RESEARCH ARTICLE

Effects of temperature and salinity on larvae adhesion of four ascidians

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In order to explore the influence mechanism of early biofilm and ecological factors on larvae adhesion and population establishment of ascidian in the aquaculture pond of *Stichopus japonicus*, laboratory simulation experiment was adopted to investigate the effects of temperature and salinity on the adhesion of four ascidians' larvae including *Styela Clava*, *Ciona Savignyi*, *Ciona Intestinalis*, and *Molgula manhattensis* on the basis of field investigation. The results showed that biofilm exhibited positive effect on the adhesion of four larvae, and the adhering rates of four larvae all increased with the increasing temperature in the range from 12℃ to 27℃, while salinity showed little effect on the fixation from 29‰ to 32‰. Under the same environment, *M. manhattensis* showed higher adhesion competitiveness than the other three species, which might explain why *M. manhattensis* was the dominant species in the sessile ascidians community in the ponds farming *S. japonicus*. The study was helpful to understand the dialectical relationship between environmental factors and biological factors in the aquaculture environment and provided a theoretical basis for exploring the mechanism of marine biofilm formation.

Keywords: ascidians; adhesion; larvae; temperature; salinity.

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Introduction

In recent years, the cultivation of *Stichopus japonicus* has become the pillar industry of aquaculture in China, especially in northern coastal areas. Seaside pond cultivation including pond cultivation and cofferdam cultivation is the dominant mode [1]. The artificial adherents placed in the pond provide not only a secluded habitat for the sea cucumber, but also an attachment surface for the fouling organisms [2]. Ascidians are important members of the fouling organisms in the culture environment of sea cucumbers. Attached ascidians grow rapidly, which can not only occupy the living space of sea cucumbers, but also compete with sea cucumbers for bait and dissolved oxygen, so that inhibits the growth of *S. japonicus* [3]. The strong water filtration capacity of ascidians improves the transparency of the aquaculture water and increases the light intensity at the bottom, which is unfavorable to the *S. japonicus* [4].

An ongoing ecological investigation was carried out on the aquaculture pond of *S. japonicus* in Yantai, Shandong, China (37°22′ N, 121°25′ E) from 2020 to 2021. Common fouling organisms in the surrounding waters such as *Bugula sp.*, *Mytilus edulis*, *Barnacles*, *Membranipora*, *Oysters*, *Hydroides elegans*, and *Tubularia mesembryanthemum* rarely appeared in the aquaculture ponds of *S. japonicus* in the coastal intertidal zone [5]. Ascidians were the dominant species of sessile organisms in the pond, whose

species, biomass, and distribution were significantly different from those in the nearby sea. The dominant ascidians in Yantai coastal water were *Styela Clava*, *Ciona Intestinalis*, and *Ciona Savignyi*. Larvae of these ascidians could be detected in the water of aquaculture ponds and nearby waters from February to November, but were rarely occurred in the community of sessile organisms in ponds. *S. clava* was occasionally found on nylon nets, and the individuals were significantly smaller than those in the surrounding sea. The dominant sessile ascidian in the pond were identified as *Molgula manhattensis*. This ascidian is the most abundant one from June to October every year, and mainly appears on farming equipment such as ground cages and nylon nets.

The investigations on population dynamics of ascidians in the pond farming *S. japonicus* during 2020 to 2021 preliminarily determined that the main environmental factors affecting ascidians population were water temperature, salinity, and levels of nutrients. The biofouling of ascidians began with adhesion of larvae. The biofilm played an important role in the attachment procedure of marine invertebrate larvae [6]. Previous research found that temperature and salinity showed great influences on microorganisms and microalgae communities in water and sediment, which were likely to influence the formation and dynamic succession of biofilm. In this study, biofilms were developed under controlled indoor conditions. The fixation of artificially hatched ascidians larvae on the biofilm was investigated in order to clarify the effect of temperature and salinity on early formation of ascidium community. It was of great significance to control the biofouling of ascidians in the culture process of *S. japonicus*.

Materials and Methods

Seawater

The seawater used for the study was taken from the pond farming *S. japonicus* (Shandong Oriental Ocean Sci-Tech Co., Ltd., Yantai, Shandong, China) and was filtered through a 20 μm cellulose acetate filter (Merck-Millipore, Darmstadt, Germany) with a salinity of 32‰ and pH7.5 ~ 7.9.

Attachment plate

The polyethylene plates of ground cage commonly used in the culture of *S. japonicus* were used as attachment materials. The plates were cut into 150 mm × 100 mm pieces, cleaned by immersing the plate in 75% ethanol for 3 mins, then rinsed twice with ultra-pure water before sterilized at 121℃ for 15 mins and dried naturally on a clean bench.

Develop of biofilm

The experiments were carried out in 36 aquariums of 400 mm × 300 mm × 240 mm with 200 mm water in depth. Seawater was renewed every day. 7 pieces of the treated attachment plate were hung uniformly in each tank, and the upper end was 50 mm below the water surface. Biofilms were developed on the surface of plate under the static. Six parallels were set for each treatment. According to the results of early field investigation, the levels of temperature in this study were set as 12, 15, 18, 21, 24, and 27℃, and the levels of salinity were set as 29, 30, 31, and 32‰, respectively. One piece of attachment plate was taken out from each tank at 1, 2, 4, 6, 8, 15, and 30 days, respectively, to analyze the adherence of ascidians' larvae.

Fixation of ascidians' larvae on biofilms

Live specimens of four ascidians including *Styela clava*, *Ciona savignyi*, *Ciona intestinalis*, and *Molgula manhattensis* were collected from unpolluted shallow water along Yantai coast of the Yellow Sea, China (37°22' N, 121°25' E). The ascidians were taken down along with their adherences to avoid being hurt, and all debris and epibiota were afterwards cleared carefully from the adherences. 200 individual ascidians per cubic meter were cultured in the laboratory at the temperature ranging from 18℃ to 22℃ under continuous illumination and fed on microalgae *Nitzschia closterium* which were cultivated by Microalgae Laboratory of Yantai

University, Yantai, Shandong, China twice daily. The ascidians reached their sexual maturity in 7-15 days. Then the mature spermatovum hatched into tadpole larva at 20℃ for 9 ~ 15 hrs and attached themselves to nylon rope within 12 hrs. Sterilized seawater was added to every 250 mm × 250 mm × 250 mm fiber reinforce plastic (FRP) tank at a water depth of 200 mm. Two pieces of attachment plates that had formed biofilm at different times were uniformly hung in each tank, and one piece of blank attachment plate without biofilm was set as a blank control. Two hundred larvae of ascidian were added into every tank, and the environmental factors were controlled to be the same as corresponding film-forming conditions. After 12 hours, the attached plates were taken out to detect the number of attached larvae. 9 parallels were set for every treatment group of each ascidian, respectively.

Statistical analysis

All the results of the experiment were shown as mean ± standard deviation (SD). SPSS 11.0 (IBM, Armonk, New York, USA) was used for variance analysis. One-way ANOVA and Duncan was used for the comparative analysis of the adhesion rate of the ascidians. $P < 0.05$ was accepted as the significant difference.

Results and discussion

The larvae of ascidians remain in a swimming state for only a few hours, no more than 24 h, after which the larvae begin to settle and metamorphosis [7]. The previous studies showed that the attachment rate of larvae increased sharply with the extension of time, and the rate of settlement at 12 h was significantly higher than that at 6 h ($P < 0.01$). After 12 h, the adhesion rate remained stability, indicating that the settlement was basically completed within 12 h [8]. Combined with previous research results, 12 h was selected as the attachment time of larvae in this study.

Effect of water temperature on the adhesion of ascidian larvae

The attachment rates of the four ascidians larvae on the surface of the attached plates under different water temperatures were shown in Figure 1. The adhering rate of the four larvae on the surface of attachment plates gradually increased with the increase of water temperature. 21℃ and 24℃ were the most suitable temperatures for larvae to adhere, and then, the adhering rates decreased with the increase of water temperature. At the same temperature, the adhesion rate of *M. manhattensis* was higher than that of the other three ascidians larvae. In natural sea areas, the adhesion of ascidians larvae mostly occurs in June to September with higher water temperature, and a large number of ascidians larvae die when the water temperature decreases [9]. According to the research on the ascidians in Yellow Sea, China and Bohai Sea, China, there are two reproductive peaks. The first peak occurs from late May to July, and the second peak occurs from October to mid-November. The water temperatures of the two periods are 18-24℃, and the adhering ability of *M. manhattensis* was higher than that of other kinds of ascidians in this temperature range. These results indicated that *M. manhattensis* had stronger adhesion competitiveness when four species of ascidians larvae coexisted in water. The previous investigation of the pond farming *Stichopus japonicus* also showed that *M. manhattensis* was the dominant species in attached ascidians, which was consistent with the results of this study. The adhering rate of the four larvae also increased with the increase of biofilm's age in days. When the biofilm's age exceeded 14 days, the adhering rate of larvae tended to be stable with a slight decrease. The adhering rates of four larvae on the surface formed biofilm were significantly higher than that of the blank material ($P < 0.01$), indicating that the biofilm had positive effects on the attachment of larvae.

Effect of salinity on the adhesion of ascidian larvae

According to the preliminary field survey, the salinity of water in the sea cucumber culture

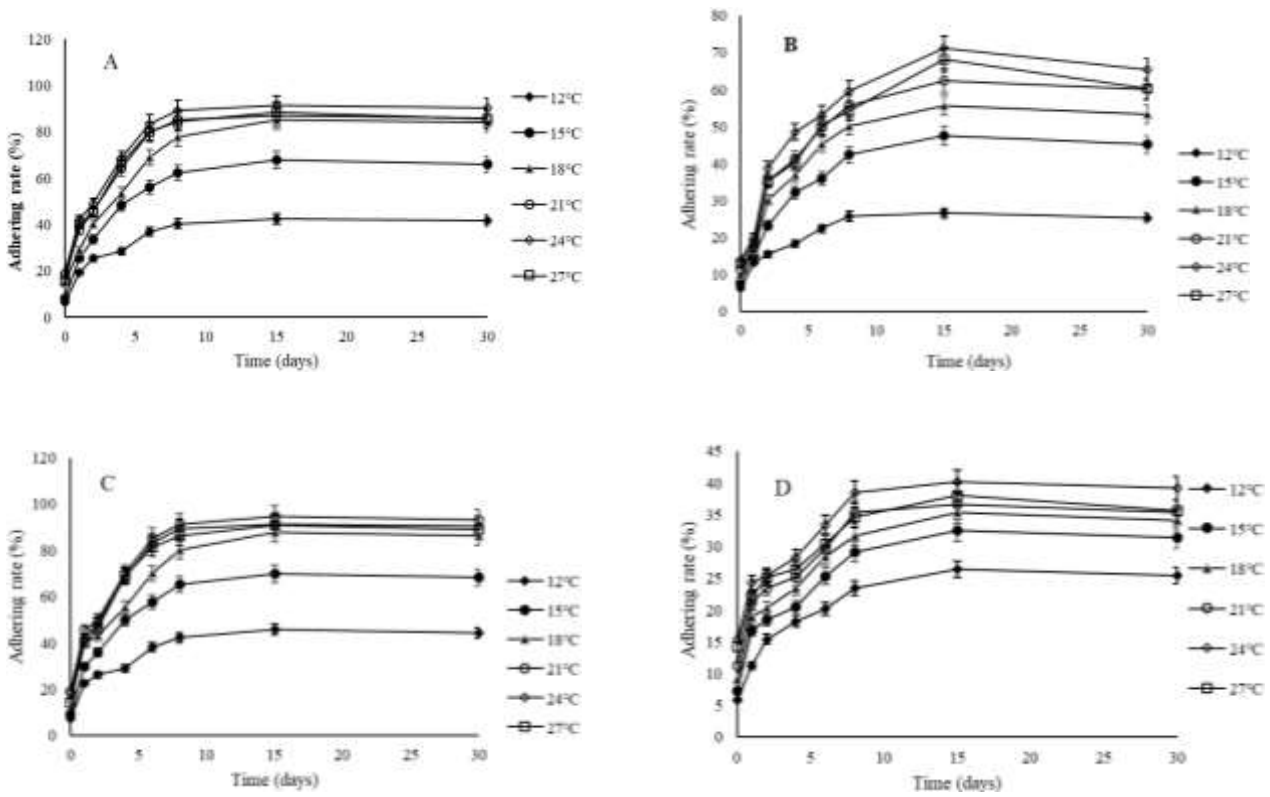

Figure 1. Effects of water temperature on the adhesion of ascidians larvae. **A.** *Styela clava*. **B.** *Ciona intestinalis*. **C.** *Molgula manhattensis*. **D.** *Ciona savignyi*.

pond was 29-32‰ in a year, so the salinity levels were set as 29, 30, 31, and 32‰ in this study. Figure 2 showed the adhering rates of four ascidian larvae on the surface of the attached plate under different salinity conditions. The adhering rates of four larvae on the surface formed biofilm were significantly higher than that of the blank material ($P < 0.01$) and increased with the increase of biofilm's age in days. The adhering rates of four ascidians larvae on the surface of the attached plates increased slightly with the increase of salinity ($P > 0.05$). It could be seen from the results that the salinity set in this study showed no significant effect on the adhesion of ascidians larvae, which was different from the results of field investigation, and the specific reasons needed to be further studied.

Biofouling is an ecological succession process [10, 11], and the adhesion of larvae on the biofilm is the starting point of fouling and sessile population formation of ascidians. This study confirmed that the biofilm had a positive effect on the adhesion of larvae, and the adhering rates of larvae on the surface formed biofilm were significantly higher than that of the blank attachment material ($P < 0.01$). Under the same conditions, the adhering rates of *M. manhattensis* was the highest in the four larvae, followed by *S. clava*. The adhesion of *C. intestinalis* and *C. savignyi* were significantly lower than that of the other two species. That may explain why *M. manhattensis* are the dominant species in the sessile ascidians community in the ponds farming *S. japonicus*, because it had higher adhesion competitiveness than the other three species in the aquatic environment. It had been identified through early field survey that the main environmental factors affecting the sessile ascidians population in the ponds farming *S. japonicus* were water temperature and salinity. These factors also are the main factors influencing the water floating

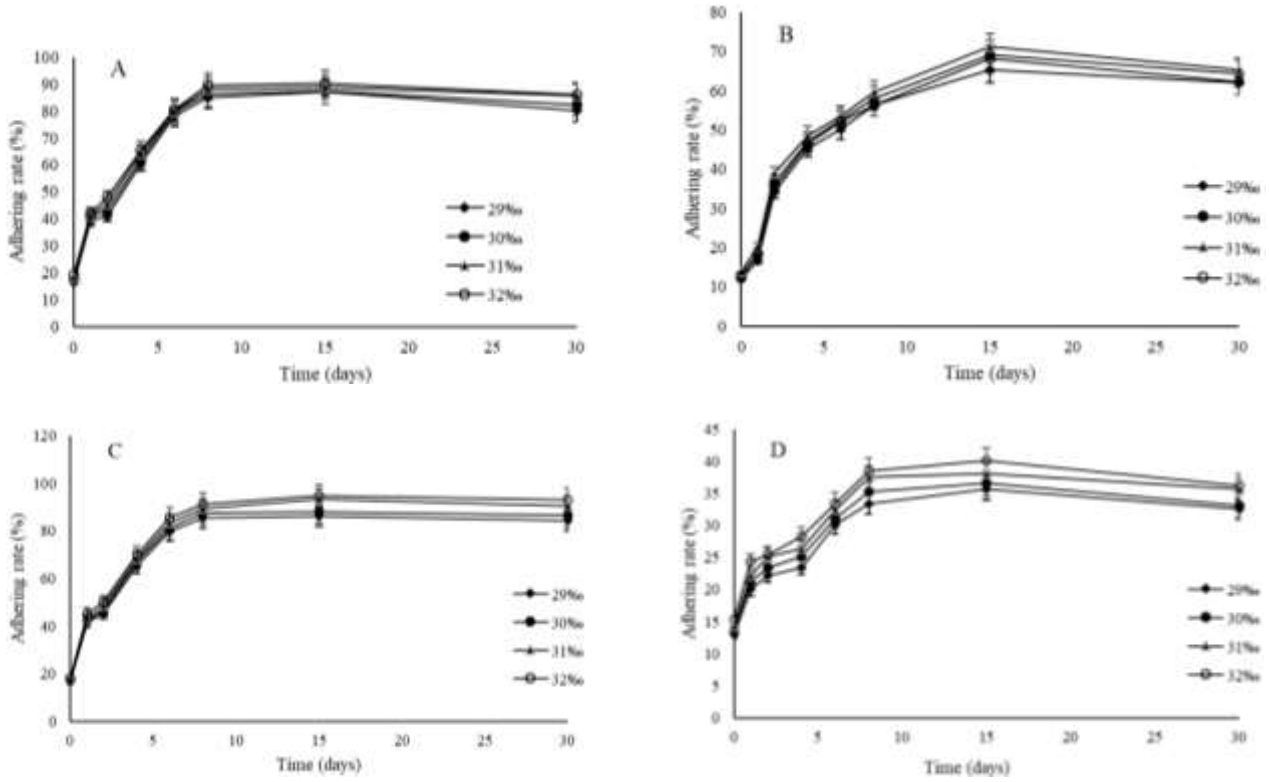

Figure 2. Effects of salinity on the adhesion of ascidians larvae. **A.** *Styela clava*. **B.** *Ciona intestinalis*. **C.** *Molgula manhattensis*. **D.** *Ciona savignyi*.

microbial communities [12]. The results of this study showed that water temperature showed a significant effect on the adhesion of four kinds of ascidians' larvae, while salinity had little effect on the fixation of the four larvae. The preliminary field investigation found that temperature and salinity of aquatic pond had significant influence on the microorganisms and phytoplankton. The interaction of environmental factors with biotic factors resulted in the formation of sessile ascidians community different from the surrounding sea areas, and the influencing mechanism needs to be further studied.

Conclusion

This study investigated the effects of temperature and salinity on the adhesion of four ascidians' larvae including *Styela clava*, *Ciona savignyi*, *Ciona intestinalis*, and *Molgula manhattensis*. The biofilm exhibited a positive effect on the adhesion of four larvae, and the adhering rates of four larvae all increased with the increasing levels of temperature. Salinity had little effect on the fixation of larvae. Under the same environment, *M. manhattensis* showed higher adhesion competitiveness than the other three species, which might explain why *M. manhattensis* were the dominant species in the sessile ascidians community in the ponds farming *S. japonicus*.

Acknowledgements

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