

## Microbial deterioration of tsunami-affected paper-based objects

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### Abstract

Huge numbers of cultural objects were damaged by microorganisms after having been soaked with seawater from the tsunami that followed the Great East Japan Earthquake on March 11, 2011. Among the cultural objects that were damaged, paper-based objects in the tsunami area were severely affected. In the present study, on-site investigations and a culture-based analysis were carried out to determine the extent of the microbial deterioration of paper-based objects (i.e., administrative documents and historical documents). A unique discoloration, mainly black- and light red-spotted alterations produced by microbes, was observed on many paper-based objects that had been left wet for several months. We also observed the results of the adhesive tape method applied to the black-spotted areas on damaged documents: *Stachybotrys*, *Chaetomium* and *Cladosporium*. We identified dark-colored fungal isolates from black-spotted paper as *Stachybotrys chartarum*, which was observed to grow significantly in mineral salt medium supplemented with cellulose as the sole nutrient source; maximum growth was shown in 10% NaCl. Two isolates from light red-spotted paper were related to *Streptomyces* sp. and *Myxotrichum* sp., respectively. These isolates generated red pigments in the mycelia and on the culture plates. *Penicillium*-related isolates were dominant in the sample tested, and they showed a higher level of NaCl tolerance. With regard to the discoloration found on tsunami-affected paper-based objects that had remained wet for several months, *Stachybotrys* and *Streptomyces* sp. and *Myxotrichum* sp. seem to be responsible for the black- and light red-spotted paper alterations, respectively.

### Introduction

Many historical and cultural important objects are at risk of microbial deterioration, and no material is immune to microbial attack. Microorganisms have been shown to play a role in the deterioration of historic paintings, woods, paper, textiles, waxes, polymers and coatings, glass, metals, and stone (Mitchell and McNamara 2010). In most cases, the prevention of microbial deterioration aims at reducing the water surrounding the cultural objects, because the availability of water is considered the main factor for microbial activities (Caneva and Ceschin 2008). However, undesirable water from artificial accidents (e.g., pipe breaks, rain infiltration, air-conditioning system trouble) or unexpected natural disasters (e.g., floods, tsunamis, hurricanes) can enable microorganisms to colonize very rapidly on objects.

When the Great East Japan Earthquake triggered a tsunami in March 2011, a large number of cultural objects and documents were severely damaged at the Pacific coast side of the Tohoku region in Japan. The objects that were left wet for several months after having been soaked with seawater from the tsunami suffered significant microbial deterioration as well as direct tsunami damage (e.g., loss and breakage). Among the cultural objects damaged by the tsunami, typical alterations of paper by microbes (mainly black- and light red-spotted alterations) were commonly observed in tsunami-affected areas. The microbial deterioration of paper generally causes different types of damage, depending on the organisms responsible for the attack. Some paper-degrading fungi and bacteria are capable of dissolving cellulose fibers through the action of cellulolytic enzymes, and they may produce pigments or organic acids which cause serious damage to paper-based objects (Nyuksha

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1983, Ciferri et al. 2000, Michaelsen et al. 2009, Sterflinger and Pinzari 2012).

The present study focused on the microbial deterioration of tsunami-affected paper-based objects such as historical documents and administrative documents. Studies on the isolation, identification and characterization of microbes on tsunami- or other seawater-affected paper-based objects seem to be rare, and it is expected that the findings of such studies will provide fundamental information about both the initial conservation steps to take after disasters and the potential health risks to the individuals who salvage or restore the damaged objects. This report was written based on our previous paper published in the International Biodeterioration & Biodegradation (Sato et al. 2014).

## Materials and Methods

### *On-site investigation in the cities of Kamaishi and Rikuzentakata*

The cities of Kamaishi and Rikuzentakata, situated in the southeastern section of Iwate Prefecture in the Tohoku region, at the Pacific coast, were severely damaged by the earthquake and tsunami. A large number of administrative documents at the Kamaishi City Office were left wet for two months after having been soaked with seawater. The documents were moved from the basement of the Kamaishi City Office to the classrooms of the unused Kamaishi Daiichi junior high school building. The wet documents were then air-dried and stored temporarily in the classrooms. In the city of Rikuzentakata, administrative documents in the City Office remained wet and abandoned for over a year after the tsunami.

We carried out an on-site investigation of microbial deterioration of administrative documents on August 7–8, 2012 by visual observations and the adhesive tape method (Urzi and Leo 2001). With this simple adhesive tape method it is possible to obtain information about the morphology of microorganisms on an object and their relationship with the colonized material surfaces without damaging the object (Urzi and Leo 2001). In brief, a strip of transparent adhesive tape is gently attached to the surface of some deteriorating papers and then placed on a slide glass. The preparations were then brought back to the

laboratory and observed under a light microscope (BX53, Olympus, Tokyo) equipped with a charge-coupled device (CCD) camera (DP72, Olympus).

### *Microbial analysis of deteriorating paper samples*

#### *a) Sample collection*

Two types of deteriorating paper samples were obtained from tsunami-affected areas in the Tohoku region. One is Japanese paper in the underlining of a painted folding screen (the “IJP” sample, Fig. 1) which had been soaked with seawater by the tsunami at a private home in the city of Ishinomaki located in the northeastern section of Miyagi Prefecture. The other is a paper envelope (the “KEP” sample, Fig. 2) from the basement of the Kamaishi City Office mentioned above.



Fig.1 The Japanese paper in the underlining of a painted folding screen from a private home in the city of Ishinomaki (northeastern Miyagi Prefecture) (Sato et al. 2014).



Fig.2 A envelope paper from the basement of Kamaishi City Office (Sato et al. 2014).

#### *b) Light microscopic and scanning electron microscopic analysis*

For light microscopic observation, the KEP sample was mounted on a slide glass with a drop of

lactophenol cotton blue mounting medium. The preparations were sealed with nail polish and observed under a light microscope (BX53, Olympus) as described above. For the scanning electron microscopic (SEM) analysis, a small amount of the KEP sample was examined by SEM (model S-3700N; Hitachi, Tokyo) under low vacuum (LV) conditions inside the sample chamber.

*c) Fungal and bacterial isolation and morphological observations*

Fungal and bacterial strains were isolated from deteriorating spots of the KEP and IJP samples by using direct inoculation methods. Each paper sample was placed on half-strength cornmeal agar (CMA; BD, Franklin Lakes, NJ, USA), 8.5gL<sup>-1</sup>; agar (BD), 7.5gL<sup>-1</sup> and potato dextrose agar (PDA; BD, 39gL<sup>-1</sup>). Fungal and bacterial isolates were identified on the basis of cultural microscopic morphology with light microscope and on the basis of their specific gene sequences, as described in the next section.

*d) DNA extraction and PCR conditions of the 18S-26/28S internal transcribed spacer (ITS) region and 16S rRNA gene sequences*

Pure cultures of isolates were grown on the half-strength CM medium for 5–10 days at 25°C prior to DNA extraction. DNA was extracted using ISOPLANT (Nippon Gene, Toyama, Japan) per the manufacturer's instructions. We assessed the extracted DNA using standard electrophoresis through a 1% (w/v) agarose gel (Solana; Rikaken, Nagoya, Japan) followed by staining with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

Polymerase chain reaction (PCR) amplification was carried out in a reaction mixture containing 100ng of template DNA, *Ex Taq* buffer (Takara, Otsu, Japan), dNTPs (Takara), *Ex Taq* DNA polymerase (Takara) and each of the two primers. The PCR primers for the fungal 18S-26/28S internal transcribed spacer (ITS) region sequences were ITS1-F (Gardes and Bruns 1993) primer (5'-CTTGGTCAATTTAGAGGAAGTAA-3') and ITS4 (White et al. 1990) primer (5'-TCCTCCGCTTATTG ATATGC-3'), and those for bacterial 16S rRNA gene were 10F primer (5'-AGTTTGATATCCTGGCTCAG -3', corresponding to positions 10–27 of the *E. coli* 16S rRNA gene) and 1541R primer (5'-AAGGAGGTG ATCCAGCCG-3', positions 1524–1541). PCR was

done in a PCR Thermal Cycler Dice (Takara) using the following parameters: for fungi, 4 min at 94°C followed by 35 cycles each of 35 sec at 94°C, 55 sec at 52°C, and 2 min at 72°C and final extension at 72°C for 10 min; for bacteria, 5 min at 95°C followed by 25 cycles each of 30 sec at 95°C, 1 min at 60°C, and 1 min at 72°C.

*e) Sequencing and phylogenetic analysis*

We performed the sequencing of PCR products using a DNA sequencer (model 3130x, Applied Biosystems, Foster City, CA) and an ABI PRISMTM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The determined sequences were analyzed using Molecular Evolutionary Genetics Analysis software (MEGA, version 5.05) (Tamura et al. 2011) and aligned together with reference sequences obtained from the GeneBank, EMBL and DDBJ using the CLUSTAL W program included in MEGA 5.05. The evolutionary distance matrix of the aligned sequences was calculated using the two-parameter model for multiple substitutions (Kimura 1980). Phylogenetic trees were constructed using the software MEGA 5.05 based on the neighbor-joining method (Saitou and Nei 1987, Tamura and Nei 1993). The reliability of the tree topology was estimated by the bootstrap method (1,000 replicates, Felsenstein 1985).

*f) Sodium chloride (NaCl) tolerance and cellulose-utilizing capability*

The effect of sodium chloride (NaCl) on the growth of the isolates was determined in yeast-malt extract agar [yeast-malt extract agar (BD), 38gL<sup>-1</sup>] supplemented with a graded series of NaCl concentrations [0%, 1%, 2%, 3%, 4%, 5%, 7%, 10%, 13% (w/v)]. Five-day-old yeast-malt extract agar plate cultures were used to inoculate the plate of the yeast-malt extract agar-NaCl medium. Cultures were incubated for 4 weeks at 23°C. We determined the maximum NaCl concentration at which any growth occurred (i.e., the colony dia. was < 2 mm at 4 weeks).

For the examination of cellulose-utilizing capability, representative strains were cultured in 20-mL test tubes containing 5mL of mineral salt (MS) medium with a strip of quantitative filter paper (No. 5C; Advantec, Tokyo) as the sole nutrient source. The MS medium contained the following ingredients (mg

L<sup>-1</sup>): NaNO<sub>3</sub> (500), K<sub>2</sub>HPO<sub>4</sub> (1000), MgSO<sub>4</sub>·7H<sub>2</sub>O (500), KCl (500), and Fe(SO<sub>4</sub>)·7H<sub>2</sub>O (10), and the pH of the medium was adjusted to 7.3 (Dubos 1928). Growth was checked after a 4-week incubation at 23°C by looking for colonies that had increased in size.

## Results and Discussion

### *General tendency of the damage: the on-site investigation*

A photograph of the administrative documents stored at the Kamaishi Daiichi junior high school is given in Figure 3. Of the documents, 153 were taken at random for visual observation. In 83% (n=127) of the 153 documents, some type of microbial deterioration was detected, and in the other 17% (n=26) of the documents, no microbial growth was observed. These 26 documents had been situated on the top shelf or a second shelf of a cabinet when they were affected by the tsunami (Fig. 4). On the documents with microbial deterioration (n=127), a high degree of black- and light red-spotted alterations was observed (Fig. 5). The frequencies of the appearance of black- and light red-spotted alterations were 100% (n=127) and 97.6% (n=124), respectively. Fifteen samples were taken by the adhesive tape method from the black-spotted points on 15 documents selected at random, and observed under a light microscope. Ten samples showed the typical morphological characteristics of the genus *Stachybotrys*, three samples showed *Chaetomium*-like fungus, and two samples showed *Cladosporium*-



Fig.3 The administrative documents stored in the classrooms of the unused Kamaishi Daiichi junior high school building.



Fig.4 The administrative documents situated in a cabinet in the basement of the Kamaishi City Office.

like fungus (Fig. 6).

The administrative documents from the Rikuzentakata City Office were also subjected to the adhesive tape method. Fifteen samples from the black-spotted area on 15 randomly chosen documents were observed: *Stachybotrys*-like fungus, 11 samples; *Aspergillus niger*-like fungus, three samples; *Chaetomium*-like fungus, one sample (picture not shown).

*Stachybotrys*, *Chaetomium*, *Aspergillus* and *Cladosporium* are commonly found on paper-based objects (Valentín 2003), and some species belonging to these genera produce cellulase (Thomas 1956, Lakshmikanth and Mathur 1990, Abrha and Gashe



Fig.5 Black- and light red-spotted alterations on administrative documents from the Kamaishi City Office (Sato et al. 2014).

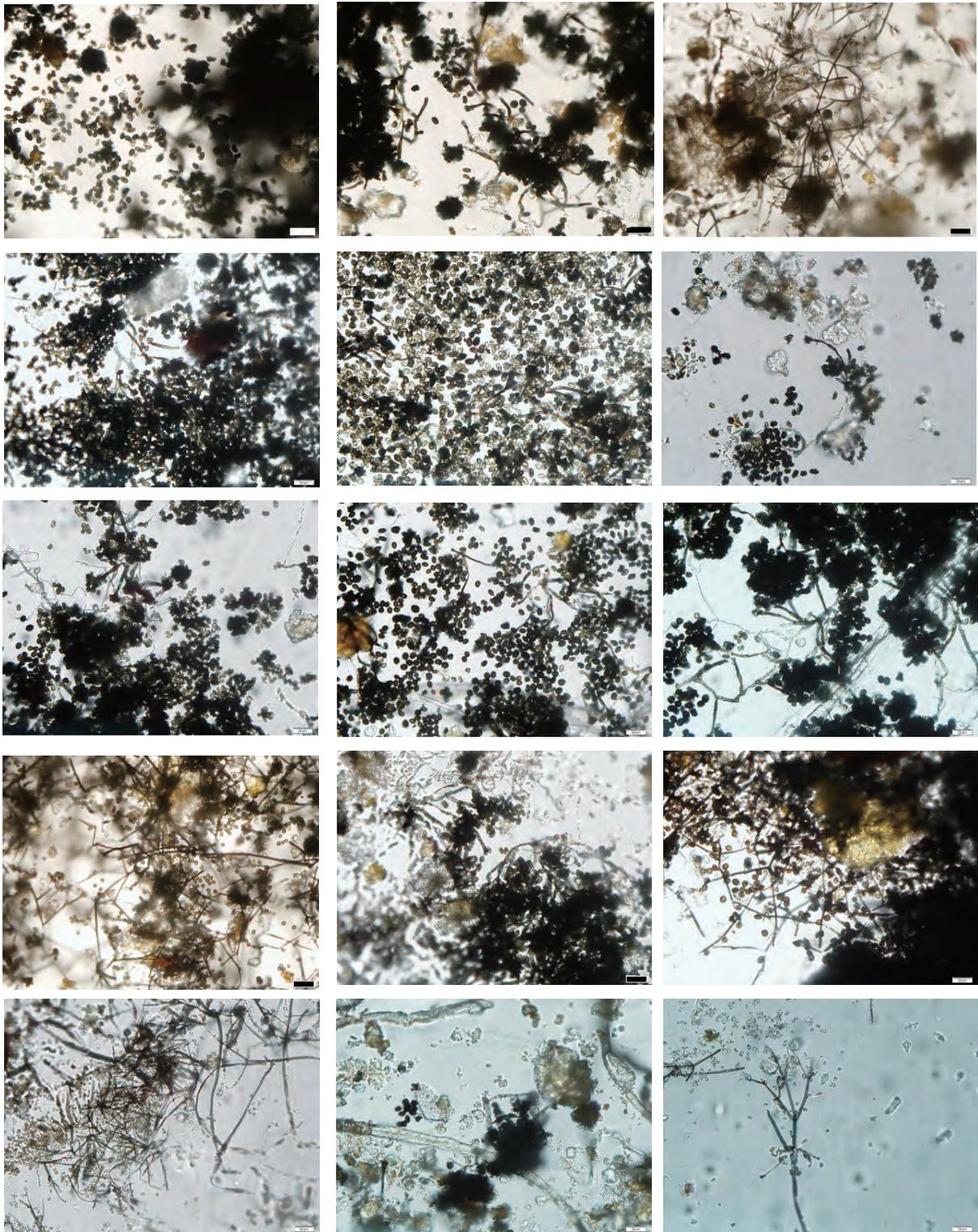


Fig.6 Light microscopic observation following the adhesive tape method of a black-spotted area of the administrative documents from the Kamaishi City Office building. Bars, 20 $\mu$ m.

1992). Although the numbers of samples observed were too small to make a sufficient assessment, fungal diversity colonized on the seawater-damaged paper appeared to be relatively low compared to freshwater-

damaged paper. The salts in seawater may suppress the growth of salt-sensitive microorganisms which could potentially colonize on wet paper-based objects (Higashijima et al. 2012).

### **Microscopic observations of collected sample**

Because the microbes seemed to be strongly attached to the damaged paper in the light red area, the sampling could not be done properly by the adhesive tape method. Therefore, a small number of samples of red spotted paper (from the KEP sample) was collected by light microscopy and LV-SEM. The light microscopic observation of a small amount of the KEP sample altered to light red revealed the presence of very thin mycelia along the paper fibers (Fig. 7). The mycelia contained light red pigments. When a small amount of a non-discolored area of the KEP sample was observed by light microscopy, no red-colored mycelia were observed but some achromatic mycelia were present on the paper fibers (picture not shown).

The LV-SEM analysis revealed that the mycelial filaments, most of which were less than 1 $\mu$ m in dia., were present on the paper fibers of the light red-spotted area of the KEP sample (Fig. 8). Considering their morphological characteristics, especially the



Fig.7 Light microscopic observation of the paper envelope from the basement of the Kamaishi City Office building. Bar, 20 $\mu$ m (Sato et al. 2014).

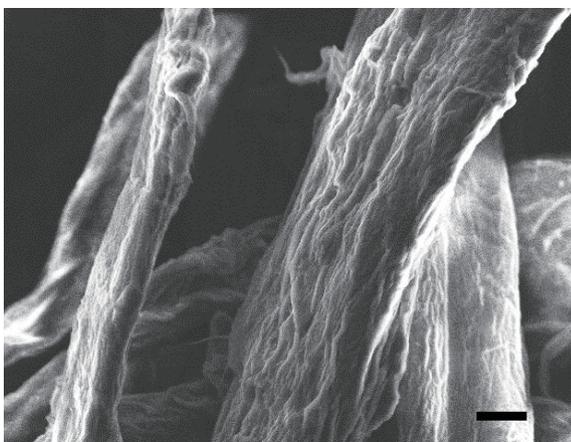


Fig.8 Micrograph of the paper envelope from the basement of Kamaishi City Office building, by SEM under low-vacuum conditions. Bar, 5 $\mu$ m (Sato et al. 2014).

size of their mycelia, it appeared that this was a filamentous bacterium belonging to the order *Actinomycetales*. The production of pigments as secondary metabolites is a well-known physiological characteristic of the species belonging to the order *Actinomycetales*. More specifically, red- and pink/purple-colored stains were reported to be produced by *Streptomyces* species of the order *Actinomycetales* (Karbowska-Berent and Strzelczyk 2000, Pinna and Salvadori 2005).

### **Isolation and identification from microbes deteriorated paper samples**

#### **a) Isolates from red-colored papers**

We selected two paper fragments from the KEP sample, one from a light red-spotted area and the other from a non-discolored area, for isolation. A total of 17 strains were isolated from the sample of the light red area, and these were fungi (16 isolates) and bacteria (1 isolate): nine *Penicillium echinulatum*-related isolates, five *Penicillium* sp. BMP3038-related isolates, one *Penicillium vancouverense*-related isolate, one *Myxotrichum deflexum*-related isolate, and one *Streptomyces fulvoviolaceus*-related isolate (Table 1). All of the 12 isolates from the samples of the non-discolored area were fungi and fell into three species of the genus *Penicillium*: seven *Penicillium chrysogenum*-related isolates, three *Penicillium* sp. BMP3038-related isolates, and two *Penicillium commune*-related isolates (Table 1).

These results showed that *Penicillium*-related isolates were dominant in both the light red-spotted area (88.9% of the isolates) and the non-discolored area (100% of isolates) of the KEP sample. With respect to red pigment production, none of the *Penicillium* isolates produced red pigment inside the mycelia or on culture media, although red-pigment production has been observed in some species of *Penicillium* (Curtis and Grove 1947, Méndez et al. 2011, Hailei et al. 2011).

In contrast, a bacterial strain, *Streptomyces fulvoviolaceus*-related strain KA-C5, and a fungal strain, *Myxotrichum deflexum*-related strain KA-C6A, isolated from the light-red area of the KEP sample produced significant amounts of red pigments in the mycelia and on the culture plates (Fig.9). In Figure 10, a phylogenetic tree based on the 16S rRNA gene sequences of the strain KA-C5 (approx. 1,450 bp) is

shown. Strain KA-C5 belongs to the genus *Streptomyces* and falls into one distinct subclade with *S. phaeochromogenes* NRRL B-1517 (GeneBank accession no. EU594474), *S. noboritoensis* NBRC 13065<sup>T</sup> (AB184287) and *S. miyazakiensis* NBRC 13791<sup>T</sup> (AB184482).

The isolate had 97.2% sequence similarity with *S. phaeochromogenes* NRRL B-1517, 97.2% similarity with *S. noboritoensis* NBRC 13065<sup>T</sup>, and 97.4% similarity with *S. miyazakiensis* NBRC 13791<sup>T</sup>. The isolate might be a new species, but further taxonomic studies are needed for an exact assignment of the isolate. The phylogenetic trees constructed with the 18S-26/28S ITS region sequences of the strain

KA-C6A and their phylogenetic relatives showed that this strain is related to *Myxotrichum* sp. BF38, *Myxotrichum* sp. 2008119 and *M. deflexum* UAMH6365 (Fig. 11). The *Myxotrichum deflexum* (*Eidamella deflexum* is an obsolete synonym of this species: Hambleton et al. 1998) belonging to the class Ascomycetes, grows slowly, forms yellowish-white to light gray colonies, often develops pink or wine red patches, and expresses a pink diffusible pigment on some media (Howard 2003). *M. deflexum* is commonly found on paper-based materials (Pasquariello et al. 2005) and is recognized as a chromogenous fungus (Nugari 2005).

With regard to the red alterations on

Table 1 Phylogenetic identification of fungal and bacterial strains isolated from a paper envelope\* (Sato et al. 2014)

Strain name	Closest BLAST match (accession no.)	Identity (%)
Light red-colored area		
KA-C1	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	99
KA-C2	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	100
KA-C5	<i>Streptomyces fulvoviolaceus</i> NBRC14148 (AB184573)	98
KA-C6A	<i>Myxotrichum deflexum</i> UAMH6365 (AF062814)	99
KA-C7	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	100
KA-C8	<i>Penicillium vancouverense</i> CBS126323 (JN617675)	100
KA-C9	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	99
KA-P1	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	99
KA-P2	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	100
KA-P3	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	100
KA-P4	<i>Penicillium</i> sp. BMP3038 (HQ832995)	99
KA-P5	<i>Penicillium</i> sp. BMP3038 (HQ832995)	100
KA-P6	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	100
KA-P7	<i>Penicillium</i> sp. BMP3038 (HQ832995)	100
KA-P8	<i>Penicillium</i> sp. BMP3038 (HQ832995)	100
KA-P9	<i>Penicillium echinulatum</i> JCM22836 (AB479316)	100
KA-P10	<i>Penicillium</i> sp. BMP3038 (HQ832995)	99
Non-discolored area		
KAC-C2	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
KAC-C8	<i>Penicillium chrysogenum</i> A096 (JQ015265)	99
KAC-C9	<i>Penicillium commune</i> P8.1 (EU833215)	99
KAC-P1	<i>Penicillium</i> sp. BMP3038 (HQ832995)	100
KAC-P2	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
KAC-P3	<i>Penicillium</i> sp. BMP3038 (HQ832995)	100
KAC-P4	<i>Penicillium</i> sp. BMP3038 (HQ832995)	100
KAC-P5	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
KAC-P6	<i>Penicillium commune</i> P8.1 (EU833215)	99
KAC-P7	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
KAC-P9	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
KAC-P10	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100

\* The paper, which had been left wet for two months after the March 2011 tsunami, was collected from the basement of the Kamaishi City Office building.

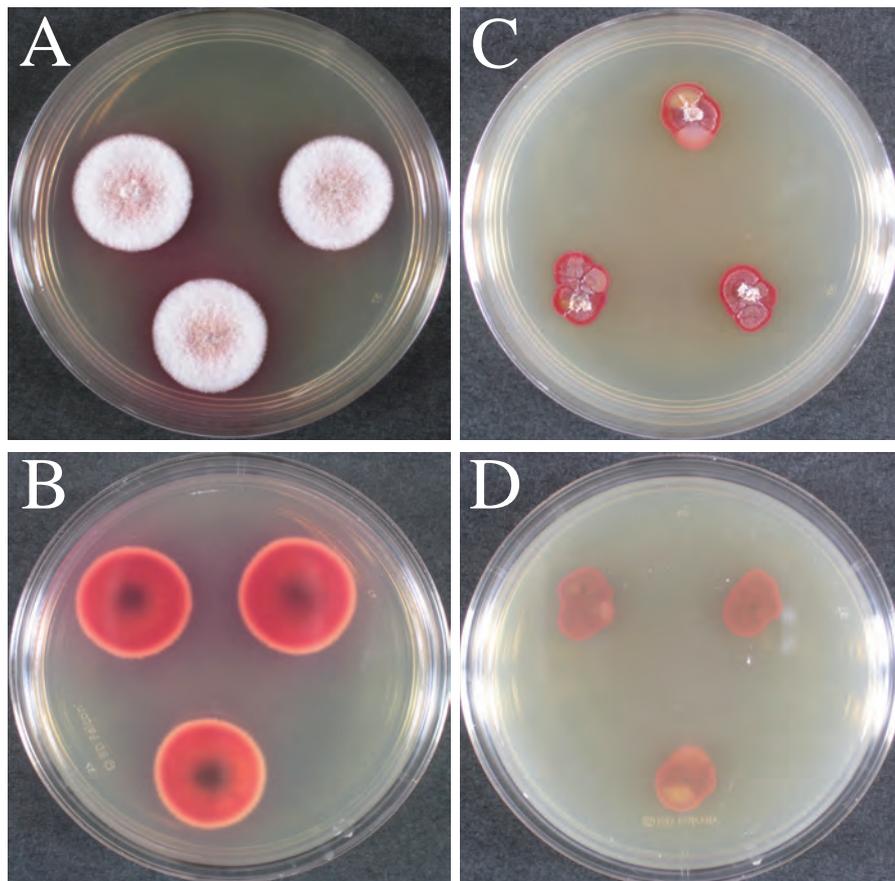


Fig.9 Colony morphology of strains KA-C6A (A and B) and KA-C5 (C and D) on half-strength cornmeal agar. Top row: the upper surface of the Petri dish. Bottom row: the lower surface (Sato et al. 2014).

administrative documents, the *Streptomyces* isolate seemed by microscopic analysis to be related to the red-colored filamentous bacteria observed on the paper in the light red-spotted area of the KEP sample. However, the *M. deflexum*-related strain KA-C6A also produced red pigments on culture media (Fig. 9). Further analyses—such as the detection of specific microorganisms by molecular approaches and microscopic observations with increased sample numbers—are necessary for linking certain microbial species to the red alteration.

#### b) Isolates from yellow and black-colored Japanese paper

Two fragments of paper from yellow- and black-altered areas were used for isolating microorganisms from the IJP sample. A total of 15 strains and four strains were isolated from yellow- and black-colored areas, respectively (Table 2). The 15 strains isolated from yellow areas were related to *Penicillium chrysogenum*, five isolates; *Geomyces destructans*, five isolates; *Penicillium solitum*, two isolates;

*Penicillium commune*, one isolate; *Aspergillus versicolor*, one isolate; *Penicillium citreonigrum*, one isolate (Table 2). Among the isolates, older colonies of *Geomyces*-related strains exhibited yellow exudates on the surface of the culture plates (picture not shown). The closest known relatives of the isolates was *Geomyces destructans* (97% sequence similarity), which causes white nose syndrome (WNS), an emerging disease in North America that has caused a substantial decline of hibernating bats (Gargas et al. 2009).

All four of the isolates from the black-colored areas of the sample were affiliated with *Stachybotrys* species and had 100% sequence similarity with their closest neighbor, *Stachybotrys chartarum* UAMH7900 (Table 2). A phylogenetic analysis based on 18S-26/28S ITS region sequences showed that the four isolates fell into the same clade with *S. chartarum* and *S. chlorohalonata* (Fig. 12). Morphological observation of the four isolates revealed that they had the typical characteristics of *S. chartarum* (Fig. 13). From these results, the four isolates were identified as

*S. chartarum*, which is ecologically known as a common soil fungus and to be cellulose-decomposing; it is frequently found on some paper-based materials as mentioned above. Moreover, *S. chartarum* has been known as a possible agent of a variety of health

effects, especially pulmonary hemorrhage, among occupants of water-damaged buildings (Centers for Disease Control and Prevention 1994, 1995, 1997) and has been shown to produce a number of mycotoxins, including satratoxins (Jarvis et al.

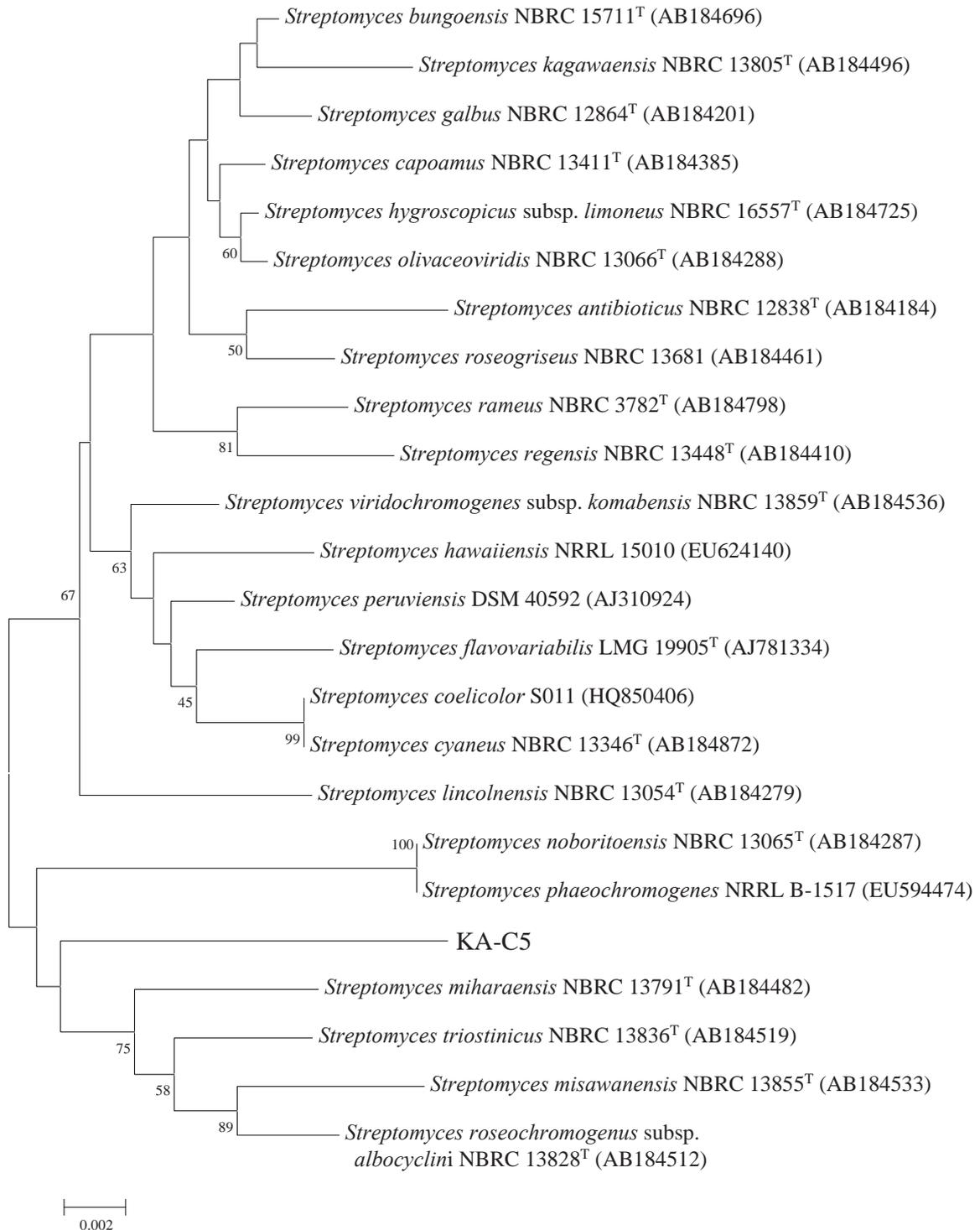


Fig.10 Neighbor-joining tree of *Streptomyces* sp.strain KA-C5 based on 16S rRNA gene sequences (approx.1,450 bp), including representatives of the most closely related strains and additional members of the genus *Streptomyces*.Bootstrap values above 50% are shown.Bar, 0.02 substitutions per nucleotide position (Sato et al. 2014).

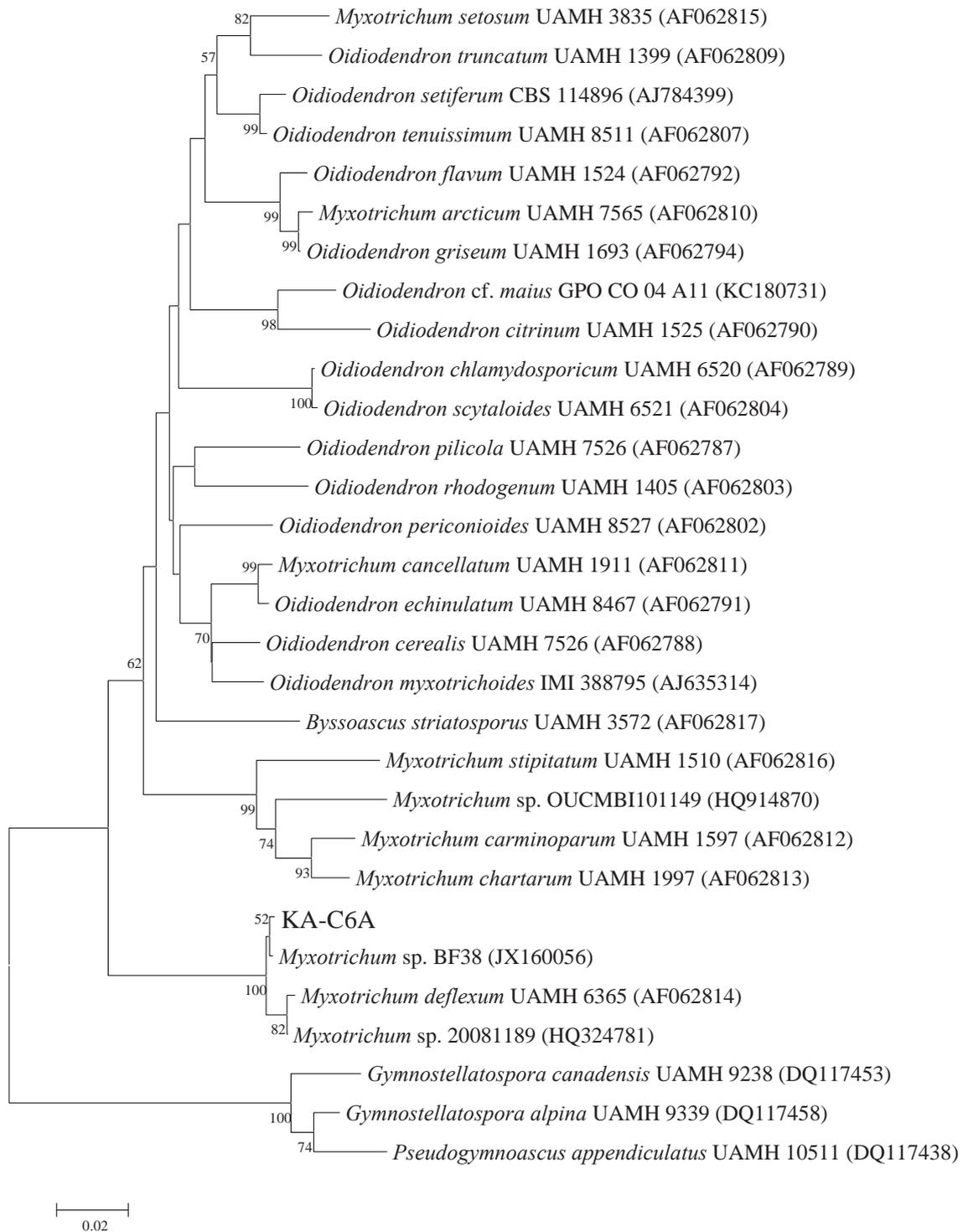


Fig.11 Neighbor-joining tree of *Myxotrichum* sp.strain KA-C6A based on 18S-26/28S internal transcribed spacer region sequences (approx.470 bp), including representatives of the most closely related strains and additional members of the related genus.Bootstrap values above 50% are shown.Bar, 0.02 substitutions per nucleotide position (Sato et al. 2014).

1998).

#### **Physiological characteristics of the isolates**

Sodium chloride (NaCl) tolerance was tested for 48 species isolated from the KEP and IJP samples in

the range of 0% to 13% (w/v) NaCl (Table 3). All of the *Penicillium*-related isolates (77% of all of the isolates) and the single *Aspergillus versicolor*-related isolate grew even at the highest concentration of NaCl in this test, 13% NaCl. These strains are thus thought

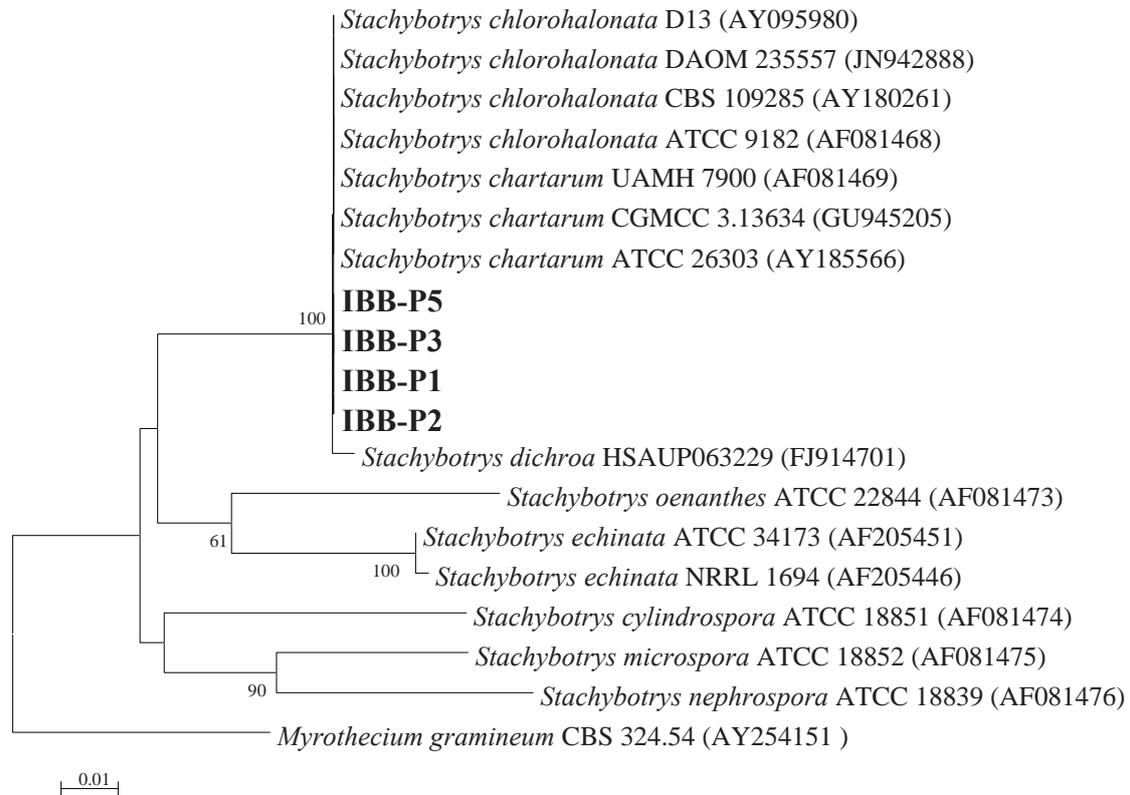


Fig.12 Neighbor-joining tree of *Stachybotrys* isolates based on the 18S-26/28S internal transcribed spacer region sequences (approx.470 bp), including representatives of the most closely related strains and additional members of the genus *Stachybotrys*. Bootstrap values above 50% are shown. Bar, 0.01 substitutions per nucleotide position (Sato et al. 2014).



Fig.13 Light microscopic photograph of *Stachybotrys chartarum* strain IBB-P1. Bar, 10 $\mu$ m.

to tolerate higher concentrations of NaCl, and thus a retest using higher concentrations of NaCl is necessary with these strains. The *S. chartarum* isolates and *G. destructans*-related isolates could tolerate 10% NaCl as the maximum; the *M. deflexum*-related isolate could grow at the maximum of 4% NaCl. The *Streptomyces*-related isolate could not tolerate 2% NaCl.

A survey of NaCl tolerance has been done for terrestrial fungi selected from the major taxonomic classes by Tresner and Hayes (1971). In their survey, species of *Penicillium* and *Aspergillus* were notably the most resistant to high concentrations of NaCl. The majority of the species were able to grow even in the presence of 20% or higher NaCl (Tresner and Hayes 1971). It can be inferred that environmental factors, especially NaCl derived from seawater, influenced the predominance of the *Penicillium* species on the damaged paper.

Because three strains (*S. chartarum* IBB-P1, *M. deflexum*-related strain KA-C6A, and *Streptomyces*-related strain KA-C5) seem to be responsible for the very characteristic feature of deterioration on tsunami-affected paper (i.e., black- and light red-discolorations), we selected them as representative strains for a further physiological experiment to determine the strains' cellulose-utilizing capability. *S. chartarum* strain IBB-P1 showed remarkable growth on and degradation of a strip of the quantitative filter paper, whereas the *M. deflexum*-related strain KA-C6A and the *Streptomyces*-related strain KA-C5 could not grow in

the mineral salt medium supplemented with cellulose as the sole carbon source after a 4-week incubation (Fig. 14).

## Conclusions

Our on-site investigation of microbial deterioration revealed that marked black- and light red-spotted alterations were observed as typical microbial discoloration of the seawater-soaked, paper-based objects left wet for several months in tsunami-affected areas. We used the adhesive tape method on 15 randomly selected black-spotted areas on the damaged documents, and *Stachybotrys*, *Chaetomium* and *Cladosporium* were identified. These fungi would be related to paper degradation, because some species belonging to these genera produce cellulase as one of their metabolic products. All four of the isolates from a small amount of black-altered samples were identified as *S. chartarum* by their phylogenetic and morphological characteristics. A representative isolate of *S. chartarum* strain IBB-P1 showed significant growth with filter paper as the sole

nutrient source. It may be concluded that *S. chartarum* was one of the key species causing the black alterations found on tsunami-affected, paper-based objects left wet for several months.

With regard to the light red alteration, microscopic observations of collected samples showed the presence of red-colored mycelia on the paper's fibers, which is thought to be filamentous bacteria belonging to the order *Actinomycetales*. From the results of the isolation and identification of microbes colonizing on the red-altered paper samples, we isolated the red pigment-producing microbes *Streptomyces* sp. and *Myxotrichum* sp. These microbes seemed to be involved in the red-spotted alteration on the damaged paper, although further investigations are still needed to elucidate the relationship between these microbes and such unique discoloration.

The *Penicillium*-related isolates were dominant on all of the paper samples tested in this study. All of the *Penicillium* isolates showed a higher level of NaCl tolerance. Although the *Penicillium*-related isolates did not seem to be directly related to the discoloration examined in this study, the NaCl tolerance of these isolates might be advantageous for the high salt conditions on tsunami-affected, paper-based objects.

Finally, 'salt stress' is thought to contribute to the growth suppression of salt-sensitive microorganisms which could potentially colonize on wet paper. However, we found that the objects that were left wet for several months after having been soaked with seawater suffered significant microbial deterioration. In light of our observations, it would appear that the most effective initial responses to conserve paper documents that have been damaged by seawater are rapid air-drying or freezing and subsequently drying in a vacuum, if possible.

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Fig.14 Growth of *Stachybotrys chartarum* strain IBB-P1 in the mineral salt medium supplemented with cellulose as a sole carbon source after 4-week incubation (B, C). A fungus-free paper tube served as the negative control (A).

Table 2 Phylogenetic identification of fungal strains isolated from Japanese paper in the underlining of a painted folding screen at the private home in the city of Ishinomaki, Japan (Sato et al. 2014)

Strain name	Closest BLAST match (accession no.)	Identity (%)
Yellow-colored area		
IBY-C1	<i>Penicillium solitum</i> 20-01 (JN642222)	100
IBY-C2	<i>Penicillium solitum</i> 20-01 (JN642222)	100
IBY-C3	<i>Penicillium commune</i> aurim1124 (DQ093692)	100
IBY-C4	<i>Geomyces</i> sp. F12 (JF439475)	100
IBY-C5	<i>Aspergillus versicolor</i> UOA/HCPF8709 (FJ878627)	100
IBY-C6	<i>Geomyces</i> sp. F12 (JF439475)	100
IBY-C7	<i>Geomyces</i> sp. F12 (JF439475)	99
IBY-C8	<i>Geomyces</i> sp. F12 (JF439475)	99
IBY-P1	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
IBY-P2	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
IBY-P3	<i>Penicillium citreonigrum</i> 35 (EU497942)	100
IBY-P4	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
IBY-P5	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
IBY-P6	<i>Penicillium chrysogenum</i> A096 (JQ015265)	100
Black-colored area		
IBB-P1	<i>Stachybotrys chartarum</i> UAMH7900 (AF081469)	100
IBB-P2	<i>Stachybotrys chartarum</i> UAMH7900 (AF081469)	100
IBB-P3	<i>Stachybotrys chartarum</i> UAMH7900 (AF081469)	100
IBB-P5	<i>Stachybotrys chartarum</i> UAMH7900 (AF081469)	100

Table 3 The effect of sodium chloride (NaCl) on the growth of multiple strains isolated from tsunami-affected papers (Sato et al. 2014).

Taxonomical relative of isolates	Total no. of isolates tested	NaCl [% (w/v)]									
		0	1	2	3	4	5	7	10	13 or >	
KEP* sample: light red-colored area											
<i>Penicillium echinulatum</i>	9	+	+	+	+	+	+	+	+	+	+(9)
<i>Penicillium</i> sp. BMP3038	5	+	+	+	+	+	+	+	+	+	+(5)
<i>Penicillium vancouverense</i>	1	+	+	+	+	+	+	+	+	+	+
<i>Myxotrichum deflexum</i>	1	+	+	+	+	+	-	-	-	-	-
<i>Streptomyces fulvoviolaceus</i>	1	+	+	-	-	-	-	-	-	-	-
KEP sample: non-discolored area											
<i>Penicillium chrysogenum</i>	7	+	+	+	+	+	+	+	+	+	+(7)
<i>Penicillium</i> sp. BMP3038	3	+	+	+	+	+	+	+	+	+	+(3)
<i>Penicillium commune</i>	2	+	+	+	+	+	+	+	+	+	+(2)
IBJ** sample: yellow-colored area											
<i>Penicillium chrysogenum</i>	5	+	+	+	+	+	+	+	+	+	+(5)
<i>Geomyces destructans</i>	5	+	+	+	+	+	+	+	+	+	+(5) -
<i>Penicillium solitum</i>	2	+	+	+	+	+	+	+	+	+	+(2)
<i>Penicillium commune</i>	1	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus versicolor</i>	1	+	+	+	+	+	+	+	+	+	+
<i>Penicillium citreonigrum</i>	1	+	+	+	+	+	+	+	+	+	+
IBJ sample: black-colored area											
<i>Stachybotrys charutrum</i>	4	+	+	+	+	+	+	+	+	+	+(4) -

\* KEP: a paper envelope from the basement of the Kamaishi City Office.

\*\* IBJ: Japanese paper in the underlining of a painted folding screen from a private home in the city of Ishinomaki, Japan.

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