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Effect of *Stenotrophomonas maltophilia* Bacteria and Environmental Factors on the Thallus of Red Seaweed *Kappaphycus alvarezii*

Marlina Achmad[1,2†*], Alimuddin Alimuddin[3†] and Sukenda Sukenda[3]
[1]*Aquaculture Science, Postgraduate School, Bogor Agricultural University, Bogor, Indonesia*
[2]*Fisheries Department, Faculty of Marine Science and Fisheries, Hasanuddin University, South Sulawesi 90245, Indonesia*
[3]*Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Bogor, Indonesia*
[*]For correspondence: marlina.achmad@unhas.ac.id; marlinachmad83@gmail.com
[†]Contributed equally to this work and are co-first authors
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Abstract

Interaction between environmental factors, particularly temperature and salinity with bacteria is considered one of the main triggers of disease infection in various hosts including seaweed. Therefore, this study aimed to evaluate the effects of interactions between temperature and salinity with pathogenic bacteria on the manifestation of ice-ice disease in red seaweed Kappaphycus alvarezii. Red seaweed was weighed to 50-51 g for each treatment replicate, incubated at 25°C and 28°C, then infected with *Stenotrophomonas maltophilia* bacteria at a concentration of 10^0-10^6 cfu/mL in a culture medium (30 g/L salinity). Salinity treatment was conducted in variations of 28, 30 and 35 g/L with the same bacteria concentration (10^6 cfu/mL) and temperature (28°C). The results showed that seaweed infected by *S. maltophilia* and incubated at 28°C had a higher reduction in weight and greater thallus bleaching compared to the 25°C treatment. A significant difference was observed in the percentage of bleaching on the secondary thallus in both temperature treatments. The onset of disease transmission affected *K. alvarezii* morphological structure, which showed severe condition on day 4. In conclusion, both temperature and salinity affected ice-ice disease infection in red seaweed. However, this study could not fully elucidate the interactions between biotic and abiotic factors and the effect on ice-ice disease. Further investigations are needed to examine the effect of the interaction.

Keywords: *Stenotrophomonas maltophilia*; *Kappaphycus alvarezii*; Temperature; Salinity; Thallus; Bleaching

Introduction

Disease occurrence is an important factor in marine ecology and appears to be increasing along with the extreme environmental change. Several marine organisms including seaweed have experienced mass mortality caused by disease. Red seaweed, also known as *Kappaphycus alvarezii*, reported experiencing bleaching when the water temperature exceeds 30°C (Largo *et al*. 1995b). This phenomenon starts with the disappearance of pigment in the thallus. Bleaching can also be found in other red algae species such as *Delisea pulchra* (Fernandez *et al*. 2012).

Ice-ice in *K. alvarezii* was initially suspected to be a non-infectious disease triggered by inadequate environmental conditions such as extreme temperature, high light intensity and low salinity. However, studies have reported that pathogenic bacteria infection also causes severe ice-ice disease in macroalgae (Gachon *et al*. 2010). A previous study found that opportunistic pathogenic bacteria such as *Vibrio* sp. (P11) and *Cytophaga* sp. (P25) were successfully isolated from *K. alvarezii* seaweed manifesting ice-ice disease (Largo *et al*. 1999). Other bacteria isolated include *Stenotrophomonas maltophilia*, a Gram-negative bacteria found in the natural environment (Brooke 2012). It was initially isolated in 1943 as booker bacteria with the name *Pseudomonas maltophilia* (Hugh and Leifson 1963). Immersion of seaweed in this bacteria could trigger bleaching of seaweed (Achmad *et al*. 2016).

Previous studies suggested that bleaching phenomenon was caused by combinations of bacterial infection and environmental factors. During stress conditions, seaweed exudes chemicals that attract bacteria, inducing bleaching and stiffening of branches. Some thallus remained healthy, while those infected experienced depigmentation and eventually damage (Naguit and Tisera 2009). The initial factor that may cause symptoms of ice-ice disease in *K. alvarezii* seaweed is environmental stress. The main environmental stressors are 30-35°C temperature,

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≥ 20 g/L salinity and high light intensity (Largo *et al*. 1995a). Studies focusing on the effect of environmental stress in *K. alvarezii* have continued to attract attention, including the relationship between the growth and high chlorophyll concentration when maintained at 20-24°C temperature and 25-35 g/L salinity (Araújo *et al*. 2013). The range of salinity that supports *K. alvarezii* growth cultured *in vitro* (Hayashi *et al*. 2011) and transgenic seaweed (Triana *et al*. 2016) has been recorded at 25-45 g/L.

Symbiotic or commensal interactions between bacteria and algae tend to occur at a narrow temperature range. This is because environmental temperature could increase the susceptibility of the macroalgae to opportunistic pathogen infection. Opportunistic pathogens are considered commensal to macroalgae and would take advantage when the host is weakened, ultimately causing disease. In general, disease manifestation in algae is often correlated with the presence of bacteria that secrete cell wall-destroying enzymes such as agarases, alginases, cellulases, pectinase and fucoidanase. These enzymes disrupt the integrity of the cell wall, allowing bacteria to gain access into the algae living tissue (Goecke *et al*. 2010). Bacteria that are pathogenic to eukaryotic microalgae have been reported to attach the surface of macroalgae at a density of 104 cfu (Largo *et al*. 1995a; Vairappan *et al*. 2009). However, there are only a few reports on the interaction of environmental factors and bacteria that cause ice-ice disease and the effects on tissue conditions in *K. alvarezii* seaweed.

In general, tissue age affects the carrageenan level in seaweed (Mendoza *et al*. 2006). Galactant composition in *K. alvarezii* seaweed increased along with thallus diameter (Lechat *et al*. 1997). Tissue age is represented by important parts of the thallus, namely basal part, representing old tissue and apical part, representing young tissue. According to different studies, young thallus has the highest gel strength (Craigie and Wen 1984). The highest agar quantity is also found in the young parts of *Gelidium pristoides* seaweed (Carter and Anderson 1986). This indicates that tissue age might influence seaweed biology especially galactant quality, which is produced by several agarophyta. The difference in chemical characteristics between apical and basal parts is an interesting aspect to study, particularly in relation to the pathogenic infection of *K. alvarezii* seaweed.

Limited progress has been made in elucidating the cause of seaweed disease through microscopic pathology (Work and Meteyer 2014). Methods such as microbe culture, molecular testing and histopathology are used to determine the relationship between pathogen and the injured tissue (Work and Meteyer 2014; Qu-er-e *et al*. 2015). Histological analysis has also been used to confirm the relationship between bacteria and ice-ice disease in red algae *Delisea pulchra* (Fernandes *et al*. 2012). Therefore, this study aimed to evaluate the effect of interactions between temperature, salinity and pathogenic bacteria on macroscopic and microscopic changes in *K. alvarezii* thallus.

Materials and Methods

Bacteria culture

Bacteria isolates of *S. maltophilia* with high pathogenicity were selected as samples. The isolates were cultured in solid seawater complete (SWC) medium at 28°C for 24 h followed by subsequent culture in liquid SWC, which was shaken at 140 rpm for 24 h. The suspension was then harvested and used in the following experiments.

K. alvarezii seaweed preparation

Seaweed used in this experiment was obtained from a farmer in Lampung, Indonesia. Before use, seaweed was sterilized following the procedure described by Sulistiani and Yani (2014). About 50-51 g wet weight was weighed and then rinsed with sterile seawater for cleaning purposes. Subsequently, seaweed was submerged in a solution containing 1% povidone iodine 1% (10 mL Betadine dissolved in 90 mL sterile seawater) for 3-4 min. The sample was placed in sterile seawater and subsequently distributed in each replicate aquarium that has been equipped with aeration. However, there was no water replacement during the experiment.

Temperature-pathogenic bacteria interaction test

This study used factorial design, with two factors, namely temperature (25 and 28°C) and *S. maltophilia* bacteria at concentrations of 10^0, 10^2, 10^4 and 10^6 cfu/mL. Different bacteria concentrations were prepared by serial dilution. Temperature was controlled by a heater and each treatment had three repetitions.

The temperature used in this study was low and normal, in line with Vairappan *et al*. (2001) who reported that the increase of injury levels on *Laminaria religiosa* seaweed occurred at a temperature up to 10°C, by lowering salinity from 30 g/L into 12 g/L within a week. The low temperature used was to simulate the environmental change caused by rainfall. In different temperature treatments, salinity was set at normal conditions, which was 30-32 g/L. Other than different temperature treatments, this study also observed the effect of salinity on the thallus of *K. alvarezii*. Salinity treatment was carried out at 28, 30 and 35 g/L. In the experiment with different salinities, the temperature was set at normal condition of 28°C with and without *S. maltophilia* bacteria infection at 10^6 cfu/g concentration. Subsequently, seaweed was incubated for 7 days and morphological changes were observed every day.

Experimental parameters

Experimental parameters measured in this study included wet weight changes and the percentage of bleaching in thallus. Weight change observation was carried out by

weighing seaweed at the beginning until the end of the experiment (from day two until day five). The number of branches with bleaching symptoms was counted at every part. The thallus was divided into two regions, namely basal (old tissue) and apical (young tissue). Generally, red algae group is bordered by apical meristem, which causes basal-apical gradient through the branch. Sub-apical generates thallus branches, where the apical region of the thallus represents young tissue (Mendoza *et al*. 2006). In this study, thallus region was separated into primary (basal), secondary (apical) and tertiary (sub-apical) (Fig. 1).

Histopathology

Seaweed tissue structure observation was conducted before and after cohabitation/transmission test. The thallus tissue was exposed to different temperatures of 25°C and 28°C along with *S. maltophilia* bacteria concentration of 10^6 cfu/mL. Histological images were collected from healthy seaweed tissue and those infected by ice-ice disease followed by a descriptive analysis.

Data analysis

Weight changes and the number of thallus showing pathogenic bacteria infection symptoms were analyzed descriptively. The effect of interactions between the two factors tested was analyzed by analysis of variance followed by Duncan's post hoc test. Statistical analysis was performed using the statistical software SAS program v. 9.1 portable Windows.

Results

Effect of temperature and bacteria concentration

The results showed that temperature and bacteria concentration significantly affected seaweed weight changes as shown in Table 1. The weight reduction of seaweed infected by *S. maltophilia* maintained at 25°C (0.50-1.10%) was lower compared to that of 28°C (1.23-1.71%). Temperature had significant effects on the number of thallus with bleaching symptoms in the primary, secondary and tertiary regions (Table 1). At a temperature of 28°C, more infections were observed on all thallus regions compared to 25°C.

Pathogen treatment, namely exposure to *S. maltophilia* bacterial infection 10^4 and 10^6 cfu/mL showed a significant difference in changes in wet weight (1.11-1.64%), the amount of bleaching in the primary thallus (0.59-1.72%), secondary thallus (1.09-4.26%) and tertiary thallus (2.17-7.09%) of seaweed (Table 1). However, the interaction between temperature and bacteria gave the same effect on changes in the wet weight of seaweed, the amount of bleaching in the primary thallus, secondary thallus and tertiary thallus. This indicates that the influence of a single factor, either temperature or bacteria, can trigger the symptoms of ice-ice disease in seaweed and if both factors work together, they cannot trigger the symptoms.

Temperature and seaweed thallus morphological condition

Seaweed thallus morphology after infection showed a striking difference on each day (Fig. 2). Ice-ice disease symptoms started to appear on day one after infection showing the presence of bleaching spot on thallus. On day two after infection, bleaching started to be clearly seen in several areas of the thallus and on day three, the bleaching area started to spread. Bleaching had spread all over the thallus on day four. On day five following infection, some thallus experienced bleaching while others were found broken indicating the mortality of the thallus with bleaching spread all over.

Histology of seaweed tissue exposed to different temperatures

The histopathological profile of seaweed thallus infected with ice-ice disease is presented in Fig. 3. The thallus tissue condition assessed before (I) and after infection (II) of ice-ice disease was categorized into control (IA and IIA), medium severe condition (IB and IIB) and highly severe (IC and IIC). The indicators used to distinguish the three tissue conditions were determined from epithelial cell condition, the number of protoplasm and the distance between cells. In the infected tissue, epithelial cell was reduced, cell protoplasm content was decreased, cell wall was incomplete and the gap between cells was widened.

Salinity and *K. alvarezii* wet weight changes

A reduction in *K. alvarezii* wet weight after *S. maltophilia* infection was observed as one of the responses to different salinities (Table 2). The statistical analyses showed that salinity had no significant effect on seaweed wet weight reduction. This indicated that 28, 30 and 35 g/L treatment salinity had the same effect on weight reduction. The data were analyzed by two-way analysis of variance. Different superscripts in the same column indicate significant differences ($P < 0.05$). The damaged and bleached seaweed thallus due to the effects of *S. maltophilia* infection at different water salinity is shown in Table 2.

The statistical results showed that the percentage of bleaching was similar between primary thallus maintained in 28 and 30 g/L salinity is 0% (no visible ice-ice symptoms). However, the results were lower compared to those maintained in 35 g/L salinity (54.23-70.83%). The salinity of 28 and 35 g/L showed the same effect on the percentage of leaching in the secondary thallus, namely 100% and 97-100% respectively (Table 2). The tertiary thallus was similarof bleaching (100%) at 28 and 35 g/L salinity, both of which were higher compared to that of 30 g/L salinity (40.89-51.41%). These results indicate that water salinity and bacteria infection influence bleaching percentage. The effect of 28 and 35 g/L salinity on the percentage of bleaching was high, particularly in secondary and tertiary thallus. Secondary and tertiary thallus are part of

Table 1: Wet weight reduction and the number of seaweed thallus infected by ice-ice disease (bleaching) on *K. alvarezii* immersed in *S. maltophilia* bacteria suspension and incubated at different temperatures

Treatments	Changes in wet weight (%)	The number of seaweed thallus bleaching (%)		
		Primer Thallus	Secondary Thallus	Tersier Thallus
Temperature (°C)				
25	0.80 ± 0.30 [a]	0.74 ± 0.24[a]	1.61 ± 0.52[a]	2.93 ± 1.02[a]
28	1.47 ± 0.24 [b]	1.47 ± 0.39[b]	3.38 ± 0.99[b]	6.28 ± 1.93[b]
Concentration (cfu/mL)				
10^0	0.87 ± 0.41 [a]	1.03 ± 0.56 [a]	1.86 ± 0.71[a]	4.26 ± 2.38[a]
10^2	1.15 ± 0.64 [ab]	1.01 ± 0.46[a]	2.24 ± 0.88[ab]	4.29 ± 2.66[a]
10^4	1.30 ± 0.19 [b]	1.13 ± 0.54[b]	3.10 ± 1.16[c]	5.11 ± 1.98[b]
10^6	1.22 ± 0.42 [b]	1.25 ± 0.47[bc]	2.78 ± 1.69[bc]	4.77 ± 2.60[a]
Interaction of Temp. and bacteria concentration	*P > F = 0.84*	*P > F = 0.89*	*P > F = 0.10*	*P > F = 0.93*

Different superscript letters in the same column indicate significantly different results in Duncan's further test (*P < 0.05*)

Table 2: Wet weight reduction and the percentage of seaweed *K. alvarezii* thallus showing ice-ice disease (bleaching) symptoms upon immersion with *S. maltophilia* bacteria 10^6 cfu/mL and incubation at different salinities

Treatments	Wet weight reduction (%)	The number of seaweed thallus bleaching (%)		
		Primer Thallus	Secondary Thallus	Tersier Thallus
Salinity (g/L)				
28	33.00 ± 12.52[a]	0.00 ± 0.00[a]	100.00 ± 0.00[b]	100.00 ± 12.52[b]
30	36.40 ± 10.25[a]	0.00 ± 0.00[a]	46.37 ± 6.89[a]	46.15 ± 5.26[a]
35	36.78 ± 2.30[a]	62.50 ± 8.33[b]	99.11 ± 1.79[b]	100.00 ± 0.00[b]
Interaction of salinity and bacteria concentration	*P > F = 0.58*	*P > F = 0.42*	*P > F = 0.46*	*P > F = 0.97*

Different superscript letters in the same column indicate significantly different results in Duncan's further test (*P < 0.05*)

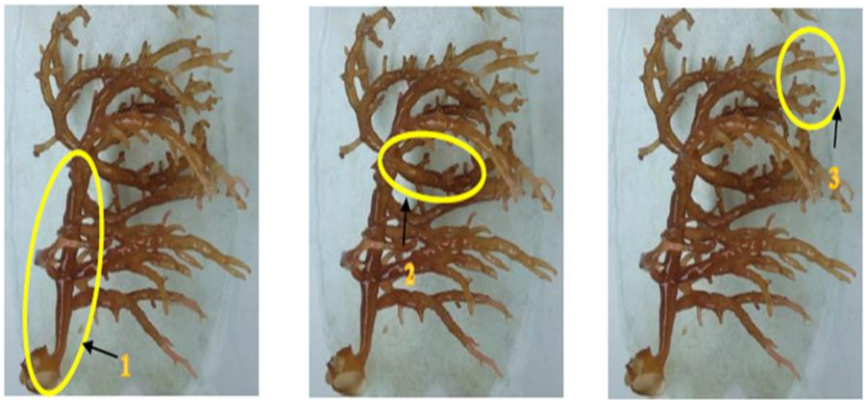

Fig. 1: Thallus segment of *K. alvarezii* seaweed: (**1**) primary thallus (basal region, left image); (**2**) secondary thallus (apical region, middle image); (**3**) Tertiary thallus (subapical region, right image)

the apical region which is prone to ice-ice disease. Moreover, the results of the interaction of salinity and bacterial factors showed no significant difference, either in changes in seaweed weight or the amount of bleaching. This indicates that the symptoms of ice-ice disease in seaweed are not a collaboration between environmental factors and pathogens simultaneously.

Thallus morphological condition following exposure to different salinities and *Stenotrophomonas maltophilia* bacteria

The results showed that seaweed thallus infected by *S. maltophilia* and exposed to 28 g/L, 30 g/L and 35 g/L salinities showed ice-ice disease symptom clearly visible after four days (Fig. 4). This also suggests that environmental factors especially salinity has greater potential to rapidly cause death in *K. alvarezii* thallus compared to temperature. However, the severity level was the same in each treatment on day four. As the culture time progressed, the severity of ice-ice disease increased. The morphological condition clearly indicated infection on day 4.

Discussion

Low temperature has been suggested as a factor that supports the survival of algae tissue (Mantri *et al*. 2011;

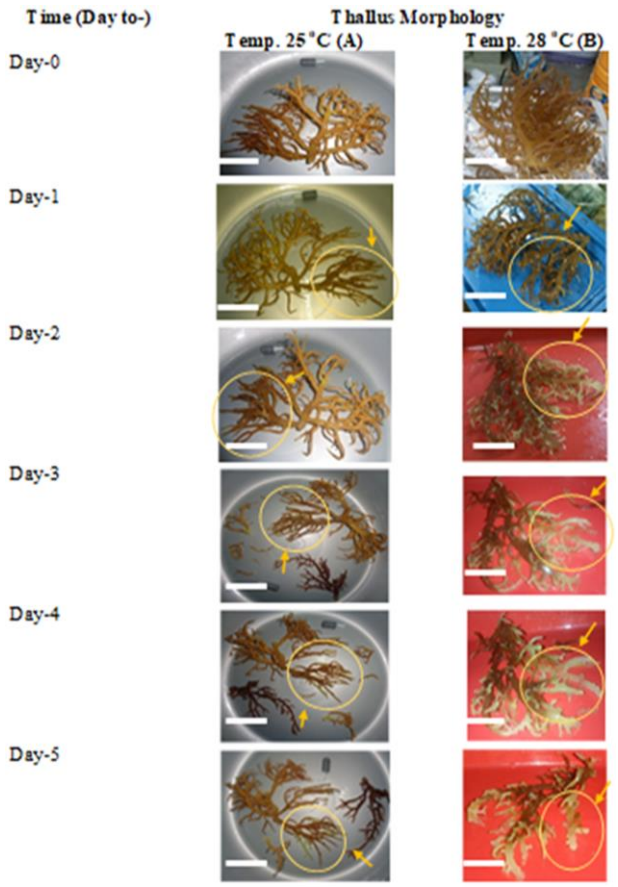

Fig. 2: Seaweed thallus morphological changes following bacteria infection: seaweed maintained at 25°C (A) and 28°C (B). (0) thallus still appeared healthy (before infection); (1) bleaching spot appeared only on certain areas on the surface and the tip of thallus; (2) bleaching started to appear on the thallus surface; (3) bleaching started to spread over the thallus areas; (4) severe bleaching was observed along the thallus areas; (5) part of the thallus was broken by bleaching. The number of each image indicates the day of thallus observation; the arrow and circle represent the infected area on the thallus

Mandal *et al*. 2015). This may be related to the lower activity of pathogenic bacteria at 25°C water temperature compared to 28°C. In this study, symptoms of ice-ice disease (IID) first appeared in seaweed maintained at 28°C (Table 1). Generally, 25°C is the optimal growth temperature for some seaweed such as *Fucus vesiculosus* and *Gracilaria vermiculophylla*, while temperatures starting at 26°C promote the proliferation of certain bacteria within seaweed host (Düsedau *et al*. 2023).

At seawater temperatures above 26°C, the number and stability of antimicrobial compound in coral mucus were depleted, increasing sensitivity toward pathogens (Orland and Kushmaro 2009). Wet weight reduction was calculated as the proportion of decrease during the experiment relative to the initial weight. Meanwhile, the percentage of bleaching was the proportion of infected thallus group with

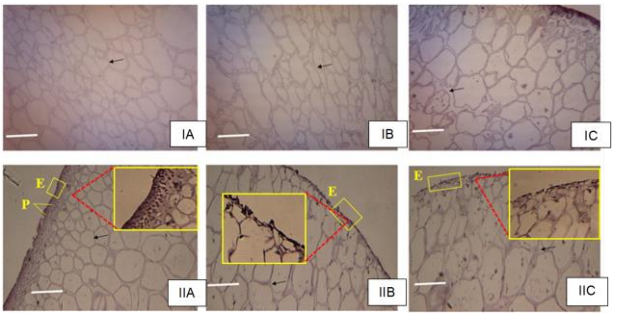

Fig. 3: Histology of thallus infected by ice-ice maintained at 25°C (B) and 28°C (C) incubation temperature. (IA) control, non-treated with stress, (IB) medium severe thallus condition, (IC) highly severe condition. (E = epithelial region; P = protoplast). Cell gaps (black arrow) appear very tight (IIA); relatively widening gap (IIB); wider gaps between the cells (IIC). (40x magnification; bar scale 100 μm)

bleaching symptoms relative to the total number. The data were analyzed by two-way analyses of variance. Different superscript in the same column indicates significant differences ($P < 0.05$). The results showed that different bacteria concentrations only significantly affected ($P < 0.05$) the percentage of bleaching in secondary thallus, but had no effect on primary and tertiary (Table 1).

The decrease in seaweed wet weight may be caused by the reduction of photosynthetic pigment in the thallus due to ice-ice disease infection. Generally, disease in algae is characterized by visible and prominent morphological changes. This includes tumors caused by abnormal cell growth, unusual pigmentation showing spots, rust, or bleaching due to destruction and loss of pigment, as well as impaired photosynthesis function (Gachon *et al*. 2010; Goecke *et al*. 2010).

Thallus bleaching process is related to the presence of pathogenic bacteria. The adhesion of disease agents (bacteria) to the host organisms followed by extracellular substance production plays a key role in tissue degradation and weight reduction, primarily caused by the lyses of host cells (Salyers and Whitt 1994). Largo *et al*. (1995a, b) reported depletion of haloamine in thallus, caused by the use of this substance by bacteria, making the seaweed more vulnerable to ice-ice disease.

Bacteria concentration showed significant effects on seaweed weight reduction. The concentration of 10^6 cfu/mL resulted in the highest seaweed weight reduction, ranging from 1.11-1.64%. The surface of algae was covered by 10^6 bacteria cell/cm^2 to 10^7 bacteria cell/cm^2 (Tujula 2006). Seaweed weight loss can be attributed to nutrient competition between the bacteria on the algae surface with the host. Bacteria attach to specific hosts through adhesion mechanism and components of algae wall may become a nutrient source, facilitating proliferation on the surface (Goecke *et al*. 2010). Adhesion of bacteria on algae surface disrupts the niche condition (Egan *et al*. 2013).

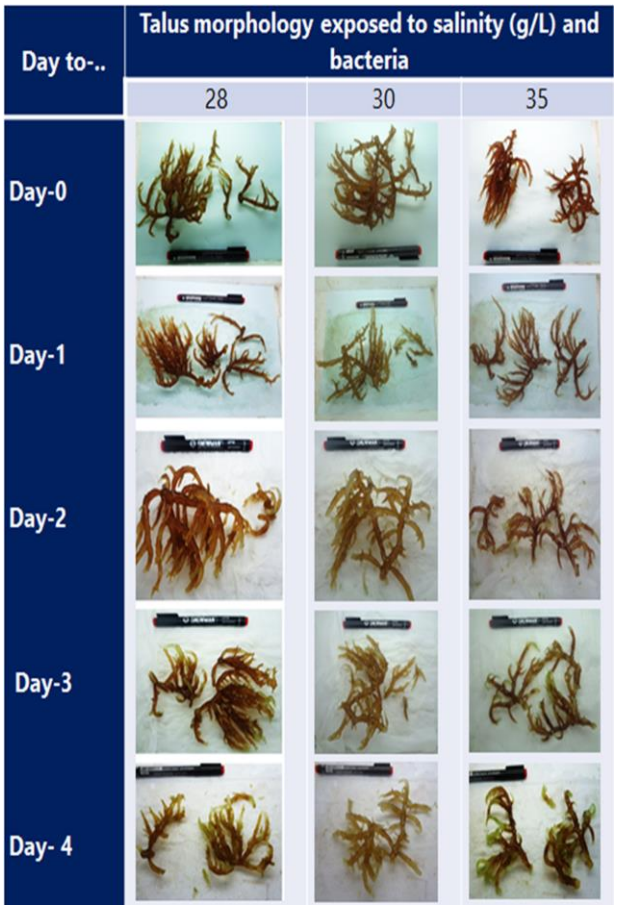

Fig. 4: Seaweed thallus morphological changes upon bacteria infection in 28 $g.L^{-1}$ salinity (A), 30 $g.L^{-1}$ (B) and 35 $g.L^{-1}$ (C). (0) healthy thallus (before infection); (1) bleaching only appeared on the surface and at the tip of the thallus; (2) bleaching started to appear on the thallus surface; (3) thallus with bleaching spread out to most of the thalus; (4) severe bleaching appeared in all over thallus and part of thallus was broken mostly caused by bleaching. (Number on each image showed thallus observation day; the arrow and circle showed the infected area)

Environmental factors have been shown to affect the prevalence of ice-ice disease. Stress caused by environmental changes including temperature (Largo *et al*. 1995a; Orland and Kushmaro 2009), salinity (Largo *et al*. 1995b) and light intensity (Largo *et al*. 1995b) potentially cause damage and bleaching on seaweed thallus. A similar phenomenon was also observed in some species of coral such as *Acropora alamata* which showed antibacterial activity at a temperature of 26°C and became more sensitive toward pathogen at a temperature above 26°C. A study by Campbell *et al*. (2011) concluded that the higher the water temperature, the more severe the bleaching in *Delisea pulchra* red algae. In this study, the 10^4-10^6 cfu/mL bacteria suspension produced the highest bleaching symptom in secondary thallus. Furthermore, at bacteria concentration 10^6 cfu/mL, several thallus had already experienced bleaching and disintegration, reducing the total count of the remaining thallus. This showed that the higher the bacteria concentration, the more severe the bleaching symptoms (Aris and Labenua 2020).

Temperature is another critical environmental factor that triggers ice-ice disease in *K. alvarezii* seaweed (Largo *et al*. 1995a). The range of temperature that could trigger disease symptoms in seaweed is 33-35°C (Ask and Azanza 2002). Temperatures under 20°C and above 30°C are reportedly dangerous for *Ulva fasciata* (Mantri *et al*. 2011; Mandal *et al*. 2015). In this study, a temperature of 25°C induced bleaching symptoms on seaweed thallus, with more severe bleaching observed at 28°C. In marine organisms such as coral, *Vibrio coralliilyticus* is virulent against *Pocillopora damicornis* at temperatures ≥ 23°C.

Bleaching on algae is mediated by bacteria abundance and influenced by environmental factors such as temperature. However, temperature alone is not sufficient to induce pathogenicity, with host immunity also playing a crucial role. The host immune response can inhibit bacteria communication through AHL (N-acyl homoserine lactones). Host immunological defense, similar to bacteria virulence, is affected by environmental conditions (Wright *et al*. 2000; Case *et al*. 2011). However, the interaction mechanism between temperature and bacteria towards bleaching still cannot be explained in detail.

The bacteria community associated with *U. australis* seaweed showed higher bacteria density at the thallus tip, reaching approximately 10^6 cell/cm^2 (Tujula 2006). The density of bacteria on the surface of macroalgae is greatly dependent on the type of bacteria, thallus region and season (Bengtsson and Øvreas 2010; Egan *et al*. 2013). The bacteria concentration in this study was still comparable to the natural environment where ice-ice was commonly found on seaweed. The surface of algae is covered by bacteria at a density of about 10^7 bacteria/cm^2 density (Armstrong *et al*. 2000). However, environmental conditions in the field are more complex compared to those of the laboratory. Further studies are needed to fully understand environmental factors contributing to ice-ice disease, as well as the impact on *K. alvarezii* immune response.

Bacteria can infect all parts of the thallus, but at a temperature of 28°C, the infection is more severe on the secondary and tertiary thallus. Both the secondary and tertiary thallus are classified as apical thallus segment (young region), known to have a lower amount and quality of galactant sulphate compared to those in basal region (Mendoza *et al*. 2006). Galactant sulphate is a cell wall component of seaweed and the composition in the basal region is more complex, making it less accessible compared to the apical region (Mendoza *et al*. 2006). In contrast, sugar content in seaweed segment is more concentrated on the apical compared to the basal region. This higher nutrient availability in the apical region is considered a key factor in bacteria preference for infection (Carter and Anderson 1986; Mendoza *et al*. 2006).

Morphological changes after bacteria infection can be used to evaluate the severity of ice-ice disease infection (bleaching) on *K. alvarezii* seaweed. The severity level was determined based on the disease manifestation observed on each day of experimentation (Fig. 2). A severity scale of 0 indicates thallus condition on the initial day, in which bleaching was still absent. A scale of 1 was used to describe seaweed condition on day 1, where small bleaching in the shape of the white spot was observed only in small parts. Meanwhile, a scale of 2 was used to describe the condition on day 2, in which bleaching spot has spread to several thallus. A scale of 3 was used to describe observation on day 3, in which seaweed condition demonstrated several shallow injuries on thallus. A scale of 4 was used to represent the condition of seaweed on day 4 showing prone thallus with big and deep injury. A scale of 5 was used to illustrate very severe bleaching, causing thallus to die and eventually break. In addition, stress signified by thallus changing color is the first symptom of seaweed suffering from ice-ice disease. As the culture period progressed, the tip gradually turned to white and the thallus eventually rotted (Sulu *et al.* 2003).

The mechanism of bacteria infection in seaweed is considered similar to that of higher plants. Weinberg *et al.* (2007) explained that bacteria enter plants through wounds or natural holes and then colonize intercellular spaces. When the thallus is injured, the area affected will become a location for bacteria to grow and multiply. At the right concentration, the bacteria will enter the thallus tissue, damage the cell walls and cause the death of seaweed (Azis *et al.* 2022).

Seaweed tissues infected by ice-ice disease showed cells with intact walls but less cytoplasmic content as indicated by the change in color intensity (Qu´er´e *et al.* 2015). In addition, dead epithelial cells have started to disappear or appear less dense (Garbary *et al*. 2013). In severely affected tissues, only outer epithelial cells showed deterioration.

Dead cell is characterized by intact cell wall devoid of protoplasm content (Qu'er'e *et al*. 2015). Histological results could confirm cell death, but cannot be used to determine whether the cell death is caused by necrosis or by programmed cell death (PCD). Necrosis is characterized by vacuolization, cell burst and tissue degradation, while PCD is marked by cell reduction and formation of body accumulation (Franklin *et al*. 2006; Dunn *et al*. 2012).

Seaweed tissue without ice-ice disease symptoms is indicated by clearly visible epithelial and protoplast layers, regular cell shapes, as well as very close cell spacing (IA and IIA in Fig. 3). Ward *et al*. (2021) explained that histologically, healthy seaweed tissue has complete, intact and dense cells arranged regularly in the medulla layer. Thallus tissue exposed to different temperatures but without bacteria injection showed good and complete condition.

In this study, seaweed exposed to different temperatures and *S.maltophilia* bacteria injection showed thallus tissue with ice-ice symptoms. The severity of infection was classified as medium (observed at 25°C) and high (at 28°C). Medium severity was characterized by visible but incomplete epithelial layer indicators, unclear protoplasts and varying cell shapes. Meanwhile, high severity led to the absence of epithelial and protoplast indicators, with cells appearing larger than normal. In the diseased tissue, the damage was visible, tissue structure was disrupted and cell lysis occurred (Riyaz *et al*. 2021). The severity of tissue damage in the thallus is due to environmental stressors followed by disease agents such as bacteria entering the tissue, damaging cell walls and causing necrosis (Spagnuolo and Genovese 2024).

The results showed that salinity levels of 25, 30 and 35 g/L are optimal for normal growth. In contrast, a water salinity of 15 g/L and 55 g/L resulted in mortality of *K. alvarezii* after three days of maintenance, with decreased growth (Hayashi *et al*. 2011). Both salinity and temperature are key environmental factors contributing to ice-ice disease in *K. alvarezii* (Largo *et al*. 1995b). Salinity exerts complex effects on plants while ionic regulation, nutritional interaction and physiological mechanism of salinity stress remain unclear. Tolerance to salinity is often dependent on the complexity of the anatomy and physiology of the plant. Under salt stress, plants regulate nutrient absorption, while preserving the uptake of toxic ions.

Furthermore, this study also shows that salinity has an effect on the amount of bleaching that appears on the seaweed thallus *K. alvarezii* which has been exposed to *S. maltophilia* bacteria 10^6 cfu/mL. Several previous studies have shown that decreasing salinity can trigger ice-ice disease in seaweed (Saraswati and Darmasetiyawana 2016; Mariño *et al.* 2019; Pang and Liu 2019). Currently, there is still a lack of reported research results related to the effects of salinity on the emergence of bleaching symptoms of ice-ice disease.

At salinity of 28 and 35 g/L showed a high amount of bleaching in secondary and tertiary thallus with a percentage of 70-100% and salinity of 30 g/L showed a bleaching presentation of 46%. This indicates that an early sign of ice-ice disease is the appearance of bleaching in tertiary and secondary thallus. In general, the seaweed thallus consists of the basal/holdfast and apical parts. In *K. alvarezii*, tersier and secondary thallus is part of the apical. The apical part is known to be the part of the thallus with high nutrients and protein and carbohydrate content compared to the basal part, including in seaweed (Mendoza *et al*. 2006; Sumandiarsa *et al*. 2022).

The effects of salt stress can be classified into two groups, namely short-term and long-term. Short-term effect occurs within days and causes growth reduction. Meanwhile, long-term effect occurs in weeks, causing excess salt in leaf development and reducing photosynthetic activity. Cases of ice-ice disease mainly occur when seaweed is exposed below or above optimal salinity levels, resulting in long-term effects.

In other species of seaweed such as *Gracilaria* spp., salinity plays an important role in photosynthesis, respiration and growth. Salinity levels below the tolerance level often inhibit growth, affect branching patterns and induce chemical composition change (Choi *et al*. 2006). On the other hand, higher salinity above the optimal range could alter biochemical reactions in photosystem, protein synthesis rate and product deposit. Reduction in agar concentration caused by photosynthesis inhibition, carbon fixation and the use of energy for ion exchange equilibrium in lower salinity has also been reported in seaweed (Li-hong *et al*. 2002).

Conclusion

In conclusion, *S. maltophilia* bacteria infection at 28°C resulted in higher weight reduction and a greater percentage of thallus bleaching with more severity than at 25°C. Meanwhile, bacteria infection at water salinity of 28, 30 and 35 g/L produced similar weight reduction effects. The pathogenic bacteria mostly attacked secondary and tertiary thallus. Ice-ice disease infection significantly caused bleaching when seaweed was exposed to bacteria concentration of 10^4. The disease reached the highest severity on day four after infection, regardless of the temperature and salinity levels. Infected tissue could be distinguished between medium and high severity by histological differences including the protoplasm content and cell gap distance.

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Author Contributions

All authors contributed to the study conception and design. Data collection and analysis were performed by MA. Visualization and data validation were performed by AA and SS. The first draft of the manuscript was written by MA. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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