TITLE: NaCl monitoring seawater influence on the growth of *Trametes versicolor*

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FOOTNOTES TO THE TITLE: Salt effects on biodegradation of Tsunami wood debris

Key Words

Trametes versicolor, tsunami, biodegradation, Wood debris, Sodium chloride (NaCl), salt effect

Abstract

There are only a few scientific data about the function of ecosystems after tsunami disasters. The ecosystems help the environment to recover after a disaster and therefore the research on its function is important. We estimated the seawater influences on wood degradation after tsunami disaster by the growth of *Trametes versicolor.* NaCl in debris was monitored to estimate the impact of tsunami on wood blocks. The debris from the

Great East Japan Earthquake on the pacific coast in March 2011 was used for the simulations. Its growth on debris was compared with those on seawater treated woods, and the amount of sodium chloride was examined to know the approximate amount of salts in the samples. As a result, this common white-rot fungus degraded wood debris in the same way as sound sapwood. Although the study was conducted at laboratory level, this is the first report from the real debris, which assessed the fungal decomposition ability of the ecosystem after tsunami disaster.

Introduction

Tsunami loads have been a huge problem in disaster areas. After the Great East Japan Earthquake on the pacific coast in March 2011, it is estimated that 20,188 thousand tons of debris accumulated. Since this type of debris was usually mixed with pollutants like anonymous chemicals and scrap metals (Karube et al. 2015), thus loads of classification and detoxification processes were required before recycling, incineration or landfill disposal for those debris (Ministry of the Environment, 2011). The processes took a time, and consequently most debris remained in disposal stations for ages. As for the Great East Japan Earthquake, in the end, 82 % was recycled, 12 % was incinerated and 6% was landfilled (Ministry of the Environment, 2015). According to the Reconstruction Agency in Japan, which was established after the Great East Japan Earthquake, half of the debris in Miyagi prefecture still remained in the cue for disposal in 2013. About 14% of over two billion tons of the debris from Miyagi and Iwate prefectures was wood debris (Ministry of the Environment, 2015). The treatments of debris from the Great East Japan Earthquake were completed in 2014. The recovery of normal life in these area is the most urgent necessity after the natural disaster (Ministry of the Environment, 2011; Chandrasekhar et al., 2015), and therefore, there has not been many case studies of recoveries of ecosystem in tsunami disaster areas.

Here, we evaluated the fungal ability on decomposition of the wood debris from this huge disaster that occurred over a short time span. All analysis with debris was conducted in 2012 just after we received the debris from Miyagi prefecture. *Trametes versicolor* was employed as a model microbe since it is a common white-rot fungus, which attacks a wide range of woods. This fungus occurs commonly in forests and mountain sides but also attacks houses and buildings. It was used for the wood decaying test for industrial purpose (Japanese Industrial Standards JIS_2010). Since after a serious disaster there is no clear single factor which will affect biodegradation efficiency, we studied the influence of salt. We compared the data with that from the samples prepared with artificial seawater based on a NaCl parameter, sodium chloride contents. Salt inhibition on fungal growth is already reported but there is no detailed data after 1970s, and also there is no study using seawater.

The information of ecological cycles after tsunami disaster, especially near the urban area, will be helpful to reconstruct the area. In this study, *T. versicolor* attack level on wood debris was measured, and the data was compared with control blocks, which were treated with artificial seawater. The NaCl contents were used to get a comparative parameter. In order to estimate the tested debris condition, the influence of seawater immersion and washing were estimated also using NaCl contents as a parameter. This study provides the novel information about biodegradation of wood debris after tsunami disaster.

Materials and methods

Trametes versicolor

A white-rot fungus, *T. versicolor* (L. ex Fr.) Quel was used for the decay test, which is available in the Deterioration Organism Laboratory (DOL) of the Research Institute for Sustainable Humanosphere, Kyoto University, Japan. *T. versicolor* was pre-cultured in a 100 ml of nutrient solution (4.0 % glucose, 0.3 % peptone, and 1.5 % malt extract) for around a week until the fungus produced granules.

Wood blocks

Standard wood blocks were prepared with sound sapwood of *Cryptomeria japonica* D. Don (20 (R) x 20 (T) x 10 (L) mm).

In addition, two groups of seawater treated wood blocks were prepared, group A as a model sample of waterborne debris such as floating driftwood or lumber and group B as a model sample of seawater-washed log after tsunami. In group A, the wood blocks were soaked in 200 ml artificial seawater (Daigo's artificial seawater SP for marine microalgae medium, Nihon pharmaceutical Co. Ltd., Japan) with 100 g quartz sands for 0, 1, 3 and 6 months. Those blocks were air-dried at room temperature for a week before use. In group B, blocks were additionally treated with artificial seawater according with the method of wood surface treatment in JIS K 1571-2010: They were immersed in the seawater for 5 hours at 25 ± 2 °C and dried for 19 hours at 40 ± 2 °C during 10 days before use.

Tsunami wood debris arrived from Natori city, Miyagi prefecture in 2012 July (kindly supplied from Dr. K. Kashimura, Chubu University, Japan). They were from a part of the debris in the cue of disposal treatment after the Great East Japan Earthquake of March 11, 2011 for 16 months. Fifteen wood debris were randomly selected and cut into 20 (R) x 20 (T) x 10 (L) mm as same as standard wood blocks (Fig. 1). The condition of the debris did not allow us to identify its wood species, however it seemed that there were at least five or six wood species in the debris. By identification on gross, the 15 debris contained four hardwoods and 11 sapwoods containing *C. japonica*.

Seawater influence on the growth of *T. versicolor*

To learn the decomposition occurrence on waterborne debris, seawater influence on growth of *T. versicolor* was examined with the wood blocks from group A. Salt immersion into the blocks was estimated by the weight change before and after soaking. Experiments were conducted with 12 repetitions for each soaking interval. A monoculture decay test was conducted according to JIS K 1571-2010. All sample blocks were weighed after oven drying at 60 ± 2 °C for 3 days and sterilized with gaseous ethylene oxide prior to the decay test. A glass jar containing 250 g quartz sand (30-50 mesh) and 80 ml nutrient solution was autoclaved before use. Three ml of pre-cultured liquid fungus suspension containing granules were inoculated to the glass jar medium and incubated till the mycelia fully covered the surface of quartz sand in the jar. Thus, wood samples were placed on the mycelial mat. They were incubated for 12 weeks at 26 ± 2 °C. Three samples were placed in one glass jar. Fungal attack to the wood samples was estimated by comparing the oven-dried masses before and after the test. Hardness was also measured by a durometer (hardness tester: Asker CL – 150H with DD2 tester, Kobunshi keiki Co. Ltd., Japan).

Debris decay test

As for debris, blocks were obtained from both edges of 15 debris because each edge could have different environmental effects such as one edge had been exposed to the air and light but the other side had been buried. Therefore total 30 blocks were used for a decay test. Sound sapwood of *C. japonica* was used as control test samples. For positive control, 5 no-treated blocks were prepared. For negative control, 5 blocks from group B were used. The monoculture decay tests were conducted as described above.

NaCl contents

Salt contents in debris and group B wood blocks were unknown, therefore they were estimated by contents of sodium ion as below. Approximately 0.1 g fractions were collected from each sample block. They were kept in a melting pot and heated at 150 °C for 3 hours in 60 % nitric acid to remove all organic materials. Ash remaining in the melting pot was dissolved in distilled water, whose amount was 100 times volume of the initial fractions. The ahs-containing solutions were filtered (DISMIC-13 cp, 0.20 μm, Advantec, Japan). NaCl contents in the wood samples were estimated by Na content in the digested solution using Inductively Coupled Plasma Optical Emission Spectrometer (ICP spectrometer) (SPS-7800 Plasma Spectrometer, SII - Seiko Instruments Inc., Japan). A small amount, about 10 cm^3, was injected into the instrument to measure its contents. The standard solutions were made by a commercial sodium standard solution (Na 1000 ppm, Nacalai tesque). To estimate the unpredictable Na contents in the seawater treated wood blocks and debris, standard curves were obtained with 0, 1, 10 and 100 ppm standard solutions to cover the wide range of concentration, and the data under 10 ppm Na contents were double-checked with 0, 1 and 10 ppm standard curves.

The Na estimation with ICP were conducted on the sample blocks of group B used for decay test and debris. The middle parts of each debris were used to measure the salt contents. 15 debris blocks were used to estimate NaCl contents in the debris.

Statistics

Kruskal-Wallis test was used to analyze the decaying rate of debris and controls. On the other hand, the weight loss and hardness change before and after decay test was analyzed with Wilcoxon test. Na contents in the debris were compared with the controls by the Mann-Whitney test. Mann-Whitney test was also applied to examine the hardness change by seawater immersion with each soaking interval. JMP 9.0 software was used for all analyses.

Results

Seawater influence on fungal attack of *T. versicolor*

This decomposition test with group A blocks was conducted to learn the impact of seawater immersion on biodegradation. Longer immersion in the artificial seawater drove salt accumulation into the wood blocks (Fig. 2A, $p < 0.001$, $X^2 = 40.976$, Kruskal-Wallis test). The weight increase of sample blocks after 1, 3 and 6 months immersion were observed and it indicated that minerals in the artificial seawater penetrated gradually into the wood blocks (Fig. 2A). On the other hand, hardness did not changed by this soaking treatment ($p = 0.485$, $X^2 = 2.446$, Kruskal-Wallis test).

T. versicolor attack caused a significant weight loss in all samples but not hardness change (Fig. 2BC) (0 month: $p < 0.001$, $X^2 = 17.280$ in weight loss parameter and $p < 0.001$, $X^2 = 11.608$ in hardness parameter, 1 month: $p < 0.001$, $X^2 = 14.533$ in weight loss parameter and $p = 0.002$, $X^2 = 9.545$ in hardness parameter, 3 month: $p < 0.001$, $X^2 = 17.295$ in weight loss parameter and $p = 0.644$, $X^2 = 0.213$ in hardness parameter, 6 month: $p = 0.008$, $X^2 = 7.053$ in weight loss parameter and $p = 0.312$, $X^2 =$

1.022 in hardness parameter, Mann-Whitney test). The intense fungal attack was observed on the surface of 1 month seawater soaking samples (Supple. 1), but their degradation level was as same as control blocks (Fig. 2B). Thought the considerable weight loss was occurred in all samples, the decay rate decreased with the immersion period ($p < 0.001$, $F = 27.580$, linear regression analysis), which reflected the hardness change with 3month and 6 month immersion samples (Fig. 2C). It meant that longer immersion in the seawater considerably made *T. versicolor* difficult to attack wood.

Debris decay test

The decay level of tsunami wood debris was compared with naïve (positive control) and group B (negative control) blocks to estimate the potential biodegradation of debris in the nature. *T. versicolor* attacked the debris without any difficulties. The mass loss from the decay test in wood debris was 17.6 ± 2.18 (average ± standard error, SE) wt%, while it was 21.2 ± 1.57 wt% in no-treated sample blocks. Hardness value decreased by fungus attack ($p < 0.001$, $X^2 = 15.196$, Wilcoxon test). There was no significant difference in decaying levels among wood debris, no-treated and seawater surface-treated wood blocks ($p = 0.194$, $X^2 = 3.282$, Kruskal-Wallis test). At the initial stage of incubation, group B wood blocks were less attacked (Supple. 2) however the these blocks even got slightly more attack after 12 weeks incubation (Mass loss, 26.5 ± 2.17 wt%).

NaCl contents in the debris

To obtain the comparative parameter, NaCl contents in the debris and group B samples were measured by the detection of sodium ion. Na contents were significantly low from the negative controls ($p = 0.001$, $X^2 = 10.714$, Mann-Whitney test). The debris samples contained 0.12 – 3.3 mg / g (average: 1.14 mg / g) Na and the group B blocks contained 15.17 – 25.71 mg / g (average: 19.32 mg / g). Na contents in non-treated wood blocks were below the measurable limit. The results were similar with 0, 1 and 10 ppm standard curve.

Discussions

This study demonstrated the salt effects on the growth of *T. versicolor.* The real debris from the Great East Japan Earthquake was also analyzed. The inhibition on the fungal growth by seawater was observed, however the results indicated that debris certainly contained salt, but not at the critical level. According to the data from ICP spectrometer, the debris contained average 1.14 mg/g Na and they were attacked by the white-rod wood decaying fungi as same as the control sound sapwood.

It is well known that salt has a function to inhibit the fungal growth (Tresner and Hayes 1971; Sato et al. 2014), on the other hand, there are few reports of tsunami influence on fungal growth the nature. Seawater normally has 3.5 % salt content. Our experiments demonstrated that wood blocks could accumulate around 5 % salt by 3 month if they were soaked into seawater. The growth rate of *T. versicolor* was significantly delayed if the wood blocks contained this amount of salt (Fig. 2, Supple. 3). Seawater contains many ions, but NaCl is one of the representative component of seawater. The results matched with the former study using 29 *T. versicolor* strains, which added sodium chloride into media (Tresner and Hayes 1971). Moreover the hardness increase of wood blocks with the salt contents was observed in this study. It would be amenable with the fact that NaCl treatment improves tensile strength of fiberboard (Uraki et al. 2005). NaCl is also known to change the water movement inside of woods (Yang et al, 2011). It seemed, however, that biodegradation process would not be really disturbed by salt after a real disaster. This is probably because that fungal tolerance to NaCl is varied in wide range (Tresner and Hayes 1971). It is highly possible that salt-sensitive microbes simply do not colonize on the debris after tsunami disaster (Higashijima et al. 2012). Actually Sato et al. (2014) isolated the fungi, which were tolerant to 15-20 % salt from the paper objects in Tohoku regions at 5 months after the Great East Japan Earthquake. Debris were often left uncontrolled in the nature after Tsunami disaster. Our samples, which were placed in debris dump for 16 months, contained about 0.001 % Na, which indicated that it contained 0.003 % salt (calculated from NaCl / Na proportion). This would be partly because of wash-off effect of rain (Pérez-Rodríguez et al. 2015; Konoplev et al. 1996; Nasrabadi et al. 2011). The wash-off function of fresh water was successfully confirmed with seawater treated wood blocks (Supple. 4), and we think that same washing-off procedure occurred on the debris due to rain.

In the test, the debris was simply classified and numbered by a chunk. Most of them were materials from residential constructions. The 15 debris seemed to contain four hardwood and 11 sapwood containing one *C. japonica*. This variation made difficult to assess the quality of these debris for their different densities and unknown wood preservation treatment before the disaster. Therefore we simply compared the data with well-studied sapwood *C. japonica* as an implementing option to evaluate the debris condition. Sapwood generally has lower natural durability and it is used as a control wood in the decaying test of JIS K 1571-2010. Even with careful consideration about this alternative, our results supported the sufficient ability of microbe to decompose the waterborne organic debris after Tsunami disaster. Decaying scale would be much larger in the nature than this experiments, however it should not be a matter because our additional test with larger wood blocks still supported this conclusion that debris would be decomposed by microbes as same as normal logs in the nature (Supple. 5).

Ecological services for human life have been highlighted nowadays and we have just realized that research on its beneficial and detrimental effects is important (Li et al., 2014). Nevertheless ecosystem service has both useful and difficult aspects for us and though it is not easy to control, it can greatly help the recovery process after a tsunami disaster (Costanza et al. 1997; Gómez-Baggethun and Barton 2013; Jansson 2013). Moreover, the knowledge of the ecological condition after the disaster will be important for encouraging victims to resume their normal life. Toyofuku et al. (2014) reported that the biotic resilience in Tohoku area was quicker than they had expected and it means that there would be active interactions between living organisms and debris. These debris will stay in nature for a certain time. Generally metal and plastic debris would have a slower impact than organic materials even though they can cause a serious environmental problem. In this study, we examined the wood debris but we must pay high attention also on the metal debris, especially on heavy metals. We must find the way to use well the ecosystem and nature to deal with a natural disaster in this modern world.

Natural disaster causes more and more serious problems nowadays because of the urbanization. Many difficulties exceed human efforts. Eventually we can not help relying on ecological purification systems to get back the healthy environment after disasters. When the scale of natural disaster is larger than our assumption, even nature itself needs a long time for the recovery. Therefore assessments after natural disaster in the city area are essential not only for the human habitability but also for the sustainability of natural eco-systems. For the prompt recovery of nature after tsunami disaster, further study on ecosystems is essential.

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Figure legends

Fig. 1 Tsunami wood debris from Natori city, Miyagi prefecture, Japan in 2012 July.

A: Arrived debris. Radioactivity was at natural level. B: 15 debris samples. C: 20 (R) x 20 (T) x 10 (L) mm sample block used for a decay test.

Fig. 2 Seawater influence on fungal attack of *T. versicolor.*

A: Salt (wt%) absorbed into the wood blocks by the immersion treatment before using for decay test (n = 12) is illustrated with □ and solid line. NaCl contents in the debris, which was calculated with ICP-indicated Na contents by NaCl molecular weight, before using for decay test is shown with ◆ (n = 30). B: The mass loss by decaying test (wt%) on *C. japonica* wood blocks immersed in the seawater for 0, 1, 3, and 6 months (n = 12). The data from debris is shown with gray column (n = 30). C: Hardness change of *C. japonica* wood block conditions after decay test. The hardness data from debris is illustrated with gray column for the data before decay test and with dotted column after decay test (n = 28). Vertical bar indicates standard error. Letters at the top of the column indicate the results of the Tukey-Kramer HSD test.

Supplemental data

Supple. 1 Photos of the decay test with 1 month seawater immersed samples to see *T. versicolor* attack. A: Fungal attack on wood block at 1 week after inoculation. Right jar was for control and left jar was for test. B: Surface of control wood block after 12 week fungus attack. C: Surface of 1 month seawater soaked wood block after 12 week fungus attack.

Supple. 2 Decay test with *T. versicolor.*

T. versicolor attacked A: sound sapwood with no treatment (positive control), B: sound sapwood treated with artificial seawater (negative control), C: wood debris from tsunami disaster (test). Scale bar at the bottom of the photos indicated 2 cm.

Supple. 3 NaCl influence on growth of *T. versicolor.*

The standard data for the comparison between debris decaying rate and control wood decaying rate based on sodium chloride contents.

A: Fungus growth (diameter) in 90 mm petri dishes on PDA medium contained 0 % NaCl: ○, 1 % NaCl: □, 3 % NaCl: ◇, 5 % NaCl: ●, 10 % NaCl: ■ Vertical bar indicates standard error. B: Photo of fungus growth at 3 month after inoculation.

To get the simplest standard of salt effects on the growth of *T. versicolor,* NaCl influence on growth of *T. versicolor* was observed on a medium. Potato dextrose agar (PDA: potato extract, 0.4 %; glucose, 2.0 %; agar, 1.5 %) contained 0, 1, 3, 5 and 10 % NaCl was prepared in 90 mm petri dishes and a pre-cultured 8 mm disk of *T. versicolor* was placed in the middle of each petri dish. Fungal growth was measured for 12 weeks at 26 ± 2 °C. Experiments were conducted with 10 repetitions for each NaCl concentration.

As it is well known that the NaCl added to PDA affected negatively on *T. versicolor* growth (Supple. 4A) ($p < 0.001$, $F = 47.808$ in the parameter of growth speed difference along with NaCl contents, linear regression analysis). It took 7 days, 3 weeks and 2 months for the mycelia of *T. versicolor* to cover the media with 0, 1 and 3 % NaCl contained media respectively. Approximately 67.5 % surface of medium was covered by mycelia at three month interval with 5 % NaCl contained PDA, and no growth was observed with 10 % NaCl contained PDA (Supple. 4B).

Supple. 4 NaCl loss test by washing to simulate the function of rain.

The wash-off effect by rain had been examined to monitor the conditional influence of the debris. A: washing condition with the indication of fractions obtained parts. B: Salt reduction rate in wood blocks after washing at 7^{th} day (n = 5). Salt quantities were estimated with Na contents detected by ICP spectrometer (SPS-7800 Plasma Spectrometer, SII - Seiko Instruments Inc., Japan). Vertical bar indicates standard error. Letters at the top of the column indicate the results of the Tukey-Kramer HSD test.

Samples treated with artificial seawater were used to simulate the washing function on salt reduction by rain. Wood sample blocks were placed on the plastic mesh so that all water was running away from a test arena. Those blocks were showered with 10 ml, 100 ml and 500 ml distilled water every day for 7 days. Fractions were obtained at 7th day and they were treated with nitric acid, and then adjusted with distilled water. Salt residue in the wood samples were estimated by Na content in the digested solution using ICP spectrometer. A small amount, about 10 cm^3, was injected into the instrument to measure its contents of Na. Standard carve was obtained with 0, 1, 10 and 100 ppm standard solution adjusted by a commercial sodium standard solution (Na 1000 ppm, Nacalai tesque). Some of detected values exceeded 100 ppm, however the data demonstrated that a water shower had a certain effect on washing out NaCl from the wood blocks ($p < 0.001$, $F = 12.100$, linear regression analysis). This wash-off effect by rain had been examined to monitor the conditional influence of the debris.

Supple. 5 Mass loss from the larger scale decay test with 20(R) x 20(T) x 110(L) mm wood block (n=5).

Debris no. 1-5 were used for tests and control samples were made with sound sapwood *C. japonica*. Decay level was same in control and test samples ($p = 0.502819$, T test).

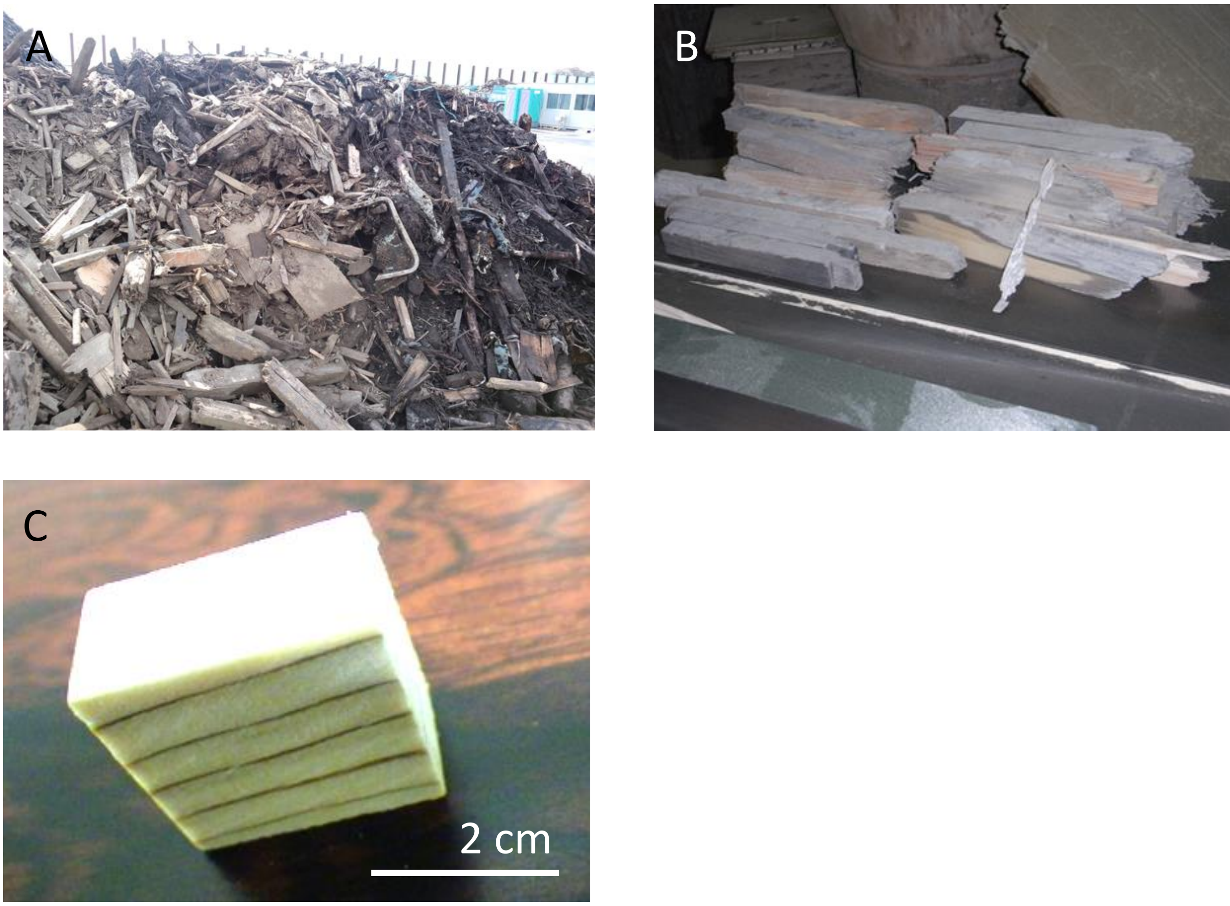


Fig 1

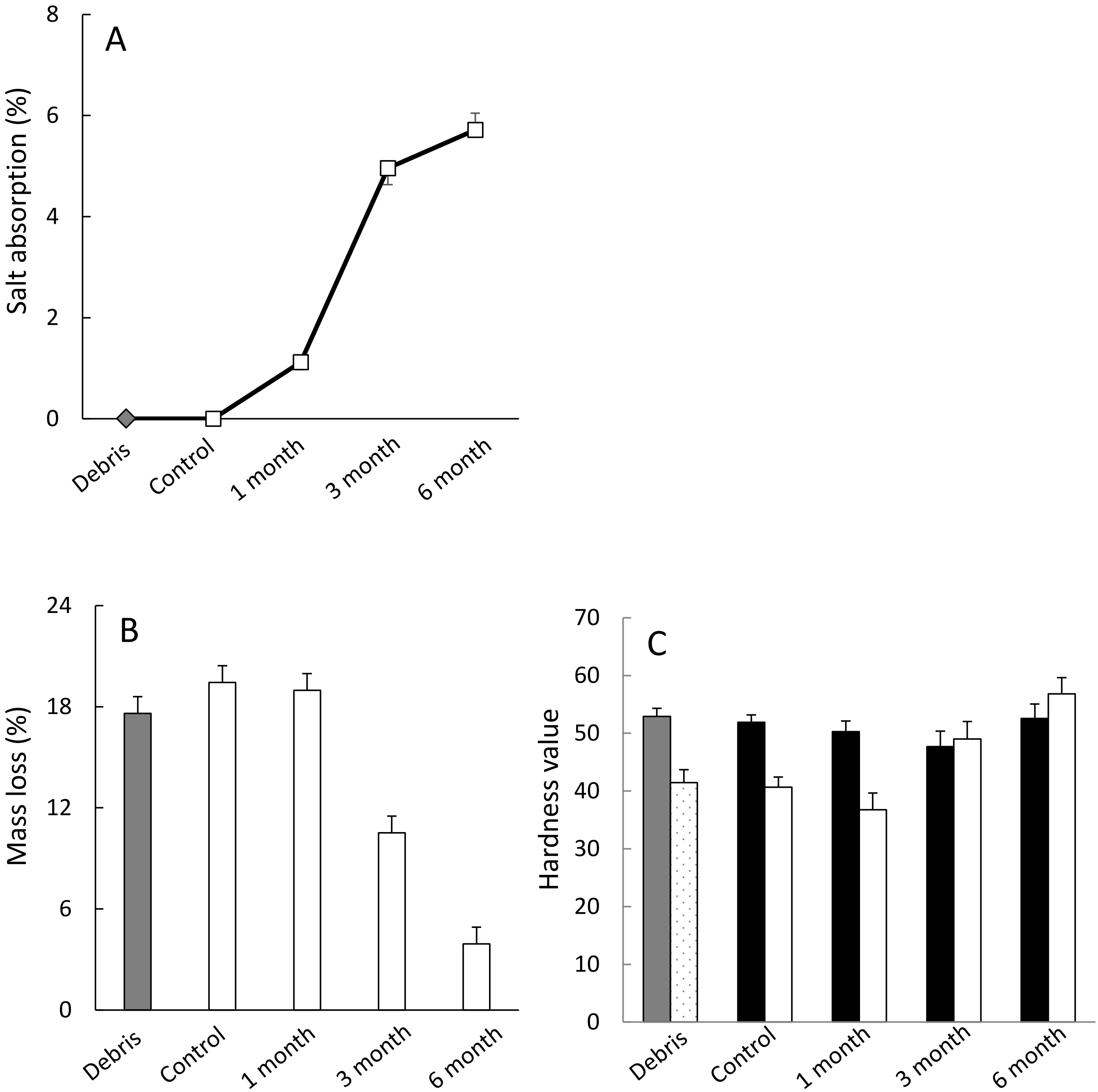
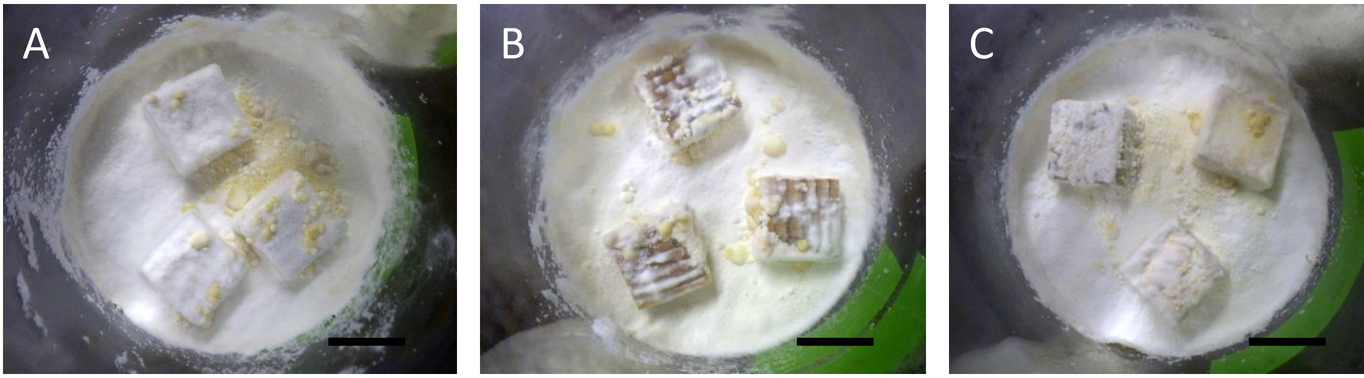
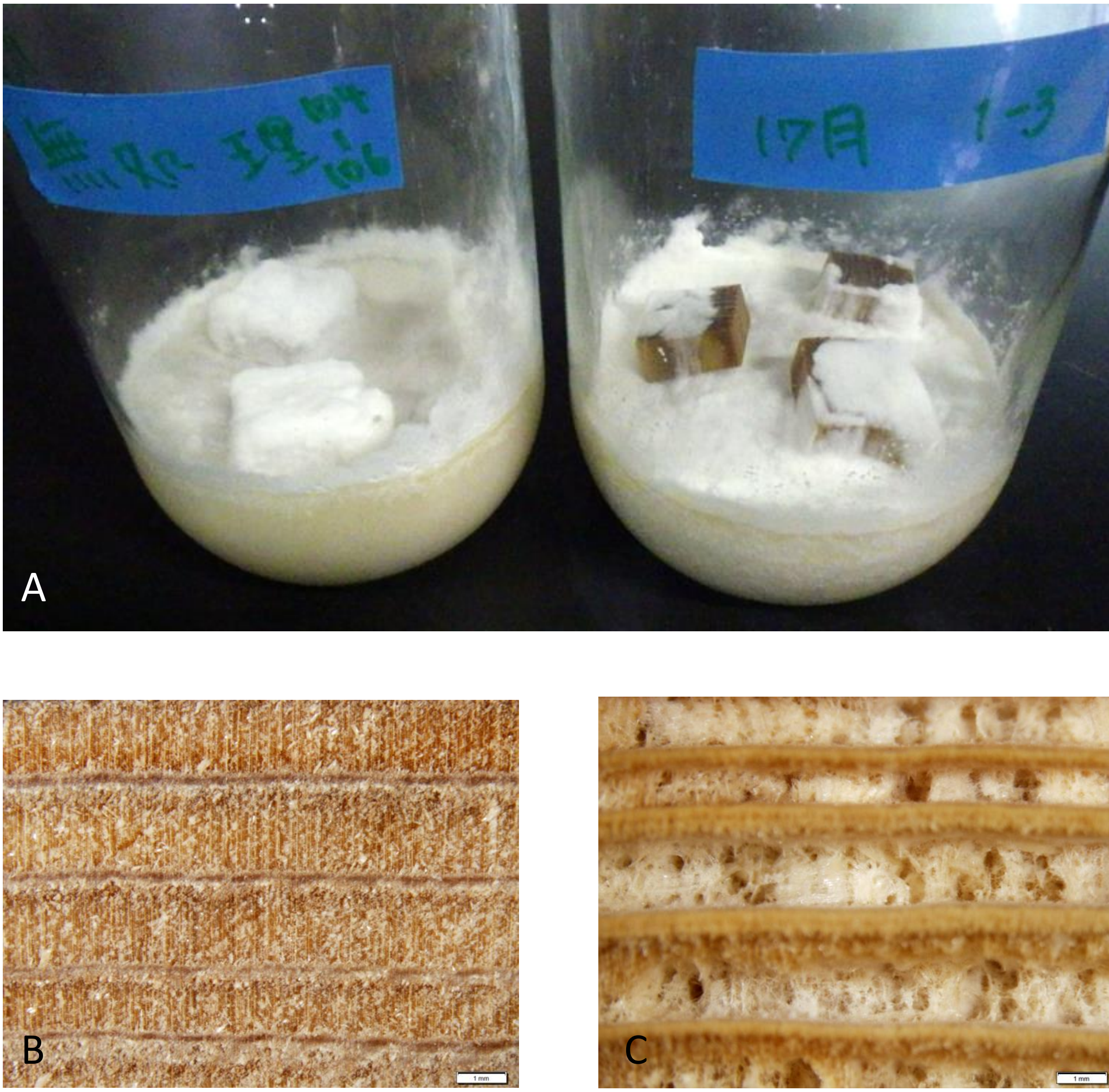

A
Salt absorption (%)
8
6
4
2
0
Debris
Control
1 month
3 month
6 month
B
Mass loss (%)
24
18
12
6
0
Debris
Control
1 month
3 month
6 month
C
Hardness value
70
60
50
40
30
20
10
0
Debris
Control
1 month
3 month
6 month

Fig 2



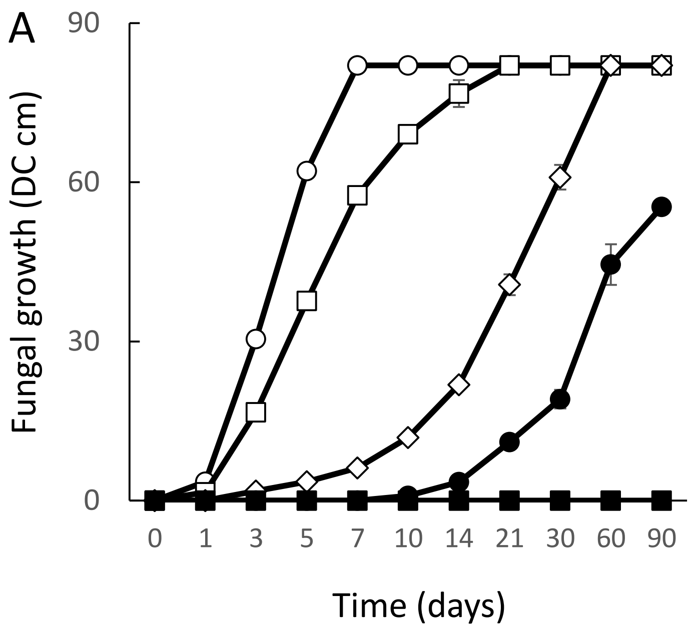
Supple 1



Supple 2

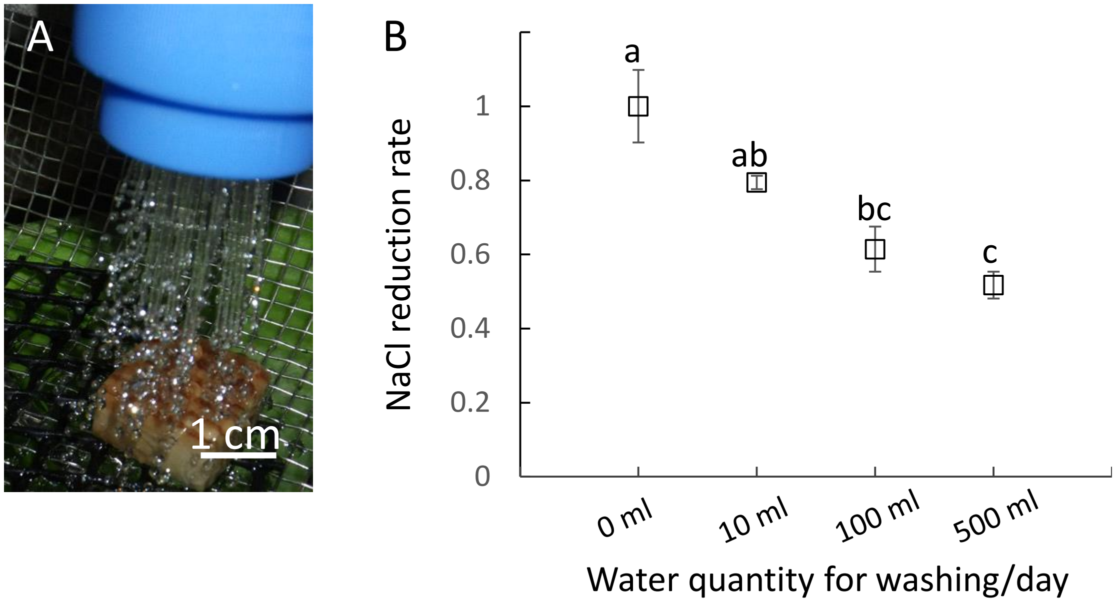
Mass loss(%) of middle sized wood blocks (n = 5)		
	Control	Debris
% (wt)	8.00	5.60
SE	0.015	0.031

Supple 3


A
90
60
30
0
Fungal growth (DC cm)
0 1 3 5 7 10 14 21 30 60 90
Time (days)


B
Na Cl
3month
1%
3%
5%
10%

Supple 4


A
1 cm
B
a
ab
bc
c
NaCl reduction rate
1
0.8
0.6
0.4
0.2
0
0 ml
10 ml
100 ml
500 ml
Water quantity for washing/day

Supple 5

Na Cl
3month
5%
10%