Effects of poly(L-lactic acid) hydrolysis on attachment of barnacle cypris larvae

著者	Ishimaru Nobuyuki, Tsukegi Takayuki, Wakisaka Minato, Shirai Yoshihito, Nishida Haruo
journal or publication title	Polymer Degradation and Stability
volume	97
number	11
page range	2170-2176
year	2012-08-27
URL	http://hdl.handle.net/10228/00006749

doi: info:doi/10.1016/j.polymdegradstab.2012.08.012

Effects of Poly(L-lactic acid) Hydrolysis on Attachment of Barnacle Cypris Larvae

Nobuyuki Ishimaru[a], *Takayuki Tsukegi*[b], *Minato Wakisaka*[a], *Yoshihito Shirai*[a,b], *and Haruo Nishida**[,a,b]

[a] Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan

[b] Eco-Town Collaborative R&D Center for the Environment and Recycling, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan

Corresponding author:

Haruo Nishida

e-mail: nishida@lsse.kyutech.ac.jp

TEL +81-93-695-6233, FAX +81-93-695-6060

Abstract:

Poly(L-lactic acid) (PLLA) applied to immersed solid surfaces in seawater inhibited colonization by barnacles due to the slow-release property of lactic acid. The effect of molecular weight of PLLA on anti-macrofouling activity was confirmed for the first time, with the lowest molecular weight PLLA producing the lowest attaching ratio of cypris larvae of *Balanus amphitrite*. From the direct addition of lactic acid into a culture of cypris larvae, it was found that the anti-barnacle settlement effect was due to the action of slow-released lactic acid to cypris larvae. The anti-macrofouling function of low molecular weight PLLA was also confirmed in a natural sea environment.

Keywords:

poly(L-lactic acid) / lactic acid / slow-release / anti-macrofouling / barnacle / cypris larvae

1. Introduction

Marine biological fouling, usually termed marine biofouling, is the undesirable accumulation of microorganisms, plants, and animals on immersed surfaces. Such accumulations can have dramatic economic and environmental costs, through increased fuel consumption to shipping, frequent dry-docking and painting for hull maintenance, the hindering of seaweed farming, etc. [1]. In preventing marine biofouling, effective tributyltin (TBT) self-polishing copolymer paints have been the most successful [2]. Following the banning of TBT as an antifoulant, several organic booster biocides have been used in conjunction with copper compounds in antifouling paints as alternative treatments [3,4].

Recently, environmentally friendly alternative methods of antifouling have been actively researched. These include treatments using natural products [5], fouling release coatings [6], antifouling topographies [7,8], and electrical antifouling systems [9].

To prevent the development of marine biofouling, especially macrofouling, by the undesirable accumulation of organisms such as barnacles, mussels, algae, etc., many kinds of systems for slow-releasing antifoulants have been developed [2,10]. These slow-release systems have been designed using surface-fragmenting/self-polishing matrices, in which the matrix polymers are hydrolyzed at ester groups of main and side chains. This allows antifoulants such as an agricultural herbicide: Diuron, cuprous oxide, and an antibacterial molecule: chlorhexidine, to be gradually released during immersion time in seawater. Recently, some biodegradable polymers [11] such as poly(3-hydroxyalkanoate)s [12], poly(ε-caprolactone) copolymers [13], poly(ester-anhydride) [14], poly(lactic acid) [15], graft copolymers containing poly(lactic acid) side chains [16], and poly(ε-caprolactone-co-lactide) [17] have been applied as environmentally benign surface-fragmenting/self-polishing matrices to controllably release the antifoulants. Langlois *et al*. indicated that the rate of biocide release was controlled by the amount of oligo(D,L-lactic acid) units in the graft copolymers [16]. On the other hand, Faÿ *et al*. reported that the lower the molecular weight of poly(ε-caprolactone-

co-lactide), the faster the release of biocide from antifouling paints, resulting in the decrease of the antifouling activity [17]. However, there has been no discussion about the direct contribution of the biodegradable polymers to the overall antifouling effect.

Barnacles and mussels are typical macrofouling organisms found in marine environments and, because their settlement involves the attachment of larvae on immersed solid surfaces, research has focused on processes necessary to their settlement, which are secretion [18-20], network formation [21,22], and cross-linking reactions of cement proteins [23]. Cypris larvae are the final larval stage of barnacles and are highly specialized to their role of locating and attaching to suitable surfaces for adult growth. The antennules of cypris larvae are their primary sensory tool. Each antennule is divided into four segments, and the third segment bears the antennular attachment disc, which measures approximately 25-30 μm (long axis) by approximately 15 μm (width) [8] and secretes cement proteins from the unicellular antennule glands [24].

In the case of a 20 kDa barnacle cement protein as one of the key materials for settlement, it was reported that its functions of self-assembly and network formation for the cementation were triggered by a salt concentration higher than that of seawater (0.55 M) and induced at pH 8-10 [22]. The cement protein was characterized by an abundance of cysteine (Cys) residues and charged amino acids. The Cys residues formed intermolecular disulphide-bonds to maintain the topology of the charged amino acids on the molecular surface, and the charged amino acids interacted with a variety of surface metals on substrata [20].

In this paper, the ability of biodegradable polyester poly(L-lactic acid) (PLLA) [25] to function as an anti-barnacle molecule was investigated. Of particular, interest was PLLA's slow-release of lactic acid [26], which, due to its acidity (p*K*a =3.86) in a similar way to acetic acid [27], may inhibit the network formation [21,22] /cross-linking reactions [20] of cement proteins at the surface layers of PLLA moldings. Effects of the molecular weight of PLLA were also examined.

2. Materials and methods

2.1. Materials

PLLA (TERRAMAC TE-2000C, M_n=3.06×10^4, M_w=6.71×10^4, *L*-unit 98.5%, T_m=175 °C) and linear low-density polyethylene (LLDPE, NOVATEC UF840, MFR = 1.5 g·10 min^{-1}, T_m 125 °C) pellets were purchased from UNITIKA Ltd. and Japan Polyethylene Corporation, respectively. Standard D/L-lactic acid (50/50 wt/wt, 90% aqueous solution) and L-lactic acid (90% aqueous solution) were purchased from Wako Pure Chemical Industries, Ltd. and Musashino Chemical Laboratory, Ltd., respectively.

Fresh seawater was drawn at the Sea of Hibiki in Japan and filtrated through a membrane filter (Millipore Corporation, Millex-HP, polyethersulfone type, pore size 0.45 μm). Artificial seawater was prepared by dissolving a Marine Art SF-1 (Tomita Pharmaceutical Co., Ltd., NaCl 22.1 g/L, $MgCl_2·6H_2O$ 9.9 g/L, $CaCl_2·2H_2O$ 1.5 g/L, Na_2SO_4 3.9 g/L, KCl 0.61 g/L, $NaHCO_3$ 0.19 g/L, KBr 96 mg/L, $Na_2B_4O_7·10H_2O$ 78 mg/L, $SrCl_2$ 13 mg/L, NaF 3 mg/L, LiCl 3 mg/L, KI 81 μg/L, $MnCl_2·4H_2O$ 0.6 μg/L, $CoCl_2·6H_2O$ 2 μg/L, $AlCl_3·6H_2O$ 8 μg/L, $FeCl_3·6H_2O$ 5 μg/L, $Na_2WO_4·2H_2O$ 2 μg/L, $(NH_4)_6Mo_7O_{24}·4H_2O$ 18 μg/L) in distilled water.

2.2. Cypris larvae

In this investigation, the cypris larvae of *Balanus amphitrite* were used to estimate the anti-macrofouling function of PLLA. The reproduction of *B. amphitrite* is confined to the summer in natural environments. Therefore, to obtain and use the cypris larvae of *B. amphitrite* throughout the year, adult barnacles were collected from the Ariake Sea in Japan and fed in the laboratory with *Artemia salina* nauplii, every 24 h, in a filtered seawater at 25 °C according to the method of Kitamura [28]. The barnacles were exposed to the sunlight for 6 h per day and the seawater was changed to fresh twice a week. The barnacles began to

release nauplii larvae after 1 day of rearing. The nauplii of *B. amphitrite* were reared with *Chaetoceros gracilis* feed in filtrated fresh seawater at 25 °C [29]. Forty to 50% of nauplii reached the cypris stage within 6 days. Cypris larvae were separated from nauplii larvae using a Pasteur pipette with the aid of an optical microscope [30].

Cypris larvae were kept in filtrated fresh seawater, which was oxygenated to saturation by aeration through bubbling with oxygen in the dark at 5 °C in a refrigerator for 3 days before being used for the adhesion test on PLLA sheet samples. While being maintained at 5 °C, the seawater was replaced with fresh seawater everyday [31].

2.3. Preparation of PLLA oligomers by hydrolysis

The hydrolysis of PLLA pellets under high-pressure steam was achieved using an autoclave (Tomy autoclave model SS-325, unobstructed capacity 55 L) at 120 °C (0.202 MPa) for 120, 180, and 240 min (Table 1). The internal temperature of the autoclave was thermostated to within ± 0.5 °C. After autoclaving, the PLLA samples were dried in vacuo at room temperature for 1 night and stored in a refrigerator at 4 °C until used in the sheet preparation.

[Table 1]

2.4. Preparation of sheet samples from PLLA and LLDPE

PLLA and LLDPE pellets in Table 1 were dried at 40 ºC for 2 h at ca. 0.1 kPa in a vacuum oven just before melt-processing. Transparent sheet samples (thickness 400 ± 30 μm) of PLLA and LLDPE were prepared by compression molding of the pellets between metal plates having mirror finished surface at 155-170 °C with a heat-pressing machine IMC-180C (Imoto Machinery Co., Ltd. (Kyoto, Japan)) at prescribed conditions listed in Table 1. Number and weight-average molecular weight values of the sheet samples were measured with a size-

exclusion chromatograph (SEC) as listed in Table 1. No significant difference in the values was detected between the pellets and the subsequently prepared sheet samples.

Test samples were prepared by cutting out squares (50×50 mm^2) from the sheet samples for the lactic acid elusion and cypris larvae adhesion tests.

2.5. Lactic acid elusion tests

Elution tests of lactic acid from the pellet samples (10 g) were carried out in flasks containing artificial seawater and distilled water (200 mL). The flasks were stirred at 100 rpm on a shaker in an oven thermostated at 25 °C. Changes in pH value of the aqueous media were monitored with a pH meter (HORIBA model F-21). The test media of 4 mL volume were sampled after prescribed periods and filtered with a membrane filter (Millipore Corporation, Millex-HP, polyethersulfone type, pore size 0.45 μm) for the elution volume analysis of lactic acid.

The elution volume of lactic acid into the medium from the sample was measured with a SHIMAZU high pressure liquid chromatograph LC-10AT system equipped with a conductivity detector CDD-6A and a Phenomenex Rezex ROA-Organic Acid H+ column at 40 ºC in a 0.0025 mM H_2SO_4 eluent (0.5 $mL \cdot min^{-1}$). The filtered solution sample (20 μL) was injected. A calibration curve for lactic acid was prepared by using the standard D/L-lactic acid in a concentration range of 0.01-100 mg/L.

The elution test was repeated 3 times with average and standard deviation values were calculated.

2.6. Cypris larvae attachment tests

Attachment tests of cypris larvae of *B. amphitrite* were carried out on (50×50 mm^2) samples of the PLLA sheet in a polystyrene Petri dish (80 mm in diameter) at 25 °C. A LLDPE sheet (50×50 mm^2) was used as a reference. The Petri dish was covered by a nylon net

(120×120 mm^2, mesh size 100 μm). The PLLA sheet sample was put on the nylon net, after which fresh seawater (20 g) was added. Cypris larvae did not attach to the nylon net, because the net's micro-texture structure prevents such attachment occurring [32].

Ten cypris larvae, produced after the newly released nauplii had passed through six ecdyses, were kept at 5 °C for 3 days and then used for each attachment test. The cypris larvae were added into the polystyrene Petri dish, and their attachment behavior was observed under an optical microscope. Attachment and survival ratios of the cypris larvae along with changes in pH value of the medium were monitored for 300 min. After the test, sample sheets were taken out from the Petri dish and the sheet surfaces were flushed for 60 s with a wash bottle. Any remaining cypris larvae were counted under an optical microscope. The test was repeated 30 times, with extreme values (top 5% and bottom 5%) eliminated before calculating average and standard deviation values. The tests on LLDPE and PLLA-31 sheets were continued for 48 h to ensure the settlement of cypris larvae.

2.7. Statistical analysis

The F-test and t-test between two samples assuming unequal variances were performed using a Microsoft Excel program. Two tailed t-statistics and p-values were calculated to estimate the null hypothesis of equal means. The attachment behaviors of cypris larvae were analyzed by fitting with the generalized linear models (GLM). The GLM fitting was performed in a program **R**, choosing the variance: Poisson and link function: log.

2.8. Preparation of tube samples

Tube samples from LLDPE/PLLA-7 (80/20 wt/wt) blend and LLDPE were prepared by an extrusion molding method at Nishi Nippon Electric Wire and Cable Co., Ltd. Each tube sample had internal diameter: 32 mm, thickness: 0.8 mm, and length: 500 mm.

2.9. Immersion test in a natural sea environment

Prepared tube samples: LLDPE/PLLA-7 (80/20 wt/wt) blend and LLDPE were fixed on surfaces of fiber-reinforced plastic (FRP) poles for the seaweed farming. The poles were immersed in the Ariake Sea in Japan for 173 days during the seaweed farming season (September 9, 2010-March 1, 2011).

2.10. Characterization

Molecular weights of PLLA samples were measured on a TOSOH HLC-8120 SEC system with refractive index (RI) and ultraviolet (UV, $\lambda = 254$ nm) detectors under the following conditions: TSKgel Super HM-H linear column (linearity range, $M_{n,PSt} = 5.89 \times 10^2$ - 2.00×10^6; molecular weight exclusion limit, $M_{n,PSt} = 4 \times 10^8$), $CHCl_3$ (HPLC grade) eluent at a flow rate of 0.6 mL·min^{-1}, and column temperature of 40ºC. Calibration curves for SEC analysis were obtained using polystyrene standards with low polydispersity values ($M_{n,PSt}$ = 5.89×10^2, 7.70×10^2, 2.43×10^3, 3.68×10^3, 1.32×10^4, 1.87×10^4, 2.93×10^4, 4.40×10^4, 1.14×10^5, 2.12×10^5, 3.82×10^5, 5.61×10^5, 2.00×10^6, Aldrich). The sample (15 mg) was dissolved in chloroform (3 mL) and the solution filtered through a membrane filter with a 0.45 μm pore size. The SEC traces were evaluated by a universal calibration method (UCM) using the published Mark–Houwink–Sakurada constants for PLLA and polystyrene at 40 ºC as follows [33]:

PLLA : $[\eta] = (2.068\times10^{-4})M^{0.734}$

Polystyrene : $[\eta] = (2.072\times10^{-4})M^{0.655}$

Based on UCM, the linearity range and molecular weight exclusion limit are $M_{n,UCM}$ = 4.41×10^2 - 1.03×10^6 and 1.6×10^8, respectively. In the following sections, $M_{n,UCM}$ and $M_{w,UCM}$ are expressed simply by M_n and M_w.

3. Results and discussion

3.1. Elution of lactic acid from PLLA samples

It is well known that the hydrolysis behavior of PLLA proceeds in distilled water [34]; in acidic [35], basic [36], and phosphate-buffered solutions [37]; by enzyme [38]; in steam [39,40]; and *in vivo* [41]. PLLA releases L-lactic acid (LA) during hydrolysis according to the autocatalytic degradation kinetics in water [42] and steam [40]. On the other hand, in a stronger acidic solution than lactic acid, or in a basic solution including an excess amount of basic compounds, PLLA hydrolysis proceeds according to the non-autocatalytic random degradation kinetics [43]. Langlois *et al*. reported the hydrolysis behavior of graft copolymers containing oligo(D,L-lactic acid) side chains in artificial seawater for releasing Cu_2O [16], there is, however, no report about hydrolysis behavior of PLLA homopolymers in seawater for releasing the L-lactic acid only.

In Figure 1, pH changes of distilled water and the artificial seawater during the adding of LA in a dropwise fashion are illustrated. In distilled water, the pH value rapidly decreased to 3.9 after the addition of 50 mg/L of LA. Contrastively, in artificial seawater, due to its buffering action the pH value steadily decreased with the addition of LA, reaching 6.3 at 100 mg/L of LA from the initial value of 8.3. Further addition of LA into the seawater caused a shift to a more acidic region of around 4.0 at 1000 mg/L of LA.

[Figure 1]

To confirm the effects of molecular weight of PLLA on the LA elution rate, the elution test was carried out in artificial seawater (200 mL) using PLLA samples (10 g) listed in Table 1. Changes in pH values and eluted amounts of LA are shown in Figure 2. Obviously, the lower the molecular weight of PLLA, the greater the decrease in the pH value and the

smoother the increase in LA elution. In 2b, rapid increases in LA elution of PLLA-1, 3, 7 at the beginning are due to the elution of accumulated LA in sheets during the steam hydrolysis.

[Figure 2]

3.2. Attachment behavior of cypris larvae on PLLA sheets

Adhesion tests of cypris larvae of *B. amphitrite* were carried out on PLLA sheet samples (50×50 mm^2). The exploration, attachment, and settlement behavior of the cypris larvae has been investigated by Maruzzo, Lagersson, and Høeg in detail [24,44]. The exploratory phase starts from the moment that the larva begins to employ its antennules for walking on a substratum. During this exploratory phase, the larva searches for a suitable area on the substratum surface to cement itself irreversibly. The cypris larva stage is a non-feeding stage specifically adapted for selecting the proper substrate for settlement and adult survival [45,46].

In Figure 3(a), attachment ratios of cypris larvae on the PLLA and LLDPE sheets are plotted. Here, the attachment by only one antennule ("close search" phase, Figure S1 in Supplementary data) was mainly observed under an optical microscope and defined as phase I in this study. The attachment of cypris larvae at phase I increased with incubation time. The highest attachment ratio (around 50 % after 6 h) was observed on the LLDPE sheet as a reference. The attachment ratio showed a tendency to decrease with the decrease in the molecular weight of PLLA, and no attachment was found on the PLLA-1 sheet having the lowest molecular weight. These results clearly indicate that a decrease in molecular weight, i.e., an increase in the elution rate of LA, directly depresses the attachment ratio of cypris larvae at phase I.

The cypris larvae in this phase (phase I in Figure 4) were washed away in running water. In Figure 4, attachment and settlement ratios of cypris larvae on the LLDPE and PLLA-31

sheets are plotted. In this stage (defined as phase II in this study), the cypris fixed with both antennules ("inspection" phase) along with the following settlement and metamorphosis of attached larvae were observed (Figure S1 in Supplementary data). These cypris larvae in phase II were never washed away even in running water. Although the relationship between phase I and II is not yet fully understood, relative positional relations exist between the phases.

In order to analyze the attachment behavior of cypris larvae at phase I, the generalized linear models (GLM) were employed to obtain a statistical fit. The GLM fitting was performed in the program **R**, choosing the variance: Poisson and link function: log. Results obtained, which were shown in Supplementary data as Figure S2, S3, S4 and S5, supported the above discussion that the decrease in molecular weight directly depresses the adhesion rate of cypris larvae. The F-test and t-test as statistical analyses were also performed. Obtained results in Table S1 in the Supplementary data supported the results of GLM fitting.

In Figure 3(b), relationships between the adhesion rate and log(t) are plotted. This plot is referred to as an Elovich equation plot, which is a well known empirical equation widely used to describe the adsorption/desorption behavior of gases onto solid surfaces [47], of pollutants onto bone char from aqueous solutions [48], and of heavy metal ions such as Cr(VI) and Cu(II) by chitin, chitosan, and *Rhizopus arrhizus* [49]. Relatively linear relationships were observed in Figure 3(b), indicating that the attachment process of cypris larvae at phase I closely follows the empirical relationship for the adsorption/desorption behaviors of various materials on solid surfaces. This result suggests that the efficacy of the eluted LA results mainly from its effect on the attachment process occurring at the attachment disc surface in each antennule of cypris larvae [45].

[Figure 3]

[Figure 4]

3.3. Additional effects of lactic acid on cypris larvae

As shown in Figure 3, the eluted LA influenced the attachment ratio of cypris larvae. If the attachment and following settlement are achieved successfully, the surviving cypris larvae can turn into adult barnacles by metamorphosis as confirmed in Figure 4. Figures 5 and 6 depict the adhesion and survival rates, respectively, of cypris larvae in the presence of various concentrations of LA directly added in fresh seawater. The attachment test was carried out in a polystyrene Petri dish without any sample sheet or nylon net, thus, the cypris larvae could adhere to the inner surface of the Petri dish.

As shown in Figure 5, the attachment ratio of cypris larvae obviously decreased with increase in the concentration of LA and the changes in attachment ratio were significant (p<0.05, see Table S2 in Supplementary data). Even in the dilute solutions of 10 and 100 mg/L of LA, the attachment ratio of cypris larvae was suppressed. On the other hand, when considering the standard deviation ranges, no statistically significant difference (p>0.05) was found in the survival ratio of cypris larvae in the LA concentration range of 0-100 mg/L (Figure 5 and Table S3 in Supplementary data). With further increases in LA concentration, the survival ratio of cypris larvae suddenly decreased with decrease in the pH value as shown in Figure 1.

Apparent LC50 and EC50 values, which were calculated from the results in Figure 6 and 5 by the probit analysis using a computer program, were 218 ppm (95% confidence limit: 162-292 ppm) and 18 ppm (95% confidence limit: 7-36 ppm), respectively. The eluted LA gradually changed into a salt form in the basic seawater environment. Thus, the obtained LC50 and EC50 values may be tentative values whose effect is confined to a narrow layer on the PLLA surface. The LD50 value of LA has been reported as being 3730 mg/kg [rat] and 4875 mg/kg [mouse] for acute toxicity by oral intake (WHO Food Additives Series No. 5). These results suggest that the anti-attachment effect of eluted LA on the cypris larvae is

dependent on a specific property of the sheet surface, and is not dependent on its general toxicity to the life of cypris larvae in the LA concentration range of 0-100 mg/L.

[Figure 5]

[Figure 6]

3.4. Settlement behavior of barnacles in a sea environment

To confirm the anti-barnacle attachment and following settlement function of PLLA, LLDPE/PLLA-7 (80/20 wt/wt) blend and LLDPE tube samples were immersed in the Ariake Sea for 173 days in 2010-2011, where PLLA-7 was selected due to its good processability for melt-blending with LLDPE used as a supporting matrix resin. Some data relating to the seawater characteristics for the immersion test such as temperature, pH, salt concentration and amount of plankton are provided as Figure S5 and S6 in Supplementary data. After the immersion in the sea, colonies of barnacles, mussels, etc. settled on the LLDPE tube surface (surface coverage ≈100 area-%). On the other hand, the settlement of organisms was scarcely observed on the LLDPE/PLLA-7 blend tube surface except for a few numbers of isolated barnacles (surface coverage ≈5.7 area-%) (Figure 7). This anti-macrofouling behavior of the LLDPE/PLLA-7 blend must be attributable to the anti-barnacle attachment property of the PLLA-7 ingredient. The immersion test in a natural sea environment is continuing to clarify the anti-macrofouling function of PLLA oligomers. The detailed results will be published elsewhere in the near future.

[Figure 7]

4. Conclusions

To examine the anti-barnacle attachment function of PLLA, the lactic acid-releasing property and the attachment/survival ratios of cypris larvae of *B. amphitrite* were investigated by using PLLA sheet samples having different molecular weights. Despite the buffering effect of seawater, the lactic acid release rate from the samples was inversely proportional to the molecular weight of PLLA. The anti-barnacle attachment property of PLLA was clearly observed as a decrease in the attachment ratio of cypris larvae with increase in the lactic acid release rate. The direct addition of lactic acid into the culture medium also showed a similar suppressing effect on cypris larvae attachment. It was suggested that the anti-barnacle attachment effect of PLLA to the cypris larvae was dependent on a specific property of slow-released lactic acid from the sheet surfaces, rather than the direct and general effect of toxicity to the cypris larvae. The anti-macrofouling function of low molecular weight PLLA was also confirmed in a natural sea environment.

Acknowledgments

The authors gratefully acknowledge the financial support of a Grant-in-Aid for Scientific Research (No.23241030) from the Ministry of Education, Culture, Science, Sports, and Technology of Japan. The authors would like to thank the Nishi Nippon Electric Wire and Cable Co., Ltd. for the preparation of tube samples.

References

[1] Schultz MP, Bendick JA, Holm ER, Hertel WM. Economic impact of biofouling on a naval surface ship. Biofouling 2011;27:87-98.

[2] Cao S, Wang JD, Chen HS, Chen DR. Progress of marine biofouling and antifouling technologies. Chinese Sci Bull 2011;56:598–612.

[3] Thomas KV, Brooks S. The environmental fate and effects of antifouling paint biocides. Biofouling 2010;26:73-88.

[4] Voulvoulis N. Antifouling Paint Booster Biocides: Occurrence and Partitioning in Water and Sediments. Hdb Env Chem 2006;5 (Part O):155–170.

[5] Qian P-Y, Xu Y, Fusetani N. Natural products as antifouling compounds: recent progress and future perspectives. Biofouling 2010;26:223-234.

[6] Genzer J, Efimenko K. Recent developments in superhydrophobic surfaces and their relevance to marine fouling: a review. Biofouling 2006;22:339-360.

[7] Scardino AJ, de Nys R. Mini-review: Biomimetic models and bioinspired surfaces for fouling control. Biofouling 2011;27:73-86.

[8] Schumacher JF, Aldred N, Callow ME, Finlay JA, Callow JA, Clare AS, Brennan AB. Species-specific engineered antifouling topographies: correlations between the settlement of algal zoospores and barnacle cyprids. Biofouling 2007;23:307-317.

[9] Perez-Roa RE, Anderson MA, Rittschof D, Orihuela B, Wendt D, Kowalke GL, Noguera DR. Inhibition of barnacle (*Amphibalanus amphitrite*) cyprid settlement by means of localized, pulsed electric fields. Biofouling 2008;24:177–184.

[10] Kuo PL, Chuang TF, Wang HL. Surface-Fragmenting, Self- Polishing, Tin-Free Antifouling Coatings. J Coating Technol 1999;71:77-83.

[11] Tokiwa Y, Calabia BP. Biodegradability and Biodegradation of Polyesters. J Polym Environ 2007;15:259-267.

[12] Hany R, Bohlen C, Geiger T, Schmid M, Zinn M. Toward Non-Toxic Antifouling: Synthesis of Hydroxy-, Cinnamic Acid-, Sulfate-, and Zosteric Acid-Labeled Poly[3-hydroxyalkanoates]. Biomacromolecules 2004;5:1452-1456.

[13] Faÿ F, Linossier I, Langlois V, Renard E, Vallée-Réhel K. Degradation and Controlled Release Behavior of ε-Caprolactone Copolymers in Biodegradable Antifouling Coatings. Biomacromolecules 2006;7:851-857.

[14] Faÿ F, Linossier I, Langlois V, Vallée-Réhel K. Biodegradable Poly(ester-anhydride) for New Antifouling Coating. Biomacromolecules 2007;8:1751-1758.

[15] Faÿ F, Linossier I, Legendre G, Vallée-Réhel K. Micro-Encapsulation and Antifouling Coatings: Development of Poly(lactic acid) Microspheres Containing Bioactive Molecules. Macromol Symp 2008;272:45–51.

[16] Langlois V, Vallee-Rehel K, Peron JJ, le Borgne A, Walls M, Guerin P. Synthesis and hydrolytic degradation of graft copolymers containing poly(lactic acid) side chains: in vitro release studies of bioactive molecules. Polym Degrad Stab 2002;76:411–417.

[17] Faÿ F, Renard E, Langlois V, Linossier I, Vallée-Rehel K. Development of poly(ε-caprolactone-co-L-lactide) and poly(ε-caprolactone-co-δ-valerolactone) as new degradable binder used for antifouling paint. Eur Polym J 2007;43:800–4813.

[18] Waite JH. Evidence for a Repeating 3,4-Dihydroxyphenylalanine- and Hydroxyproline-containing Decapeptide in the Adhesive Protein of the Mussel, *Mytilus edulis* L. J Biol Chem 1983;258:2911-2915.

[19] Kamino K, Inoue K, Maruyama T, Takamatsu N, Harayama S, Shizuri Y. Barnacle Cement Proteins: Importance of Disulfide Bonds in Their Insolubility. J Biol Chem 2000;275:27360–27365.

[20] Kamino K. Novel barnacle underwater adhesive protein is a charged amino acid-rich protein constituted by a Cys-rich repetitive sequence. Biochem J 2001;356:503-507.

[21] Kamino K. In: Smith AM, Callow JA, editors. Biological Adhesives, Chapter 8: Barnacle Underwater Attachment. Berlin, Heidelberg: Springer-Verlag, 2006. pp. 145-166.

[22] Nakano M, Shen JR, Kamino K. Self-Assembling Peptide Inspired by a Barnacle Underwater Adhesive Protein. Biomacromolecules 2007;8:1830-1835.

[23] Yu M, Hwang J, Deming TJ. Role of L-3,4-Dihydroxyphenylalanine in Mussel Adhesive Proteins. J Am Chem Soc 1999;121:5825-5826.

[24] Maruzzo D, Conlan S, Aldred N, Clare AS, Høeg JT. Video observation of surface exploration in cyprids of Balanus amphitrite: the movements of antennular sensory setae. Biofouling 2011;27:225-239.

[25] Tokiwa Y, Calabia BP. Biodegradability and biodegradation of poly(lactide). Appl Microbiol Biotechnol 2006;72:244–251.

[26] Makino K, Arakawa M, Kondo T. Preparation and in Vitro Degradation Properties of Polylactide Microcapsules. Chem Pharm Bull 1985;33:1195-1201.

[27] Piola RF, Dunmore RA, Forrest BM. Assessing the efficacy of spray-delivered 'eco-friendly' chemicals for the control and eradication of marine fouling pests. Biofouling 2010;26:187-203.

[28] Kitamura H. Method of larval culture and water-flow assay for larval settlement of the barnacle, *Balanus amphitrite*. Sessile Organisms 1999;15:15-21.

[29] Yoshimura E, Nogata Y, Sakaguchi I. Simple methods for mass culture of barnacle larvae. Sessile Organisms 2006;23:39-42.

[30] Kado R. Method for observing the metamorphosis of cypris larvae of barnacles using an inverted microscope. Sessile Organisms 1999;15:23-25.

[31] Tsukamoto H. Growth of Barnacle, *Balanus amphitrite*, in Laboratory. Marine Fouling 1990;8:47-50.

[32] Andersson M, Berntsson KM, Jonsson PR, Gatenholm P. Microtextured surfaces: Towards macrofouling resistant coatings. Biofouling 1999;14:167-178.

[33] Yasuda N, Wang Y, Tsukegi T, Shirai Y, Nishida H. Quantitative Evaluation of Photodegradation and Racemization of Poly(L-lactic acid) under UV-C Irradiation. Polym Degrad Stab 2010;95:1238–1243.

[34] Hyon, SH. Surface Morphology for Poly-L-lactide Fibers Subjected to Hydrolysis. Sen'i Gakkaishi 1998;54:527-531.

[35] Tsuji H, Nakahara K. Poly(L-lactide). IX. Hydrolysis in Acid Media. J Appl Polym Sci 2002;86:186–194.

[36] Tsuji H, Ogiwara M, Saha SK, Sakaki T. Enzymatic, Alkaline, and Autocatalytic Degradation of Poly(L-lactic acid): Effects of Biaxial Orientation. Biomacromolecules 2006;7:380-387.

[37] Tsuji H, Ikada Y. Properties and morphology of poly(L-lactide) 4. Effects of structural parameters on long-term hydrolysis of poly(L-lactide) in phosphate-buffered solution. Polym Degrad Stab 2000;67:179-189.

[38] Tsuji H, Miyauchi S. Enzymatic Hydrolysis of Poly(lactide)s: Effects of Molecular Weight, L-Lactide Content, and Enantiomeric and Diastereoisomeric Polymer Blending. Biomacromolecules 2001;2:597-604.

[39] Nishida H, Yamashita M, Nagashima M, Hattori N, Endo T, Tokiwa Y. Theoretical Prediction of Molecular Weight on Auto-catalytic Random Hydrolysis of Aliphatic Polyesters. Macromolecules 2000;33:6595-6601.

[40] Mohd-Adnan AF, Nishida H, Shirai Y. Evaluation of Kinetics Parameters for Poly(L-lactic acid) Hydrolysis under High-Pressure Steam. Polym Degrad Stab 2008;93:1053-1058.

[41] Gilbert RD, Stannett V, Pitt CG, Schindler A. In: Grassie N, editor. Developments in polymer degradation -4, The design of biodegradable polymers: Two approaches. London: Applied Science Publishers, 1982. pp. 259-293.

[42] Pitt CG, Gu ZW. Modification of the rates of chain cleavage of poly(e-caprolactone) and related polyesters in the solid state. J Controlled Release 1987;4:283-292.

[43] Yoon JS, Chin IJ, Kim MN, Kim C. Degradation of Microbial Polyesters: A Theoretical Prediction of Molecular Weight and Polydispersity. Macromolecules 1996;29:3303-3307.

[44] Lagersson NC, Høeg JT. Settlement behavior and antennulary biomechanics in cypris larvae of *Balanus amphitrite* (Crustacea: Thecostraca: Cirripedia). Mar Biol 2002;141:513–526.

[45] Aldred N, Clare AS. The adhesive strategies of cyprids and development of barnacle-resistant marine coatings. Biofouling 2008;24:351–363

[46] Johnson WS, Allen DM. In: Zooplankton of the Atlantic and Gulf Coasts: A Guide to Their Identification and Ecology, Cirripedes: Barnacle Larvae. Baltimore: The Johns Hopkins University Press, 2005. pp. 124-125.

[47] Rudzinski W, Panczyk T. Kinetics of Isothermal Adsorption on Energetically Heterogeneous Solid Surfaces: A New Theoretical Description Based on the Statistical Rate Theory of Interfacial Transport. J Phys Chem B 2000;104:9149-9162.

[48] Cheung CW, Porter JF, McKay G. Sorption kinetic analysis for the removal of cadmium ions from effluents using bone char. Wat Res 2001;35:605-612.

[49] Sağ Y, Aktay Y. Kinetic studies on sorption of Cr(VI) and Cu(II) ions by chitin, chitosan and *Rhizopus arrhizus*. Biochem Eng J 2002;12:143–153.

Figure captions

Figure 1. Changes in pH values of artificial seawater and distilled water with the addition of lactic acid.

Figure 2. Changes in (a) pH values and (b) amounts of lactic acid (LA) eluted from PLLA samples in artificial seawater.

Figure 3. (a) Attachment ratios of cypris larvae for 300 min and (b) their Elovich plots on PLLA and LLDPE sheet samples.

Figure 4. Attachment ratios of cypris larvae for 48 h on (a) LLDPE and (b) PLLA-31 sheet samples. Phase1: Cypris larvae are fixed with one antennule and washed away by flushing. Phase II: Cypris larvae are fixed with two antennules or metamorphosed, and remained even after flushing.

Figure 5. Attachment ratios of cypris larvae in the presence of lactic acid in fresh seawater.

Figure 6. Survival ratios of cypris larvae in the presence of lactic acid in fresh seawater.

Figure 7. Immersion tests in a natural sea environment of LLDPE/PLLA-7 (80/20 wt/wt) blend and LLDPE tube samples for 173 days.

Table

Table 1. Steam hydrolysis and sheet preparation conditions of PLLA samples.

Sample	Steam hydrolysis[a]	Compression molding[b]		M_n[c]	M_w[c]
	time (min)	temp. (°C)	press. (MPa)		
PLLA-31[d]	-	160	12.4	30 600	67 100
PLLA-7	120	155	6.2	7 200	13 100
PLLA-3	180	155	6.2	2 700	9 600
PLLA-1	240	155	6.2	1 100	3 700
LLDPE	-	170	7.2	59 500	228 000

[a] at 120 °C, [b] Melting (2− 5 min) + pressing (4− 5 min) + cooling 5 min), [c] Universal calibration method, [d] Original PLLA.

Figure 1

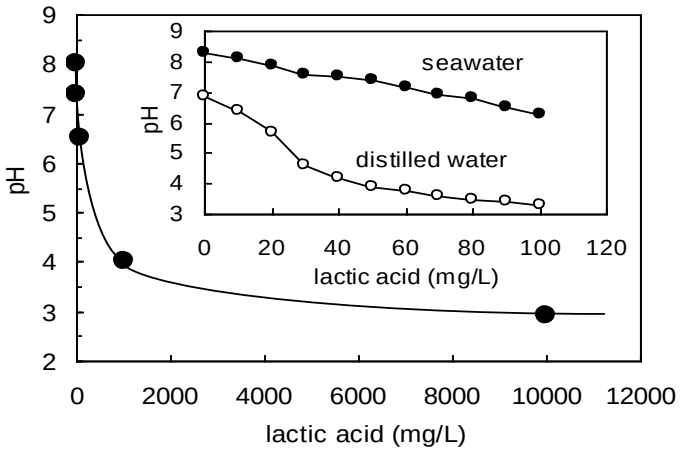

pH
lactic acid (mg/L)
seawater
distilled water
pH
lactic acid (mg/L)

Figure 2

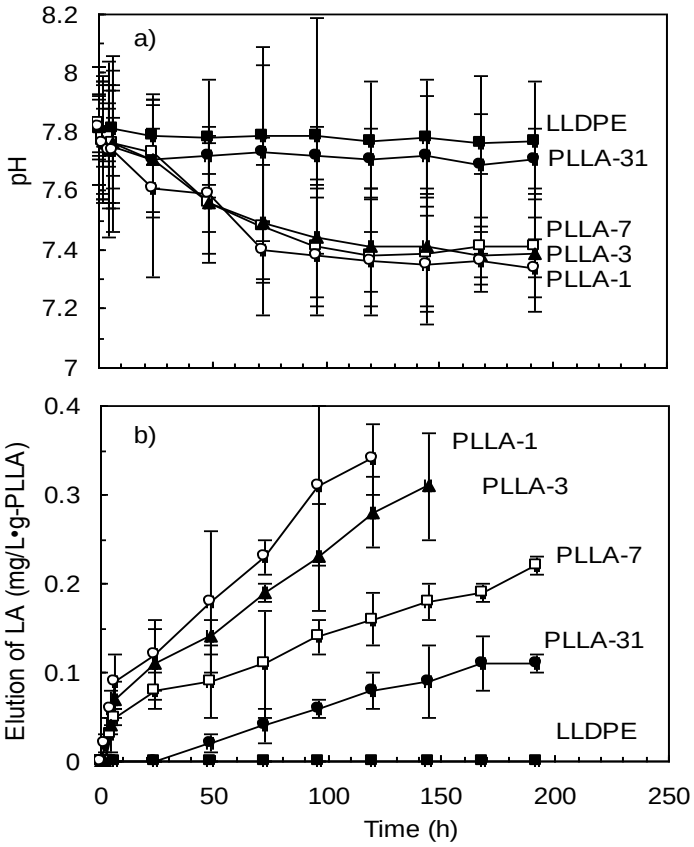

a)
pH
8.2
8
7.8
7.6
7.4
7.2
7
LLDPE
PLLA-31
PLLA-7
PLLA-3
PLLA-1
b)
Elution of LA (mg/L•g-PLLA)
0.4
0.3
0.2
0.1
0
PLLA-1
PLLA-3
PLLA-7
PLLA-31
LLDPE
0
50
100
150
200
250
Time (h)

Figure 3

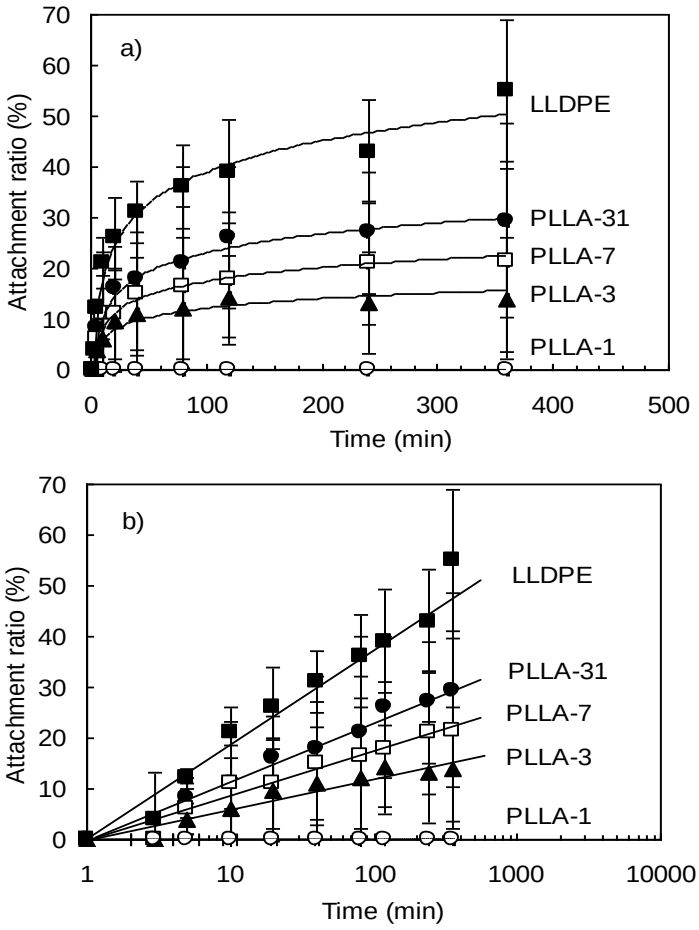

a)
Attachment ratio (%)
Time (min)
LLDPE
PLLA-31
PLLA-7
PLLA-3
PLLA-1
b)
Attachment ratio (%)
Time (min)
LLDPE
PLLA-31
PLLA-7
PLLA-3
PLLA-1

Figure 4

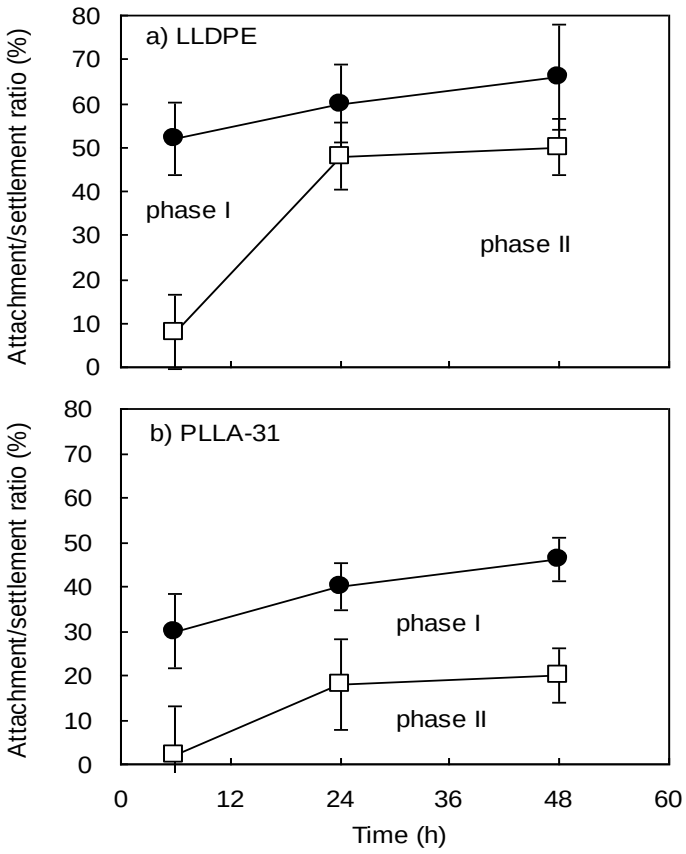

a) LLDPE
b) PLLA-31
Attachment/settlement ratio (%)
Attachment/settlement ratio (%)
phase I
phase II
phase I
phase II
0
10
20
30
40
50
60
70
80
0
12
24
36
48
60
Time (h)

Figure 5

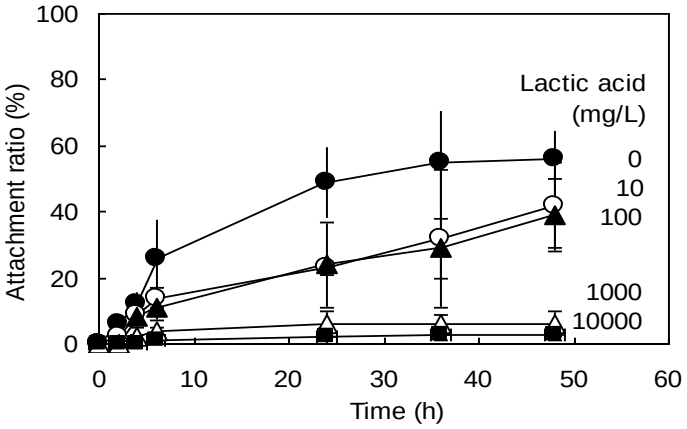

Attachment ratio (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (h)
Lactic acid
(mg/L)
0
10
100
1000
10000

Figure 6

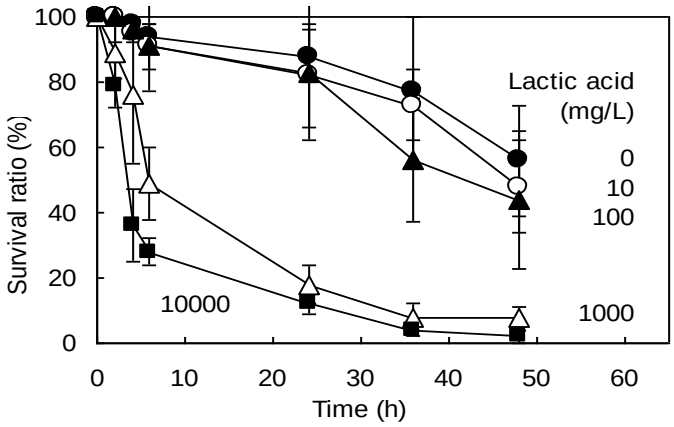
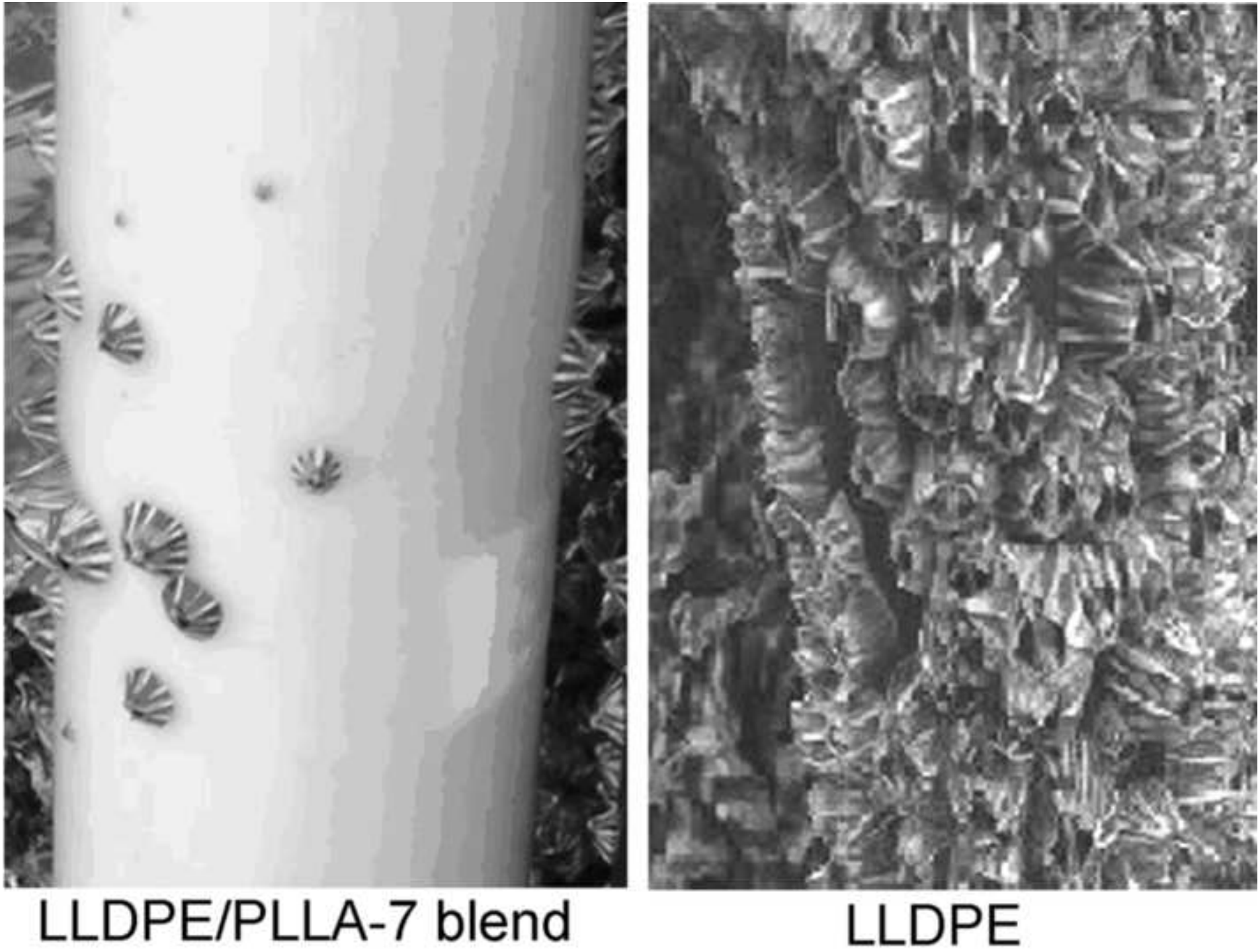

Survival ratio (%)
Time (h)
Lactic acid
(mg/L)
0
10
100
1000
10000
0
20
40
60
80
100
0
10
20
30
40
50
60

Figure 7

Click here to download high resolution image



Supplementary data

Effects of Poly(L-lactic acid) Hydrolysis on Attachment of Barnacle Cypris Larvae

Nobuyuki Ishimaru[a], *Takayuki Tsukegi*[b], *Minato Wakisaka*[a], *Yoshihito Shirai*[a,b], *and Haruo Nishida**[,a,b]

[a] Eco-Town Collaborative R&D Center for the Environment and Recycling, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan

[b] Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan

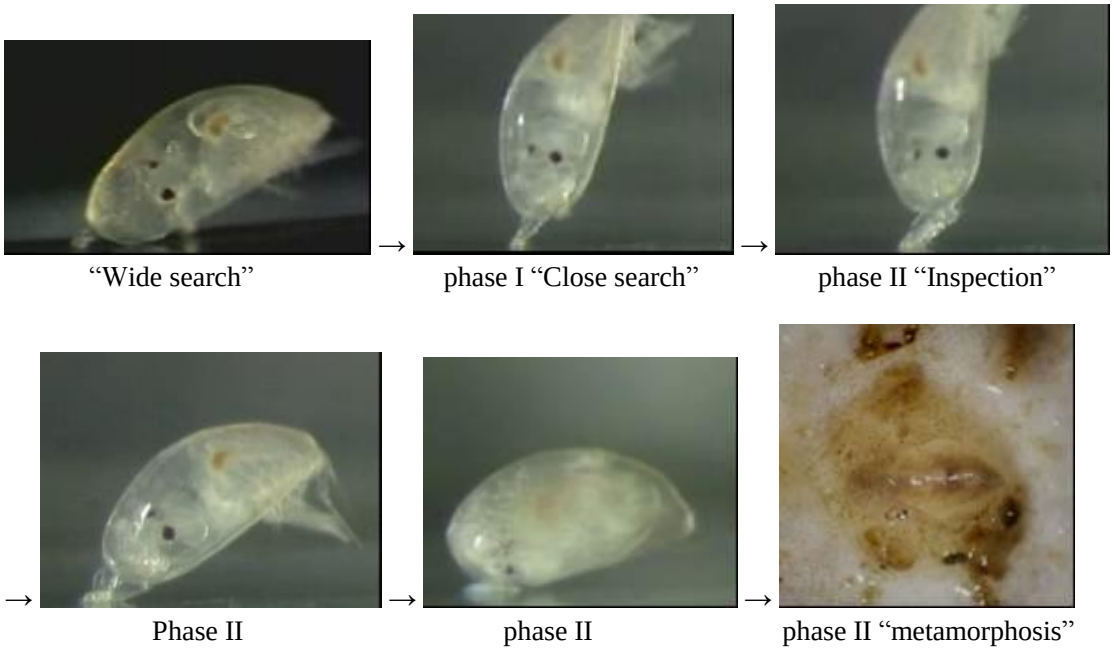


Figure S1. Attachment/settlement processes of cypris larvae on sample sheet: "close search", "inspection", and "metamorphosis" phases up to "settlement".

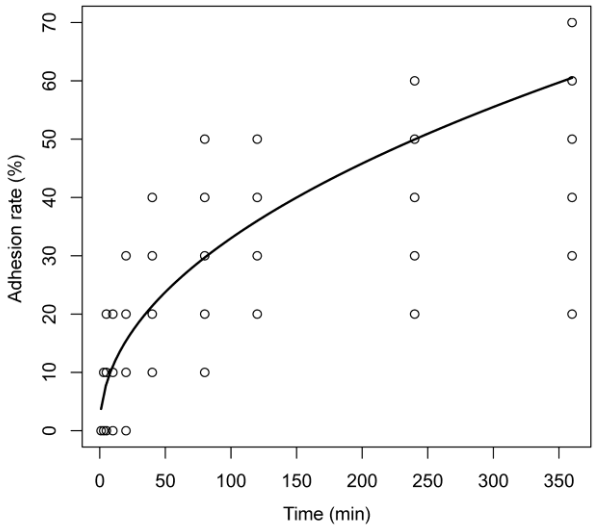


Figure S2. GLM fitting of cypris larvae attachment behavior on LLDPE sheet.

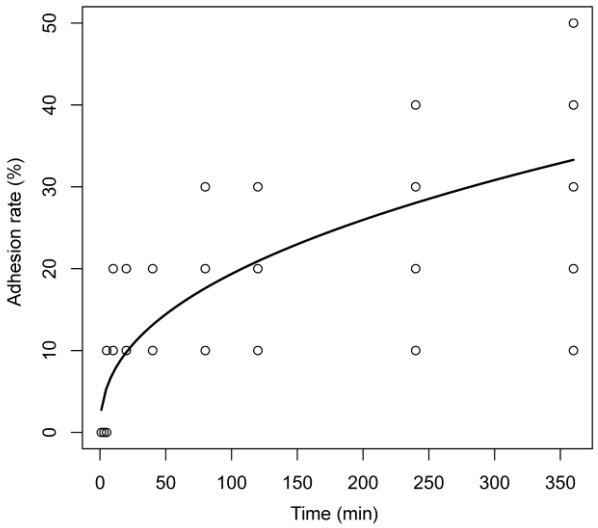


Figure S3. GLM fitting of cypris larvae attachment behavior on PLLA-31 sheet.

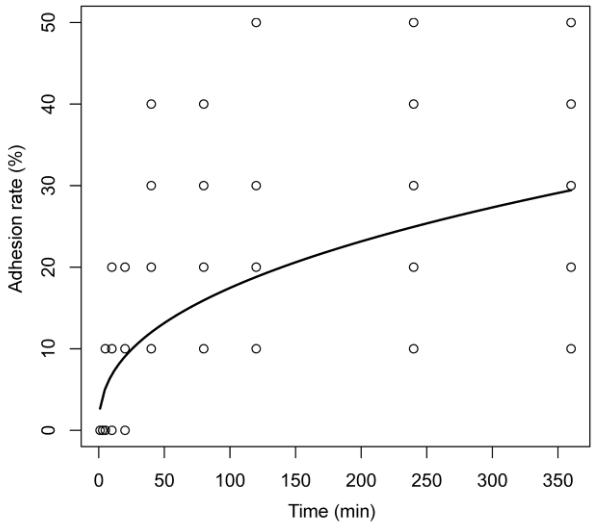


Figure S4. GLM fitting of cypris larvae attachment behavior on PLLA-7 sheet.

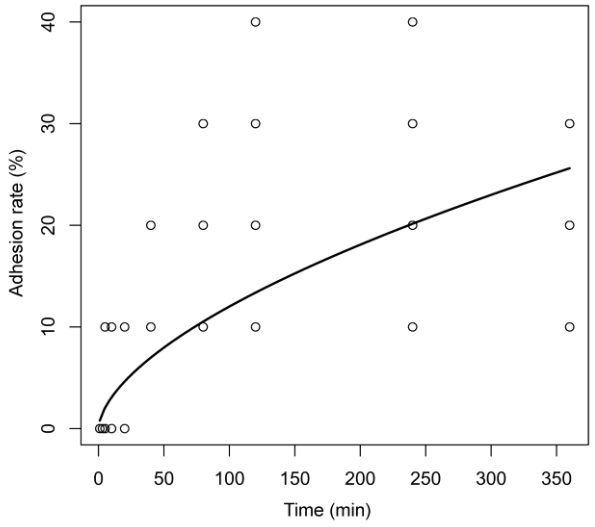


Figure S5. GLM fitting of cypris larvae attachment behavior on PLLA-3 sheet.

Table S1. Statistical analysis by the t-test of attachment ratio of cypris larvae on PLA sheets in Figure 3(a).

	Time (min)	80	120	240	360
Compared Samples					
LLDPE - PLLA-31	t statistic	6.0636	7.9738	7.1216	6.8794
	p (T<=t) two-tails	0.0000	0.0000	0.0000	0.0000
	t critical two-tails	2.0117	2.0057	2.0017	2.0017
PLLA-31 - PLLA-7	t statistic	0.0000	0.6213	1.9293	1.9136
	p (T<=t) two-tails	1.0000	0.5368	0.0586	0.0606
	t critical two-tails	2.0017	2.0017	2.0017	2.0017
PLLA-31 - PLLA-3	t statistic	2.7148	2.7277	3.2493	3.7359
	p (T<=t) two-tails	0.0087	0.0084	0.0019	0.0004
	t critical two-tails	2.0017	2.0017	2.0017	2.0017
PLLA-7 - PLLA-3	t statistic	2.4083	1.7415	1.1734	1.6435
	p (T<=t) two-tails	0.0192	0.0869	0.2454	0.1068
	t critical two-tails	2.0017	2.0017	2.0017	2.0106
PLLA-3 - PLLA-1	t statistic	12.7753	13.7295	13.1741	14.3670
	p (T<=t) two-tails	0.0000	0.0000	0.0000	0.0000
	t critical two-tails	2.0452	2.0452	2.0452	2.0452

Table S2. Statistical analysis by the t-test of attachment ratio of cypris larvae under addition of LA.

	Time (h)	6	24	36	48
Compared Samples (LA conc.)					
0 - 10 ppm	t statistic	2.4495	6.5320	6.5320	3.5777
	p (T<=t) two-tails	0.0705	0.0028	0.0028	0.0072
	t critical two-tails	2.7764	2.7764	2.7764	2.3060
0- 100 ppm	t statistic	2.5298	5.6921	6.5000	4.7068
	p (T<=t) two-tails	0.0353	0.0005	0.0002	0.0015
	t critical two-tails	2.3060	2.3060	2.3060	2.3060
10- 100 ppm	t statistic	1.0000	1.0000	3.1623	2.1381
	p (T<=t) two-tails	0.3739	0.3739	0.0341	0.0650
	t critical two-tails	2.7764	2.7764	2.7764	2.3060
100-1000 ppm	t statistic	5.6569	7.5895	6.5000	9.0000
	p (T<=t) two-tails	0.0005	0.0001	0.0002	0.0000
	t critical two-tails	2.3060	2.3060	2.3060	2.3060
1000-10000 ppm	t statistic	0.0000	0.6325	0.6325	0.6325
	p (T<=t) two-tails	1.0000	0.5447	0.5447	0.5447
	t critical two-tails	2.3060	2.3060	2.3060	2.3060

Table S3.Statistical analysis by the t-test of survival ratio of cypris larvae under addition of LA.

	Time (h)	6	24	36	48
Compared Samples (LA conc.)					
0 - 10 ppm	t statistic	0.0000	2.1381	0.0000	0.3536
	p (T<=t) two-tails	1.0000	0.0650	1.0000	0.7328
	t critical two-tails	2.3060	2.3060	2.7764	2.3060
0- 100 ppm	t statistic	1.1339	0.5000	2.1381	0.8944
	p (T<=t) two-tails	0.2897	1.0000	0.0650	0.3972
	t critical two-tails	2.3060	2.7764	2.3060	2.3060
10- 100 ppm	t statistic	2.1213	4.0000	4.0000	0.4472
	p (T<=t) two-tails	0.0667	0.0161	0.0161	0.6666
	t critical two-tails	2.3060	2.7764	2.7764	2.3060
100-1000 ppm	t statistic	8.4853	8.5732	4.5356	12.0167
	p (T<=t) two-tails	0.0000	0.0000	0.0019	0.0000
	t critical two-tails	2.3060	2.3060	2.3060	2.3060
1000-10000 ppm	t statistic	0.7559	0.8660	2.2136	0.6325
	p (T<=t) two-tails	0.4714	0.4117	0.0578	0.5447
	t critical two-tails	2.3060	2.3060	2.3060	2.3060

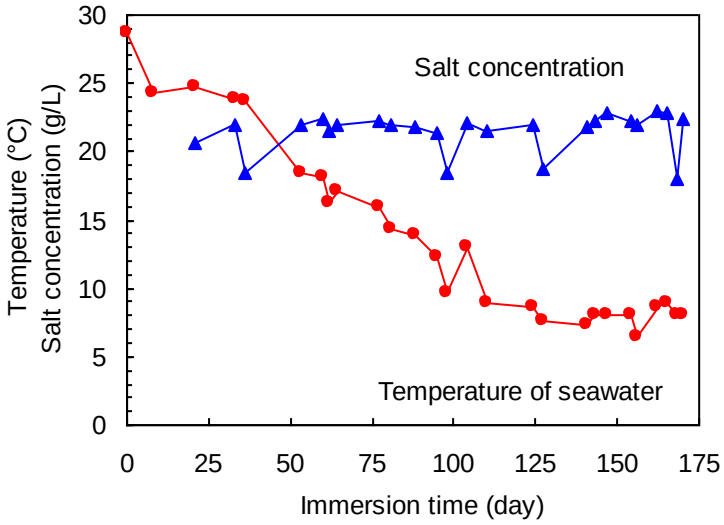


Figure S5. Changes in temperature and salt concentration of Ariake Sea water fir the immersion test.

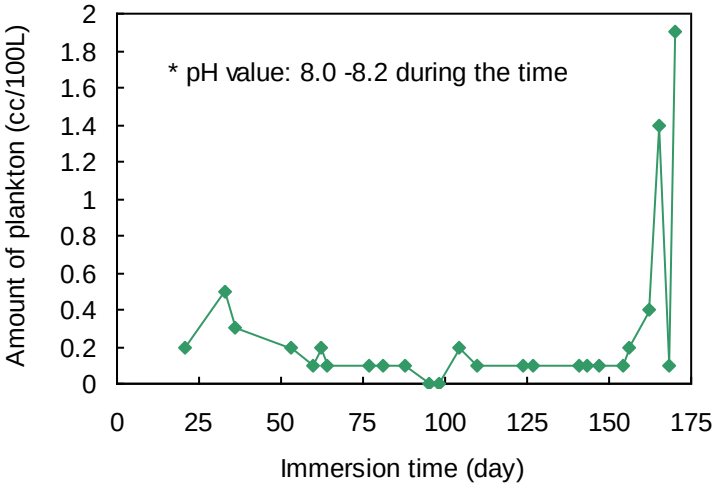


Figure S6. Changes in pH value and amount of plankton in Ariake Sea water fir the immersion test.