Original research or treatment paper

First aid for flood-damaged paper using saltwater: The inhibiting effect of saltwater on mold growth

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With the aim of developing a new easy-to-use method for rescuing flood-damaged paper, the effect of saltwater on the inhibition of fungal growth on paper was investigated. This procedure could be used instead of, or assisted by freeze drying. Cellulose-digesting *Trichoderma reesei*, three types of fungi representative of fungi growing on paper (*T. reesei*, *Aspergillus terreus*, and *Aureobasidium pullulans*) and various naturally airborne fungi were cultured on cellulosic materials in liquid media containing artificial seawater with different salt concentrations. The addition of salts successfully inhibited the growth of *T. reesei* on microcrystalline cellulose at the concentration of 3.2% (m/m) or higher. The critical salt concentration, 3.2%, is within the general range of salt content in seawater. Other solutions of salts similar to sodium chloride also inhibited fungal growth. Although the observed growth-inhibiting effect was attributed to the high osmotic pressure of the salt solution, physiological effects depending on ion species used were also considered to be possible. The growth of all three types of fungi on copy paper was inhibited effectively when the salt concentration was increased. The growth of various fungi on pure cellulose, with enough oxygen supplied to pores, was completely inhibited (as assessed by visual examination) for 24 days at salt concentrations of 3.5% (m/m) or greater. The fact that the effect of saltwater on cellulosic materials was observed even under optimum medium conditions implies that fungi would be considerably inhibited on flood-damaged paper immersed in saltwater. This method is a promising first aid measure when circumstances do not allow for flood-damaged paper to be dried immediately.

Keywords: Cellulose, Copy paper, First aid, Flood, Fungus, Osmotic pressure, Paper conservation, Salt

Introduction

In recent times, global warming has led to unusual weather conditions also resulting in heavy rainfall (Min *et al.*, 2011; Pall *et al.*, 2011). Torrential rainfall can cause flooding, which has resulted in heavy losses to life and property such as the 1966 flood of the Arno River in Florence and 2011 Thailand floods (Ogden, 1979; Turchan, 1988; Spande, 2009; Banik & Brückle, 2010). Tsunamis can damage important paper-based documents almost instantly, as was the case during the tsunami that accompanied the Great East Japan Earthquake on March 11, 2011 (Sakamoto, 2010; Kibe, 2011). The adverse effects of flood damage on paper are mold growth and adhesion of pages of books and documents (Matsushita & Kono, 2009). Mold growth on paper causes esthetic loss, difficulty in reading information, bad smell, health hazards, and acceleration in the deterioration of the physical and chemical properties of the paper (AIC Book and Paper Group, 1992, 1994; Choi, 2007). The adhesion between paper sheets causes difficulties in turning the pages of books. The freeze-drying method is currently considered to be the best way to prevent these issues (Fischer & Duncan, 1975; Masuda, 1992; Sugarman & Vitale, 1992; NARA, 1993; Waters, 1993; Patkus, 2007). However, this method requires large refrigerating chambers, a long freezing period, and expensive freeze-drying devices (Sakamoto, 2010). Hence, we are developing an

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alternative or complementary, easy-to-use method for rescuing flood-damaged paper by using salt solutions such as seawater or sodium chloride, which are expected to have an effect on mold growth (Castillo & Demoulin, 1997), possibly on paper adhesion, and on to some extent ink dissolution, due to the salting-out effect that reduces dissolution of water-soluble pastes, glues, and gums.

The Sumatra Earthquake on December 26, 2004, which had a magnitude of 9.3, caused an enormous tsunami, and approximately 16 tons of land-ownership documents in Indonesia were soaked in seawater; they remained wet, in the tropical climate, for over two months. However, no mold growth was observed on them. After the rinsing and freeze-drying processes were complete, 97% of the documents were restored without any paper adhesion or deformation (Sakamoto, 2010). This successful restoration was reported as 'the miracle of Allah' in a local newspaper, but it was presumed to be the effect of saltwater. This incident indicated that a new conservation method, which would supplement the freeze-drying method, could be developed using saltwater.

The Great East Japan Earthquake, which had a magnitude of 9.0, triggered an extremely destructive tsunami; many important papers such as administrative documents and items of cultural heritage were immersed in seawater in the Tohoku region of Japan. As in the case of the Sumatra Earthquake, no mold growth was observed on documents that were soaked in the seawater and dried after being left wet for two months (Kigawa *et al.*, 2011).

In the present study, we experimentally clarified the effect of saltwater on the inhibition of fungal growth on paper; thus, we propose an easy-to-use first aid method that involves the application of saltwater to flood-damaged paper.

Experimental

Measurement of salt concentration of seawater

Seawater was sampled 1.5 km away from a river outlet in the Aceh region's coast of Indonesia on September 29, 2010. Seawater was also sampled at two locations along the coast in the Tohoku region, Japan. One sample was taken midway between two river outlets, which were 2 km apart from each other, in Ishinomaki, near the Ishinomaki Paper Mill (Nippon Paper Industries Co., Ltd, Tokyo, Japan) on May 12, 2011. The other sample was taken midway between two river outlets, 3 km apart from each other, in Hachinohe near the Hachinohe Paper Mill (Mitsubishi Paper Mills Ltd, Tokyo, Japan) on June 21, 2011. About 500 ml of seawater was each taken within easy reach from the pier. After the samples were centrifuged at $8000 \times g$ for ten minutes, the salt concentration of seawater was measured using a seawater refractometer (MASTER-S/MillM, ATAGO Co., Ltd, Tokyo, Japan) and a temperature-corrected electric conductivity meter (YK-31SA, Lutron Electronic Enterprise Co., Ltd, Taipei, Taiwan). Five measurements were made and the mean value was calculated.

Preparation of artificial seawater

The standard artificial seawater was prepared from the mixed salts powder (Daigo SP, Wako Pure Chemicals, Ltd, Osaka, Japan) as follows: 36 g of the dry salt components dissolved in 1000 ml of pure water. Salt concentration was also measured using the same devices (seawater refractometer and temperature-corrected electric conductivity meter). The salt composition of the artificial seawater is listed in Table 1.

Test of Trichoderma reesei growth in liquid media at various salt concentrations

Microcrystalline cellulose (1.6 g, Funacel II, Funakoshi Co., Ltd, Tokyo, Japan) was used as a substrate. A constant amount (1.0×10^9 spores/l) of *T. reesei*, a cellulose-digesting fungus, was inoculated into 80 ml of a sterilized liquid culture medium (Wood's culture medium, Table 2) containing different salt concentrations of artificial seawater (Table 1) expressed in % (m/m). All the salt components dissolved completely in the medium. The Wood's culture medium is suitable for culturing cellulose-

Table 1 Composition of artificial seawater (Wood & Wood, 1992)

Type of salts	Amount (mg)
NaCl	20 747
$MgCl_2{\cdot}6H_2O$	9474
$CaCl_2{\cdot}2H_2O$	1326
Na_2SO_4	3505
KCl	597
$NaHCO_3$	171
KBr	85
$Na_2B_4O_7{\cdot}10H_2O$	34
$SrCl_2$	12
NaF	3
LiCl	1
Others	0.1042
Total	35 955

Table 2 Composition of Wood's culture medium

Component	Concentration (g per 1000 ml medium)
$(NH_4)_2HPO_4$	2.6
2,2-Dimethyl succinic acid	2.2
KH_2PO_4	1.1
$MgSO_4{\cdot}7H_2O$	0.5
$CaCl_2{\cdot}2H_2O$	0.074
$CoCl_2{\cdot}6H_2O$	0.001
$FeSO_4{\cdot}7H_2O$	0.01
$MnSO_4{\cdot}7H_2O$	0.005
$ZnSO_4{\cdot}7H_2O$	0.005
pH	5

degrading fungi (Wood & Wood, 1992). All salt concentration percentages are in % (m/m) to the total of water, salts, and medium components excluding spores and substrates. After inoculation, the cultures were incubated at 37°C under constant agitation at 150 min^{-1} (rpm) for nine days followed by the determination of the extent of fungal growth. Similarly, aqueous solutions of NaCl, KCl, $MgCl_2$, or $CaCl_2$ were also tested to compare their fungus-inhibiting ability with that of artificial seawater.

Absorbance measurement to quantify fungal growth

To determine the extent of fungal growth, we measured the absorbance of the supernatant obtained from the media by centrifugation at 15 000 × g for five minutes. Absorbance spectra in the wavelength range of 200–600 nm were measured using an ultraviolet–visible spectrophotometer (V660, JASCO Corporation, Tokyo, Japan). The absorbance of purified and deionized water (MilliQ, Millipore Corporation, Massachusetts, USA) was used as a reference against which the absorbance of the supernatant would be measured.

Culture of specific fungi on papers in petri dish

A sterilized Wood's culture medium of 20 ml with artificial seawater salt concentrations of 0, 1.0, 2.0, 3.0, 3.5, 4.0, 4.5, and 5.0% and three sheets of sterilized general copy paper (Fine PPC paper, Hokuetsu Kishu Paper Co., Ltd, Tokyo, Japan), each 50 mm × 50 mm, were placed in petri dishes as a substrate. This paper is uncoated, acid-free, and made from 100% virgin pulp of bleached hardwood. *T. reesei* and *Aspergillus terreus*, both typical cellulose-digesting fungi (Caneva *et al*., 2008), and *Aureobasidium pullulans*, a typical halotolerant fungus (Kogej *et al*., 2005; Caneva *et al*., 2008), (1.0×10^9 spores/l each) were inoculated into petri dishes. Lids were kept on them during the incubation. The relative humidity inside was kept at nearly 100%. The cultures were incubated at 25°C. After seven days, photographs were taken and the paper samples were taken out of the petri dishes and dried at 25°C for a day. The surface was observed by using a scanning electron microscope (SEM, S-4000, Hitachi, Ltd, Tokyo, Japan). The presence of visible colonies on the paper was the main criterion for determining fungal growth because the presence of unnatural colors is the most critical problem that alters the appearance of the paper.

Culturing various fungi using a cellulose sponge

Pure cellulose was also subjected to a fungal culture test to exclude the influence of additives and various pH of commercially available paper. A cellulose sponge (CA107-4W, Toray Fine Chemicals Co., Ltd, Chiba, Japan) was cut into pieces of 50 mm × 50 mm and used as a substrate; these pieces were then placed in petri dishes. The cellulose sponge employed does not contain an antimicrobial agent and is made from 100% cellulose pulp. The Wood's culture medium (60 ml) mixed with artificial seawater salt concentrations of 0, 1.0, 2.0, 3.0, 3.5, 4.0, 4.5, and 5.0% was poured on the cellulose sponge samples. The samples were kept at 23°C and 50% relative humidity. Approximately 10 ml of deionized water was added every day to compensate for the loss in water owing to water evaporation. The extent of fungal growth was determined in the same manner as in the previous experiment, using copy paper.

Results and discussion

Seawater salt concentrations in Aceh and Tohoku regions

Salt concentrations of the seawater sampled in the Aceh and the Tohoku regions and the artificial seawater measured using the refractometer and temperature-corrected electric conductivity metre are listed in Table 3. The salt concentration of the seawater sampled in the Aceh region was deemed normal. The concentrations of the saltwater samples taken at Ishinomaki and Hachinohe in the Tohoku region were lower than the normal seawater concentration levels, probably because of the mixing of water from two rivers and also because of the distance between the sampling spot and river outlets. Typically, seawater salt concentration in the open sea is 3.5% (Pidwirny, 2006). However, it varies due to water evaporation and water gain (river water inflow and rainfall) (Antonov *et al*., 2006). The relative abundance of the major salts in seawater is considered to be constant regardless of the location of the ocean (Pidwirny, 2006). Therefore, if the salt concentrations of seawater are similar, similar effects are expected for preventing fungal growth on papers.

T. reesei growth on microcrystalline cellulose

When *T. reesei* grew, fungal colonies comprised of white mycelia were observed in the media; these fungal colonies discharged a yellow metabolic substance. The fungal growth at varied salt concentrations of the artificial seawater exhibited different intensities of the yellow color of the *T. reesei* cultures in the media, as shown in Fig. 1, where the extent of yellowness indicates the amount of fungal growth. The

Table 3 Measured salt concentrations of seawater in Aceh and Tohoku regions

Sampling location	Refractometry (%)	Electric conductivity (%)
Aceh region	3.4 ± 0.1	3.32 ± 0.02
Ishinomaki in Tohoku region	2.1 ± 0.1	2.07 ± 0.04
Hachinohe in Tohoku region	2.8 ± 0.1	2.73 ± 0.02
Artificial seawater	3.0 ± 0.1	3.03 ± 0.03

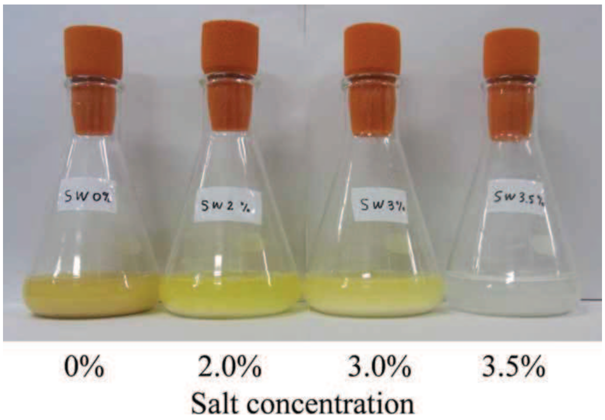

Figure 1 Photograph of the *T. reesei* culture in artificial seawater containing media at indicated salt concentrations after 9 days.

measured absorbances are shown in Fig. 2. The optical density of *T. reesei* cultures was measured at 372 nm and plotted as a function of the salt concentration, as shown in Fig. 3. *T. reesei* grew in the media with salt concentrations less than or equal to 3.0% of artificial seawater; however, no growth was observed for salt concentrations of 3.2% or greater.

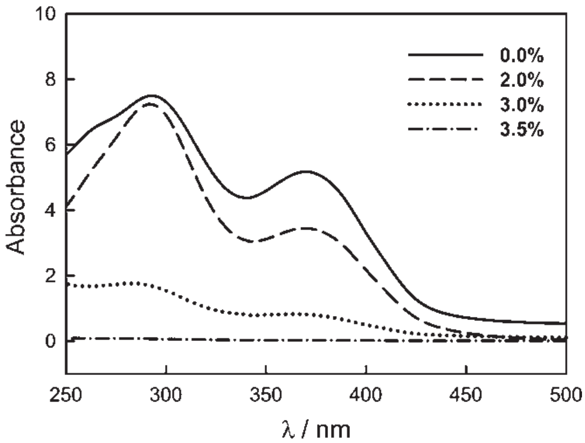

Figure 2 Absorbance spectra of supernatants of the *T. reesei* culture media.

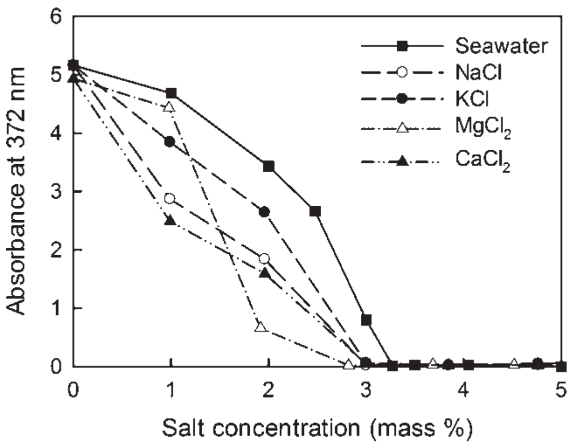

Figure 3 Fungal growth at various salt concentrations of different types of salts.

The results of the same experiment using different salt solutions of NaCl, KCl, $MgCl_2$, and $CaCl_2$ are also shown in Fig. 3. The effect of a salt solution on the inhibition of fungal growth was also confirmed for these salt solutions. The results suggest that these salts can be used as alternatives to seawater, helping to recover flood-damaged paper. All the measured single salts prove to inhibit *T. reesei* much more than the artificial seawater at concentrations lower than 3%. If flooded, a second bath comprised of an artificial salt solution (such as NaCl or others) may prove to be useful to save time. $MgCl_2$, $CaCl_2$, and partly also seawater salt, deliquesce in moist air. Consequently, a particular salt can be selected based on its efficiency of fungal growth inhibition, cost, and influence of residual salts on paper properties, after a desalination process and drying.

On the basis of osmolarity of the salt solutions, the same data are replotted in Fig. 4. Osmolarities of the solutions of seawater with the salts – NaCl, KCl, $MgCl_2$, and $CaCl_2$ – were calculated under the assumption that all the salts were ionized. Osmolarity (Osm/l) is the concentration of osmotically active particles in a solution, and it was practically calculated by adding the concentration (mol/l) of ions generated from each salt component. Fig. 4 shows that the osmolarity of the salt solutions varied from 0.8 to 1.1 Osm/ l when the absorbance at 372 nm reached zero. Although the growth-inhibiting effect is due to water being removed from cells by the high osmotic pressure of concentrated salt solutions, physiological effects, depending on the ion species used, have also been cited as a possible reason (Wen *et al.*, 2005; Duran *et al.*, 2010). The functions of salts in a cell are different from ion to ion as suggested by the function of the sodium–potassium pump (Schachtman & Liu, 1999), for example. Salts exhibit growth-inhibiting effects due to the osmotic pressure and have long been used for preservation of food such as salted fish and pickles.

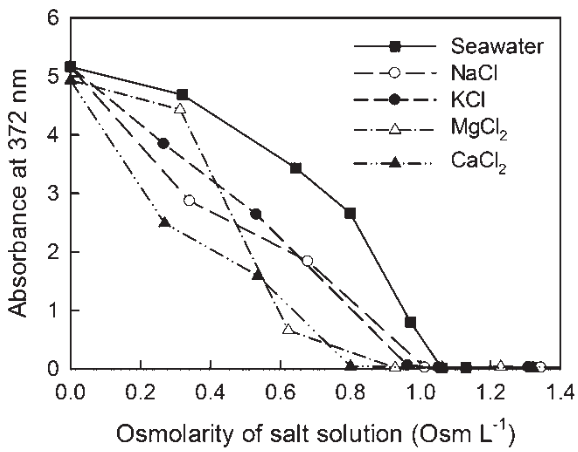

Figure 4 Fungal growth vs. osmolarity of different salts.

Specific fungal cultures on copy paper

Photographs of the copy paper samples seven days after inoculation are shown in Fig. 5. The differences in the appearance of paper samples were clearly observed among the different salt concentrations after seven days. The increase in the inhibition of the growth of *T. reesei*, *A. terreus*, and *A. pullulans* with increasing salt concentration was confirmed by visual examination. No fungal growth was observed on paper surfaces at salt concentrations of 3.5% or greater for *T. reesei* and *A. terreus*, whereas small black mycelia were observed on paper surfaces at salt concentrations of 3.5% or greater for *A. pullulans*. Even though the extent of the growth of black mycelia for *A. pullulans* does not cause inconvenience in reading books and documents, it still represents a risk to health, and diminishes the esthetic value of a document.

The SEM images of the copy papers are shown in Fig. 6. *T. reesei* is a filamentous fungus, and several mycelia were observed on the fibers, as shown in Fig. 6B. No fungal filament of *T. reesei* was observed, and only the spores inoculated initially were observed on fibers at 3.5% salt concentration, as shown in Fig. 6C. This suggests that the growth of *T. reesei* on paper was successfully inhibited at 3.5% salt concentration.

Mycelia, spores, and sporangia of *A. terreus* covered most fibers at 0% salt concentration, as shown in Fig. 6D. They also covered the fibers at 3.5% salt concentration, as shown in Fig. 6E, although they were not visible to the naked eye. However, mycelia of *A. terreus* on paper at 3.5% salt concentration were much thinner than those at 0% salt concentration. This implies that saltwater inhibits the growth of *A. terreus* as well, as is the case of *T. reesei*.

A. pullulans is a halotolerant and ubiquitous yeast-like fungus that is found in various environments (e.g. soil, water, air, and limestone). Both halotolerant and halophilic fungi survive in environments with high salt concentrations partly because salts including potassium salts are accumulated in their cells, which results in an osmotic balance being maintained between the inside and outside of the cells (Rengpipat *et al*., 1988; Prista *et al*., 2005; Gunde-Cimerman *et al*., 2009). The SEM images of *A. pullulans* in Figs. 6F and 6G show that there were more yeast-like spores and mycelia on paper at 0% salt concentration than at 3.5% salt concentration. The effect of saltwater on paper was also observed with *A. pullulans*.

The results suggest that the paper samples might have lost some weight due to the ingestion by the fungi and consequent deterioration. However, the change of mass could not be used as an exactly measure for the metabolism because the paper samples also absorbed components from the culture and some fibers were released from the paper.

Culture of various airborne fungi on cellulose sponges

After 24 days, as shown in Fig. 7, black spots having a diameter of approximately 10 mm were observed on cellulose sponge samples at 0 and 2.0% salt concentrations and the color of the surface partly changed to yellow at 3.0% salt concentration. However, at salt concentrations of 3.5% or greater, the sample remained clear and no fungal growth was observed. This suggests that the growth of various fungi in air was inhibited at salt concentrations of 3.5% and greater, although many spores of fungi other than the three sampled fungal species described in the previous section are supposed to float in air. Many pores formed in the sponge are considered to pass oxygen efficiently and absorb liquid culture medium mixed with artificial seawater salt concentrations, and hence, such a sponge is expected to provide a better environment for fungal growth than a piece

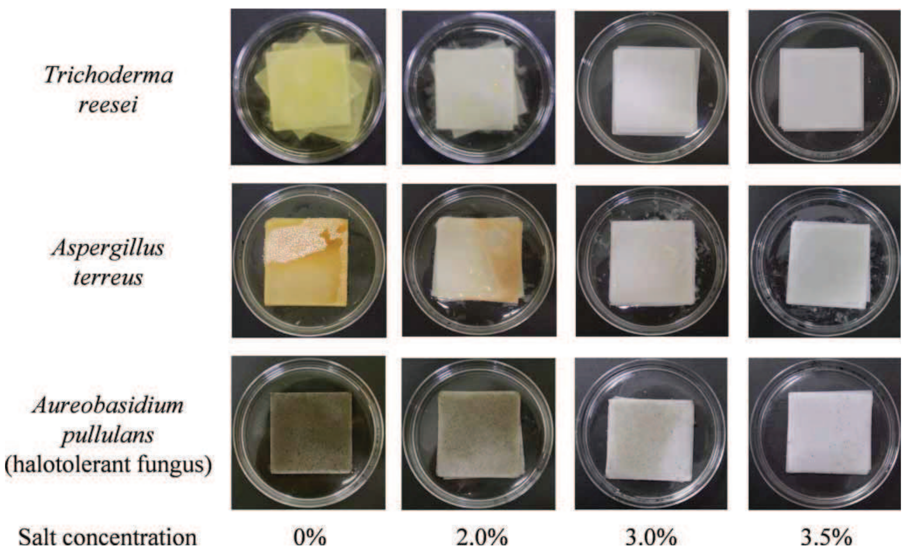

Figure 5 Photographs of copy paper samples at varied salt concentrations seven days after inoculation with fungal spores.

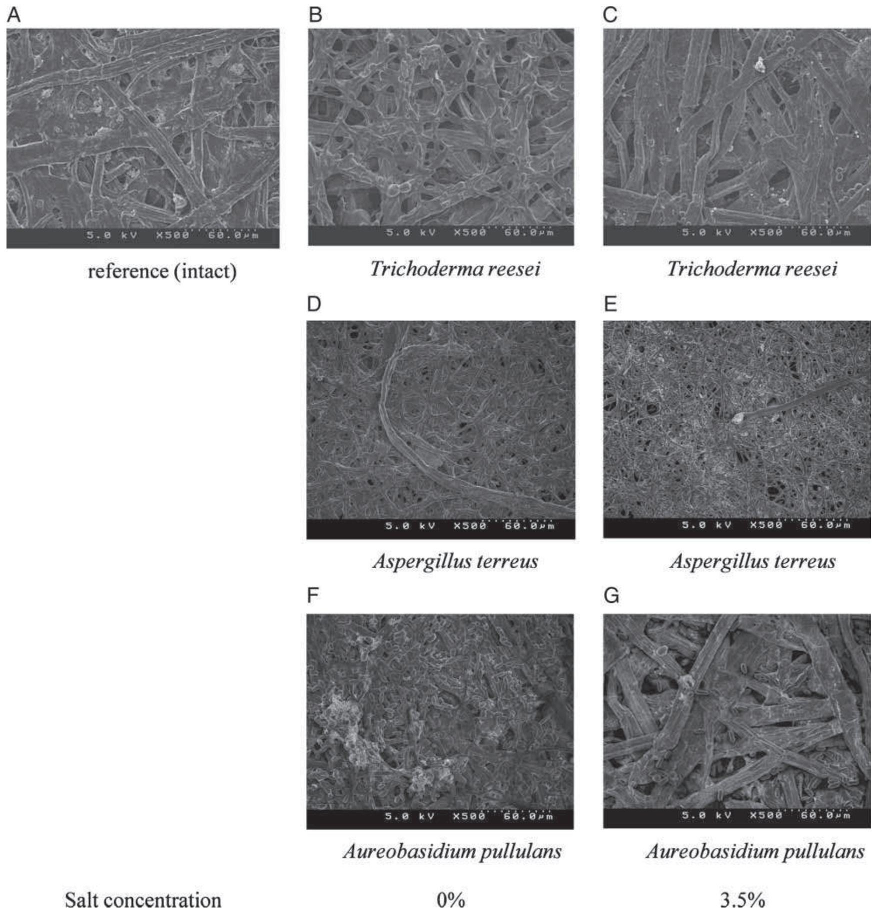

Figure 6 SEM images of copy paper: (A) intact copy paper; (B) and (C) *T. reesei*; (D) and (E) *A. terreus* at 0 and 3.5% salt concentration, respectively and (F) and (G) *A. pullulans* at 0 and 3.5% salt concentration, respectively.

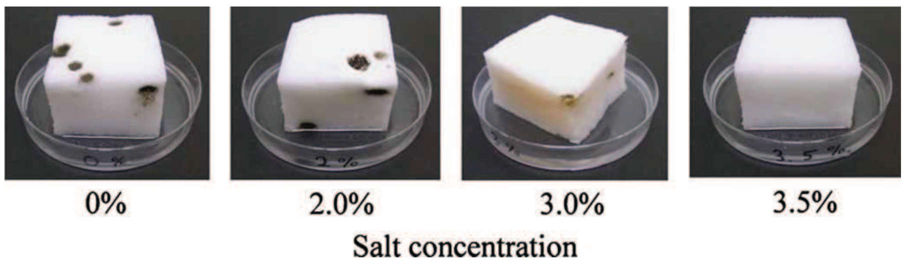

Figure 7 Photographs of cellulose sponge samples with fungal colonies after 24 days. The percentages beneath the photographs indicate the salt concentrations.

of paper. However, remarkable salt effects were observed.

At salt concentrations of 3.5% or greater, the effect of saltwater lasted for 24 days even in the optimum growth environment. Although some additives and pH of documents or book papers might have an influence on the result, this finding implies that fungal growth on flood-damaged paper would be considerably inhibited when it is immersed in saltwater having sufficiently higher concentrations.

The observed effect of salt in the tsunami-affected area and possible adverse effects of salts

Salt concentrations in seawater sampled near coasts were lower than those of average seawater in the open sea because of the inflow river water as shown in Table 3. Therefore, it is possible to claim that fungal growth would be inhibited to some degree on paper immersed in such seawater. To preserve flood-damaged paper in such cases, it would be better to use saltwater having sufficiently higher concentrations, for example, by preparing it from tap water and table salt (sodium chloride) or adding salt to diluted seawater.

In fact, when we investigated flood-damaged paper and paintings in Ofunato in the Tohoku region on April 23 and 24, 2011, no mold was observed in administrative document papers, books, and pictures at the Ofunato Municipal Sanriku Public Hall. In addition, no molds were found on a historical *sumi*-calligraphy pasted on a sliding door of the Chida family house (Fig. 8), although the sliding door was

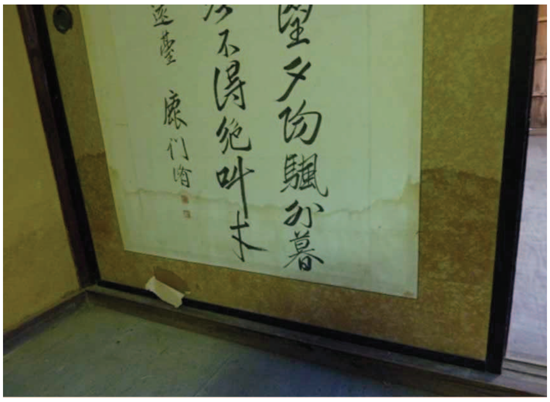

Figure 8 Photograph of historical *sumi*-calligraphy pasted on the sliding door of Chida family house.

in seawater up to approximately 40 cm from floor (as indicated by the water stain line) and was left untouched for 44 days until the photograph was taken. The temperature of the region was reported to be from −2.4 to 14.7°C in the meantime. The door and the documents are considered to have been actually wet for a few weeks. This was probably because of the effect of saltwater in addition to the effect of low temperature in the tsunami-affected area.

There are some possible problems with the saltwater method that require more research. One of them is physical damage to paper caused by growing salt crystallites as the paper dries. In this study, however, no salt crystals on dried paper were observed in the SEM images of Fig. 6. Crystallites tend to be small at high drying rates. In any case, in this saltwater method, desalination is essential before drying on a regular basis to avoid possible adverse effects of salt crystals.

Conclusion

The addition of artificial seawater salts into liquid media successfully inhibited the growth of *T. reesei* on microcrystalline cellulose at salt concentrations of 3.2% or greater. The critical salt concentration, 3.2%, is within the general seawater range. The effect of saltwater on the inhibition of *T. reesei* growth was also confirmed for salts other than sodium chloride. Although this growth-inhibiting effect mainly occurs because water is removed from cells due to osmotic pressure at high salt concentrations, physiological effects depending on the ion species used for salts are also a possible reason.

In the specific fungus culture experiment in a sterilized petri dish, the growth of *T. reesei*, *A. terreus*, and *A. pullulans* on paper was effectively inhibited with an increase in the salt concentration of the media by visual examination and only thin mycelia of *As. terreus* were observed using an SEM. In the experiment using a pure cellulose sponge substrate with enough oxygen and a liquid culture medium supplied, the growth of various fungi in air was completely inhibited by visual examination for 24 days at salt concentrations of artificial seawater of 3.5% or greater. The effect of saltwater was observed for 24 days in the cellulose culture that provided fungi with the optimum growth environment. This implies that fungal growth would be considerably inhibited on flood-damaged paper when it is immersed in saltwater of sufficiently higher concentrations. For this purpose, museums at risk should have a stock of salt available. This method using saltwater was found to be promising for effective conservation of flood-damaged paper and cellulosic materials. Further research on the influence of residual salts in paper after drying, paper adhesion, ink dissolution, desalination processes, and drying methods is required to establish a practical saltwater-based first aid for conserving flood-damaged paper.

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