasitism (Fig. 1C).

Histology also detected parasitic ciliates identified as *Ancistrocoma*-like organisms (ALOs) in the digestive tubule lumens of the oysters with the prevalence ranging from 10.0 to 13.3% (Table 1 and Fig. 2D). Unlike oysters, no Manila clams examined in this

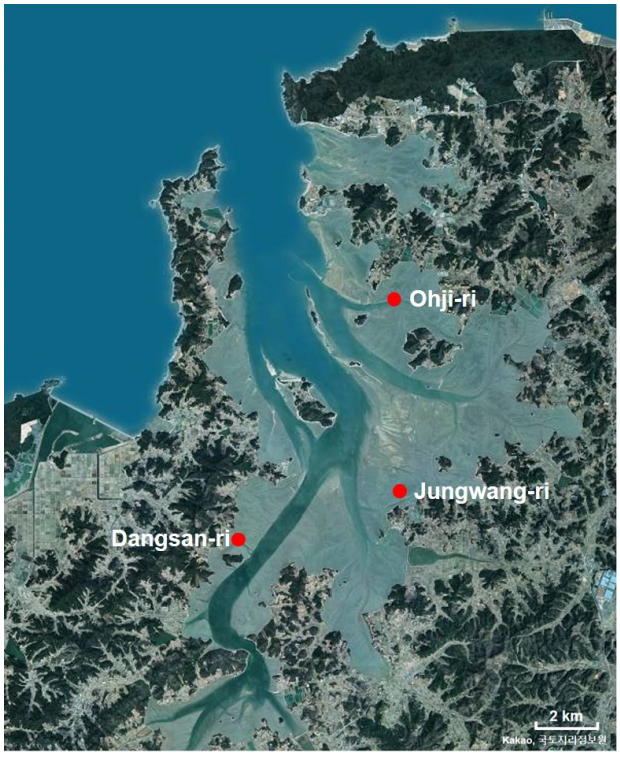

Fig. 1. Map showing the study sites in Garorim Bay on the west coast of Korea.

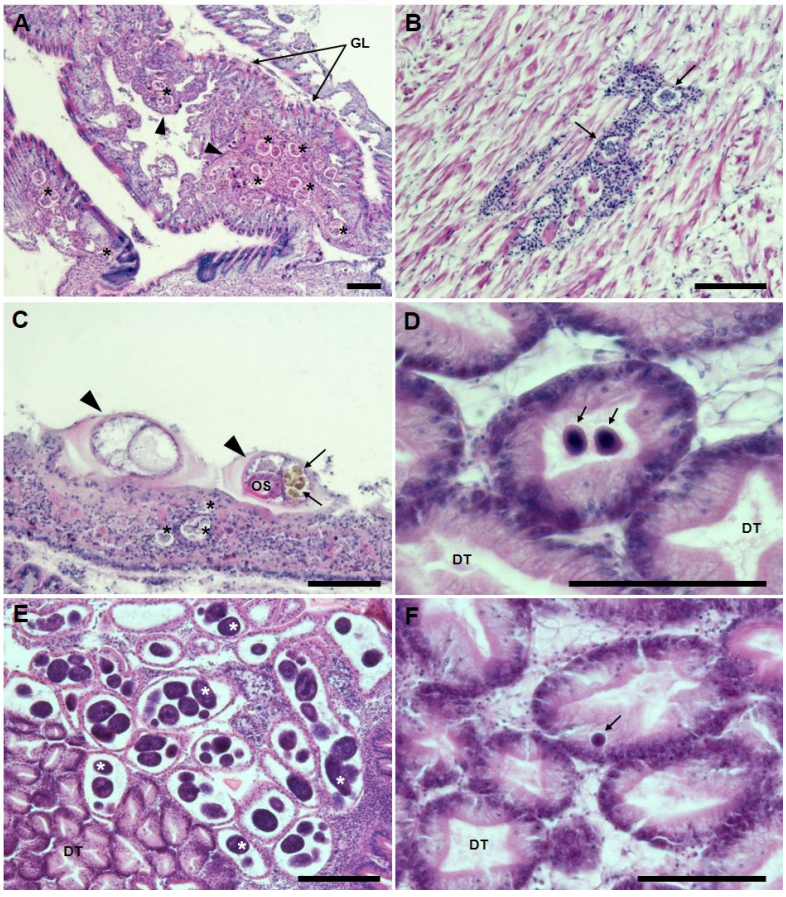

Fig. 2. Photomicrographs showing the symbiotic organisms in *Ruditapes philippinarum* (**A**-**C**, **E**) and *Crassostrea gigas* (**D**, **E**). Scale bar = 100 μm. **A**, *Perkinsus olseni* trophozoites (asterisks) surrounded by the hemocytes forming a granulomas (arrowheads) in the branchial connective tissue of the gill lamella. GL, Gill Lamella. **B**, *P. olseni* trophozoites (arrows) in the foot muscle tissue. **C**, Manila clam mantle tissue showing co-infection of the hyper-parasite *Urosporidium* sp. (arrows) in *Parvatrema duboisi* (arrowheads), and *P. olseni* (asterisks). OS, Oral Sucker. **D**, Ancistrocoma-Like Ciliates (ALCs) in the lumen of a digestive tubule. DT, Digestive Tubule. **E**, Sporocysts of *Cercaria tapidis* exhibiting the germ balls (asterisk) in the connective tissue of the gonad, resulting in gonad castration in clam. **F**, Rickettsia-Like Organisms (RLOs) in the epithelia cells of the digestive tubule.

study exhibited ALOs.

In histology, infection by *P. olseni* was evident, and most of Manila clams from the three tidal flats in Garorim bay exhibited *P. olseni* with the prevalence ranging from 80.0% (Jungwang-ri) to 95.0% (Dangsan-ri) (Table 1). *Perkinsus olseni* were observed mostly in the gill tissues (Fig. 1A) and the digestive tubule connective tissues, and rarely in the foot muscle (Fig. 1B). It was noticeable that no *P. olseni* trophozoite was observed from the Pacific oysters examined, indicating that oysters are free from *P. olseni* infection, although they occur in the same environment with Manila clam.

Fig. 3 shows the mean infection intensity of *P. olseni* determined using RFTM. The mean infection intensity of *P. olseni* in Garorim Bay in April 2015 ranged from 0.796×10^6 cells/g tissue wet weight (Ohji-ri) to 2.058×10^6 cells/g wet tissue weight (Dangsan-ri). ANOVA test indicated that the mean infection intensity in Manila clam in Dangsan-ri was significantly higher than Ohji-ri ($P < 0.05$).

We evaluated the overall health of the oysters and clams using CI, the ratio of tissue weight to the shell weight. CI of the oysters surveyed in April 2015

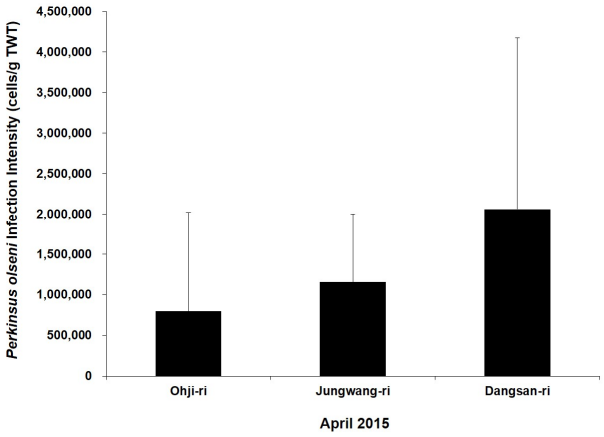

Fig. 3. The mean *Perkinsus olseni* infection intensity of Manila clams collected in April 2015. The error bars indicate the standard deviations.

ranged 23.0 (Jungwang-ri) to 32.2 (Dangsan-ri, Fig. 4). ANOVA test indicated that the mean CI recorded from Jungwang-ri was significantly lower than CI determined from Dangsan-ri ($P < 0.05$). CI of Manila clams ranged from 0.52 (Ohji-ri) to 0.64 (Dangsan-ri), and CI of Manila clams in Dangsan-ri was significantly higher than Ohji-ri (ANOVA, $P < 0.05$, Fig. 4).

Fig. 5 shows the mean DGA of the oysters and clams. The DGA of oysters ranged from 2.0 (Dangsan-ri) to 2.6 (Ohji-ri), and the DGA of Manila clam ranged from 1.4 (Jungwang-ri) to 1.8 (Ohji-ri). The mean DGA of oysters in Ohji-ri was significantly higher than the DGA of c Dangsan-ri ($P < 0.05$), suggesting that the nutritional status of oysters in Ohji-ri tidal flat is more inferior to that of oysters in Dangsan-ri. The ANOVA test also indicated that the mean DGA of Manila clam from Dangsan-ri is significantly lower than Ohji-ri ($P < 0.05$).

DISCUSSION

Both histology and the RFTM assay demonstrated that *P. olseni* infects Manila clams in the study site in high intensity. The mean infection intensity of *P. olseni*, ranging approximately from 0.7 to 2.0 million cells/g wet tissue, is considered to be substantial. Such a high level of *P. olseni* infection recorded in this study may lead to retarded growth, gonad maturation, and low production of the gametes (Park and Choi, 2004; Park *et al*., 2005). Nam *et al*. (2018) reported that the surfacing of Manila clams from the sediment to the surface is closely linked to a high level of *P. olseni* infection. The surfaced clams are unlikely to burrow back into the habitat, and the clams are possibly relocated to the upper intertidal area by the tide then perished. Waki *et al*. (2018) also reported that high infection with *P. olseni* is one of the significant causes of mortality in tidal flats in Japan, and *P. olseni* infection intensity over million cells/g tissue may be critical to the host. Several studies have

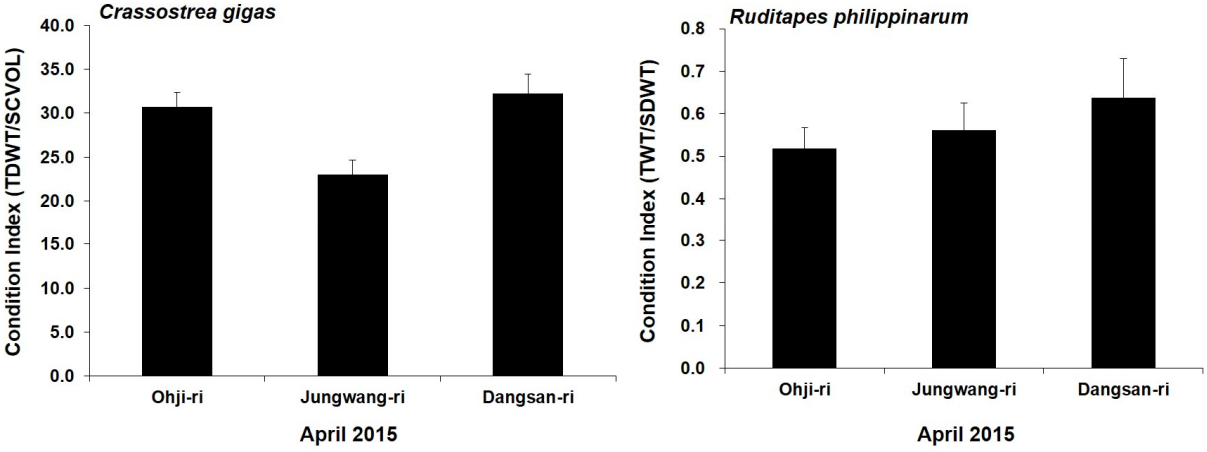

Fig. 4. The mean condition indexes of the Pacific oysters and Manila clams surveyed in April 2015. TDWT, dry tissue weight in gram, SCVOL, shall cavity volume, TWT, wet tissue weight in gram, SDWT, dry shell weight in gram. The error bars indicate the standard deviations.

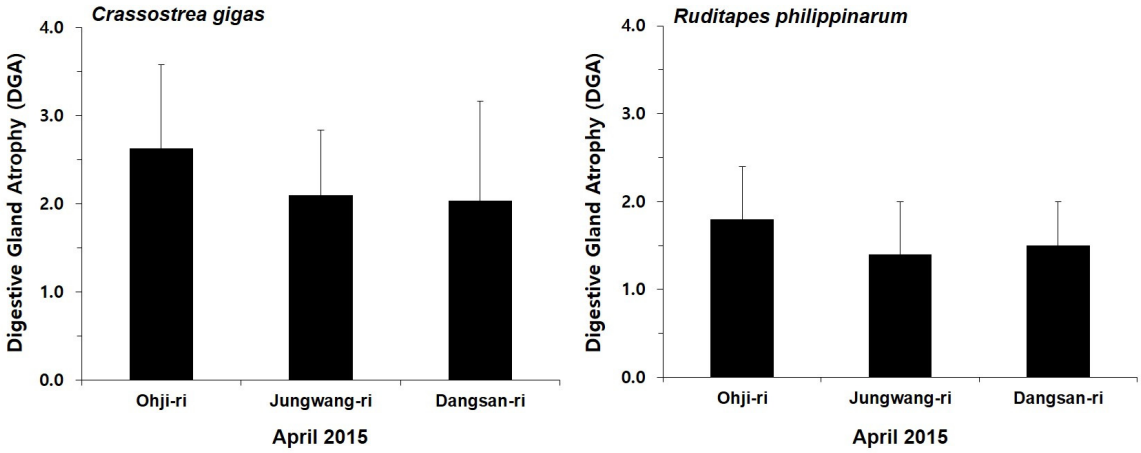

Fig. 5. The mean digestive gland atrophy (DGA) of the Pacific oysters and Manila clams. The error bars indicate the standard deviations.

reported that *P. olseni* infection intensity increase as the water temperature and salinity become high (Umeda *et al*., 2013; Waki *et al*., 2015). Park *et al*. (2006) and Yang *et al*. (2012) reported seasonal variation in the *P. olseni* infection intensity of Manila clams in Gomso Bay on the west coast. In their study, *P. olseni* infection level was significantly higher in late summer and early fall than the spring. They postulated that the observed seasonality in *P. olseni* intensity is associated with the seasonal changes in the water temperature and reproductive activity of the host. Therefore, we expect that *P. olseni* infection level in the study site could increase during summer and early fall as the water temperature increases from spring to summer.

Clams and oysters often host various types of metazoan parasites. In Korea, several studies have reported on the occurrence of trematode parasite in Manila clams and oysters (Le *et al*., 2015; Sohn *et al*., 2017). In this study, we observed the sporocyst stage of *C. tapidis* in the oyster, occupying the gonad. The sporocyst stage of *C. tapidis* was also observed from the gonad of Manila clams. Since the host clams and oysters were in the early developing stage, the impacts of the trematode infection are somewhat unclear. Several studies have reported that a high level of trematode infection in marine bivalves often results in total castration of the gonad. In this study, we observed a low level of trematode infection in the gonad, and the impacts of the trematode infection on the host population are thought to be minimal.

In summary, we surveyed the overall health and pathologic condition of the Pacific oyster and Manila clams occurring in three tidal flats in the Garorim Bay using histology. Both RFTM assay and histology confirmed a high level of *P. olseni* infection in Manila clam populations in the Bay. Histology also revealed trematode and other protozoan parasites, while the impacts of those symbiotic organisms on the host physiology were unclear. It was believed that a routine survey, as performed in this study, should be carried out to understand the impacts of the parasitic organisms on the oysters and clams for proper management of the commercially essential shellfish resources.

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