Bioremediation: How Microorganisms Are Used to Clean Up Our Mess

Overview
This lesson serves as an introduction to the topic of bioremediation. Bioremediation is the use of living things, specifically microorganisms like bacteria and fungi, to break down toxic substances (Aliotta & Colley, 2013). Students will view and summarize various resources related to bioremediation, its applications, and its limitations, then construct an answer to an overarching question: How might we use bioremediation to help fix environmental problems caused by humans?

Key Search Words
bioremediation, environment, climate change, biodiversity

Learning Objectives
After completing this lesson, students will be able to
- Define bioremediation
- Describe current and future bioremediation methods to address human impacts on the environment

Curriculum Alignment
NGSS
- HS-LS2-7 - Design, evaluate, and refine a solution for reducing the impacts of human activities on the environment and biodiversity.
- Science and Engineering Practices: Constructing Explanations and Designing Solutions

Classroom time required
50 minutes

Materials & Technology
- computers for student research
- chart paper/poster board
- markers
- Graffiti Board Teacher Guide
- Student Resource List

Safety
This activity uses common, everyday materials typically found in a classroom. Students should take all typical safety precautions, such as moving carefully around the room.

Teacher Preparation for Activity
To learn more about bioremediation, including the disadvantages and limitations of bioremediation, visit the International Information Center for Geotechnical Engineers.

Student Preparation for Activity
Before completing this lesson, students should have a basic understanding of how human activity impacts the environment. If the teacher chooses to use scientific journal articles as student resources, it would be helpful for students to have some experience reading and interpreting these types of articles.

Procedure

Lesson Activities	Approximate Time Required
Activating Strategy The teacher will… 1. Divide students into small groups (3-4 students per group). 2. Read the following Background Information aloud to students. *(Note: this is a fictional story, but may be a good time to discuss large oil spills that actually happened, like the Exxon Valdez or Deepwater Horizon spills.)* "First, I noticed that we kept pulling up dead or almost dead fish. Our nets were full of them. I knew something was seriously wrong. But soon I noticed the shine - the surface of the water had a pretty iridescent sheen to it. That's when I knew. It was oil." This quote is from a deep-sea fisherman who fishes in the Gulf of Mexico near New Orleans, LA. He noticed the oil on the water recently and made a call to the Louisiana Department of Environmental Quality, which in turn contacted the US Environmental Protection Agency (EPA). State and federal authorities investigated the source of the oil, and it has been determined that a small explosion on an off-shore oil drilling rig has caused a massive oil leak into the gulf. How will the spill be contained? How will the oil be cleaned up? 3. Ask students to brainstorm the following question with their group: **Imagine you are a scientist working for the US Environmental Protection Agency (EPA). *What are some ways you might attempt to remove the oil from the ocean water?*** 4. Ask students to share their ideas with their group. 5. Instruct students to research "bioremediation" and jot down a definition in their own words. Ask students to share their definitions with their group. 6. Explain to students that one possibility for cleaning up the oil is through bioremediation - specifically, the use of bacteria that produce enzymes that can break down oil into nontoxic products.	15 minutes
Lesson Activity The teacher will… 1. Distribute chart paper and markers to each group. 2. Explain to students that they will be conducting research to answer the following Big Question, which they should write at the center of their chart paper: **How might we use bioremediation to help fix environmental problems caused by humans?** 3. Guide students through the Bioremediation Graffiti Board activity. 4. Ask students to share their final answer to the Big Question.	25 minutes
Conclusion The teacher will… 1. Lead a discussion of answers that student groups developed. Possible discussion and extension questions include: a. As an EPA scientist, how would you explain to your boss what bioremediation is and why it would be a good idea to use it to clean up the Gulf of Mexico oil spill? b. What are some other environmental issues that we might use bioremediation techniques to combat? c. What are some of the disadvantages of bioremediation techniques? How do you think we could deal with these? d. Do you think bioremediation techniques will become more common in the coming years? Why or why not? e. Researchers at North Carolina State University have been working on using fungi attached to nanofibers to remove heavy metals from water (Park et al., 2020). What are some sources of heavy metal contamination in drinking water? Why is it a concern? What are the current techniques used to remove heavy metal contaminants from water? Why do you think these researchers are interested in using fungi?	10 minutes

Differentiation
Due to the varying difficulty levels of the resources, the teacher may choose to assign resources to students or edit the provided resource list.

Assessment/Check for Understanding
After completing the Graffiti Board activity, students will work in groups to develop and present a novel bioremediation approach to solve an environmental problem. The teacher may choose to assign specific problems (such as greenhouse gas emissions, lead in water, etc.) or allow students to choose a problem.

Required resources
- computers for student research
- chart paper/poster board
- markers
- Graffiti Board Teacher Guide
- Student Resource List

Sources
This lesson was adapted from this Graffiti Gist activity, originally by Abbey Futrell.

Rowell, L. (n.d.). *Graffiti Gist Activity*. Online and Blended Learning. https://sites.google.com/view/blendlearn/graffiti-gist-activity

Teacher Information:

Aliotta, S., & Colley, J. (2013, January 8). *Bioremediation*. The International Information Center for Geotechnical Engineers. https://www.geoengineer.org/education/web-class-projects/cee-549-geoenvironmental-engineering-winter-2013/assignments/bioremediation

Suggested student resources:

Bose, P. (2021, June 15). *Bioremediation: An Overview*. AZO Life Sciences. https://www.azolifesciences.com/article/Bioremediation-An-Overview.aspx

Chiu, S.-Y., Kao, C.-Y., Huang, T.-T., Lin, C.-J., Ong, S.-C., Chen, C.-D., Chang, J.-S., & Lin, C.-S. (2011). Microalgal biomass production and on-site bioremediation of carbon dioxide, nitrogen oxide and sulfur dioxide from flue gas using Chlorella sp. cultures. *Bioresource Technology*, *102*(19), 9135–9142. https://doi.org/10.1016/j.biortech.2011.06.091

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Lovett, B. (2020, April 9). *How Microbes Help Us Reclaim Our Wastewater*. American Society for Microbiology. https://asm.org/Articles/2020/April/How-Microbes-Help-Us-Reclaim-Our-Wastewater

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Park, Y., Liu, S., Gardner, T., Johnson, D., Keeler, A., Ortiz, N., Rabah, G., & Ford, E. (2020). Biohybrid nanofibers containing manganese oxide–forming fungi for heavy metal removal from water. *Journal of Engineered Fibers and Fabrics*. https://doi.org/10.1177/1558925019898954

Philp, R. (2015, December 8). *Bioremediation: The Pollution Sollution?*. Microbiology Society. https://microbiologysociety.org/blog/bioremediation-the-pollution-solution.html

Appendices

In addition to the Graffiti Board Teacher Guide, the text-based student resources can be found here.

Bioremediation Graffiti Board Teacher Guide

This activity is designed to be completed in steps as a whole group. The teacher will read or project the instructions for a step with all groups, allow each group to complete that step, then move on to the next step.

Students should be provided with a list of resources to choose from. A list of suggested resources is included here, but the teacher may choose to find additional resources to add to the list, replace any that may no longer be available, or choose resources that are more appropriate for their students. An electronic version of the list may be shared, or print copies of each resource can be provided (this will limit the provided list to text-only resources). Students do not necessarily need to read or watch the entire resource.

The teacher may choose to allot a specific amount of time for each step, or wait until all students have completed the step before moving on. Before beginning the activity, each student should choose ONE resource from the provided list. They should also identify a section or corner of their group's poster that will "belong" to them for this activity.

Students instructions:

Step 1: Write the Big Question at the center of your poster.

Step 2: **Read** or **watch** your chosen resource. As you read or watch, jot down notes in your corner of the poster.

Step 3: **Summarize** the overall message or importance of your chosen resource in one statement. Write and underline your statement in your corner of the poster.

Step 4: **Share** your resource with your group. In 60 seconds or less, each group member should summarize their resource and explain why they chose their underlined statement. As each group member shares, the other members will **listen** and take notes in their own corner of the poster.

Step 5: As a group, choose and **circle** the seven or eight most important words on the poster. These words may come from your statements *or* your notes.

Step 6: Combine the words you circled to compose an answer to the Big Question:

How might we use bioremediation to help fix environmental problems caused by humans?

This activity was adapted from this Graffiti Gist activity, originally by Abbey Futrell.

Bioremediation Graffiti Board Suggested Resource List

Name of Resource	Format
1. Bioremediation: The Pollution Solution?	Blog Post
2. Bioremediation: An Overview *includes disadvantages of bioremediation*	Web Article
3. Bioremediation of steel plant emissions using algae	Scientific Journal Article
4. Oil-Eating Bacteria Could Be a Solution to Spill Cleanups	Video
5. Using fungi and nanofibers to remove heavy metals from water	Scientific Journal Article
6. Community Guide to Bioremediation	Informational Poster
7. How Microbes Help Us Reclaim Our Wastewater	Web Article
8. How worms could help solve plastic pollution	Video

Resource 1:

BIOREMEDIATION: THE POLLUTION SOLUTION?

Posted on December 8, 2015 by Rebecca Philp

Over the past few months, Rebecca Philp, a PhD student from the Pirbright Institute, has been working at the Microbiology Society as our Public Affairs intern. While researching for a policy briefing, Rebecca learnt a lot about bioremediation. She explains a little about it in this blog.

Our Pollution Problem

The global population continues to rise at an astonishing rate, with estimates suggesting it will be in excess of 9 billion in 2050. The intensive agricultural and industrial systems needed to support such a large number of people will inevitably cause an accumulation of soil, water and air pollution. Estimates have attributed pollution to 62 million deaths each year, 40% of the global total, while the World Health Organization (WHO) have reported that around 7 million people are killed each year from the air they breathe. Water systems fare little better, with an estimated 70% of industrial waste dumped into surrounding water courses. The world generates 1.3 billion tonnes of rubbish every year, the majority of which is stored in landfill sites or dumped into the oceans.

We need to control our pollution; thankfully, microbes might be the answer.

Micro-organisms are well known for their ability to break down a huge range of organic compounds and absorb inorganic substances. Currently, microbes are used to clean up pollution treatment in processes known as 'bioremediation'.

The Invisible Workforce

Bioremediation uses micro-organisms to reduce pollution through the biological degradation of pollutants into non-toxic substances. This can involve either aerobic or anaerobic micro-organisms that often use this breakdown as an energy source. There are three categories of bioremediation techniques: *in situ* land treatment for soil and groundwater; biofiltration of the air; and bioreactors, predominantly involved in water treatment.

Soil

Industrial soils can be polluted by a variety of sources, such as chemical spillages, or the accumulation of heavy metals from industrial emissions. Agricultural soils can become contaminated due to pesticide use or via the heavy metals contained within agricultural products.

A visible example of where bioremediation has been used to good effect can be found in London's Olympic Park. The grounds that held the 2012 Olympics had previously been heavily polluted, after hundreds of years of industrial activity. Bioremediation cleaned 1.7 million cubic metres of heavily polluted soil to turn this brownfield site into one containing sports facilities surrounded by 45 hectares of wildlife habitats. Groundwater polluted with ammonia was cleaned using a new bioremediation technique that saw archaeal microbes breaking down the ammonia into harmless nitrogen gas. The converted park marked the London 2012 Olympic and Paralympic Games as the "greenest" and most sustainable games ever held, only possible with bioremediation techniques.

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Microbe Talk: The impact of COVID on research and career progression

While some soil cleaning techniques require the introduction of new microbes, 'biostimulation' techniques increase natural degradation processes by stimulating the growth of microbes already present. Natural biodegradation processes can be limited by many factors, including nutrient availability, temperature, or moisture content in the soil. Biostimulation techniques overcome these limitations, providing microbes with the resources they need, which increases their proliferation and leads to an increased rate of degradation.

Cleaning up oil-polluted soil is an example of where stimulating microbial growth can be used to good effect. Research has shown that poultry droppings can be used as a biostimulating agent, providing nitrogen and phosphorous to the system, which stimulates the natural growth rate of oil-degrading bacteria. Systems like these may prove cheaper and more environmentally friendly than current chemical treatment options.

Air

Air is polluted by a variety of volatile organic compounds created by a range of industrial processes. While chemical scrubbing has been used to clean gases emitted from chimneys, the newer technique of 'biofiltration' is helping to clean industrial gases. This method involves passing polluted air over a replaceable culture medium containing micro-organisms that degrade contaminates into products such as carbon dioxide, water or salts. Biofiltration is the only biological technique currently available to remediate airborne pollutants.

Water

In the UK, access to clean, potable water and modern sanitation is something we take for granted. However, there are billions of people on Earth for which this is a luxury. The WHO estimate that each year 842,000 people die as a result of diarrhoeal diseases, many of which could be prevented if they had access to clean water and proper sanitation. Around 2.6 billion people lack any sanitation, with over 200 million tons of human waste untreated every year.

Sewage treatment plants are the largest and most important bioremediation enterprise in the world. In the UK, 11 billion litres of wastewater are collected and treated everyday. Major components of raw sewage are suspended solids, organic matter, nitrogen and phosphorus.

Wastewater entering a treatment plant is aerated to provide oxygen to bacteria that degrade organic material and pollutants. Microbes consume the organic contaminants and bind the less soluble fractions, which can then be filtered off. Toxic ammonia is reduced to nitrogen gas and released into the atmosphere.

The Future

Bioremediation is not a new technique, but as our knowledge of the underlying microbial reactions grow, our ability to use them to our advantage increases. Frequently, bioremediation requires fewer resources and less energy than conventional technology, and doesn't accumulate hazardous by-products as waste. Bioremediation has technical and cost advantages, although it can often take more time to carry out than traditional methods.

Bioremediation can be tailored to the needs of the polluted site in question and the specific microbes needed to break down the pollutant are encouraged by selecting the limiting factor needed to promote their growth. This tailoring may be further improved by using synthetic biology tools to pre-adapt microbes to the pollution in the environment to which they are to be added.

Pollution is a threat to our health and damages the environment, affecting wildlife and the sustainability of our planet. Damage to our soils affects our ability to grow food, summarised in our policy briefing on Food Security. Bioremediation can help to reduce and remove the pollution we produce, to provide clean water, air and healthy soils for future generations.

Resource 2:

Bioremediation: An Overview

Download PDF Copy

By Dr. Priyom Bose, Ph.D.
Reviewed by Emily Henderson, B.Sc.

Bioremediation is a technology that employs living organisms such as bacteria and fungi to remove harmful contaminants and toxins from the environment. This reliable and eco-friendly approach has rapidly gained popularity in environmental research. Scientists have been successful in developing different bioremediation techniques to restore contaminated sites.

Bioremediation. Image Credit: OpturaDesign/Shutterstock.com

Among various other applications, this technology is used to clean up oil spills and contaminated groundwater. The microbes used in this technology can be both indigenous or non-indigenous. These microorganisms take part in the degradation, immobilization, and detoxification of various harmful chemical wastes and contaminants.

The main mechanism behind this process is to detoxify, reduce, degrade, or transform toxic substances into less toxic substances. The microbes used for bioremediation solely depend on the nature of the contaminants, for example, type of pesticides, agrochemical, xenobiotic compounds, heavy metals, plastics, organic halogens, greenhouse gases, etc. This technology is also used to process nuclear waste.

Principles of Bioremediation

The main principle behind biotechnology is the use of living organisms, especially microbes, in remediating harmful contaminants to lesser toxic substances. This technology uses bacteria, fungi, and/or plants to detoxify or degrade hazardous substances from the environment. The living organisms convert pollutants via their metabolic processes (e.g., synthesis of enzymes). This technology involves several microbes to degrade pollutants from the contamination site.

Factors of Bioremediation

Several environmental factors such as type of soil, pH, temperature, nutrients, the presence of oxygen or other electron acceptors in the soil, and the type of microbial population present in a particular contamination site play an important role in the rate of bioremediation. Thereby, for the best bioremediation process, optimization of these conditions is important. Some of the factors are discussed in details below:

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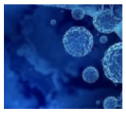
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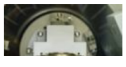
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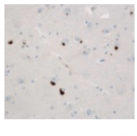
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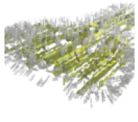
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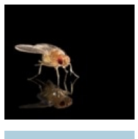
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Environmental Factors

Microbial growth depends on several environmental factors such as pH, temperature, moisture, and nutrients. Scientists practice biostimulation that involves the addition of nutrients to the contamination site which enhances the growth of microbes that assist in bioremediation.

Most of the microbes grow best at optimal conditions, thereby, amending those nutritional or biochemical conditions (e.g., pH, temperature, etc.) would aid in the growth of particular microbes. Scientists deploy different processes to alter the growth conditions, for example, if a soil is too acidic (low pH), the condition can be reversed by adding lime.

Microbial Populations for Bioremediation Processes

Microbes such as bacteria and fungi can survive varied temperatures, i.e., sub-zero to extremely high temperatures. Some bacteria exhibit a chemotactic response, where they can sense the contaminant and move towards it, thereby, leading to bioremediation. Similarly, Several fungi, involved in this process, grow their filament in the contamination site. Some of the microbes involved in this process are discussed below:

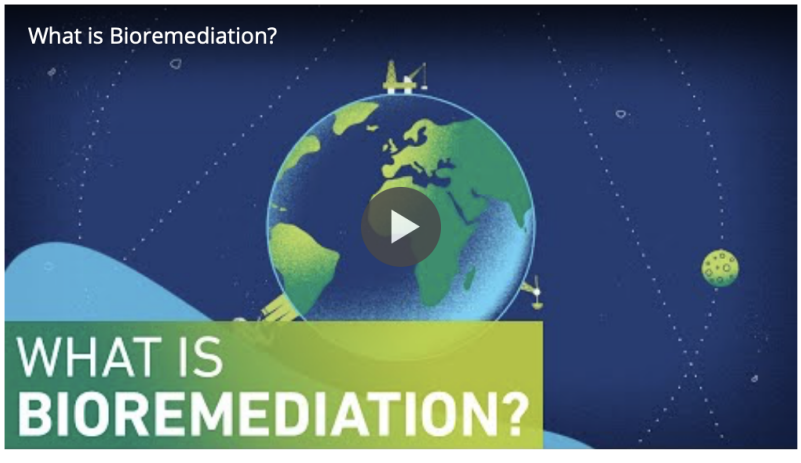
Bacteria:

For bacterial degradation, they must be in contact with the contaminant. There are two types of bacteria, namely, aerobic and anaerobic, that are used for bioremediation. Aerobic bacteria can degrade complex compounds in the presence of oxygen. These bacteria can degrade hydrocarbons, pesticides, alkanes, and polyaromatic compounds.

Some of the examples of aerobic bacteria are *Pseudomonas, Sphingomonas, Nocardia, Flavobacterium,* and *Mycobacterium.* The anaerobic bacteria functions in absence of oxygen and are not popularly used for the bioremediation process. However, some anaerobic bacteria are used in the bioremediation of polychlorinated biphenyls (PCBs), chlorinated aromatic compounds, and dechlorination of the solvent trichloroethylene and chloroform.

Fungi:

Several fungi can degrade harmful heavy metals and other hazardous components. For instance, white-rot fungus *Phanaerochaete chrysosporium* can degrade persistent or toxic environmental pollutants and *Aspergillus sp* can degrade heavy metals present in the soil.



Bioremediation Strategies

Several bioremediation strategies are employed depending on the level of saturation and aeration of the soil. These techniques can be divided into two categories, i.e., in-situ and ex-situ. The in-situ techniques are employed for the treatments of the soil or groundwater, which require minimal disturbance. Whereas ex-situ techniques are employed away from the treatment site, i.e., the contaminated soil under consideration is removed via excavation for its treatment.

In-situ treatments are mostly concentrated in the soil. Some of the examples of in situ treatments are bioaugmentation, bioventing, and biosparging. Bioaugmentation is a type of in-situ bioremediation which involves the amendment of microbes to the polluted site to increase the rate of degradation.

Bioventing is one of the most common in-situ treatments which deals with providing air and nutrients to the contaminated site via wells to trigger the growth of indigenous bacteria. Bioventing is effective for hydrocarbon degradation. In biosparging, the air is injected under pressure below the water table to enhance the groundwater oxygen concentrations and, thereby, triggering the aerobic bacteria. Landfarming is one of the simple ex-situ bioremediation techniques. This technique deals with excavating contaminated soil which is subsequently spread over a prepared bed where the indigenous biodegradative microorganisms are stimulated.

Bioreactors (slurry reactor) are also used in the ex-situ bioremediation process. This bioreactor is used for the treatment of contaminated water and soil that have been pumped up from a polluted plume. Generally, the rate of biodegradation is high in a bioreactor system.

Advantages and Disadvantage of Bioremediation

The main advantage of bioremediation is that it does not use any toxic chemicals. Typically, it utilizes nutrients such as fertilizers to activate the microbial population. Additionally, this process is less labor-intensive and is economical. Bioremediation is an eco-friendly and sustainable approach that can destroy a pollutant or convert harmful contaminants into harmless substances.

The main disadvantage of bioremediation technology is that it is restricted to biodegradable compounds. Further, researchers have revealed that sometimes the new product developed after biodegradation may be more toxic to the environment than the initial compound. Lastly, the process is time-consuming, especially for ex-situ bioremediation, which requires excavation and pumping.

Applications of Bioremediation

Bioremediation is appropriate for all states of matter, i.e., solid, liquid, and gases. This technology is highly efficient for the treatment of solids such as sediments, soil, and sludge.

In the case of liquid treatment, it is applied to remediate oil spills in the ocean, as well as, treatment of industrial wastewater and groundwater. Further, bioremediation is also widely used for the treatment of industrial air emissions.

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Resource 3:

Bioresource Technology 102 (2011) 9135–9142

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Microalgal biomass production and on-site bioremediation of carbon dioxide, nitrogen oxide and sulfur dioxide from flue gas using *Chlorella* sp. cultures

Sheng-Yi Chiu [a], Chien-Ya Kao [a,1], Tzu-Ting Huang [a], Chia-Jung Lin [a], Seow-Chin Ong [a], Chun-Da Chen [b], Jo-Shu Chang [c], Chih-Sheng Lin [a,*]

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ABSTRACT

The growth and on-site bioremediation potential of an isolated thermal- and CO_2-tolerant mutant strain, *Chlorella* sp. MTF-7, were investigated. The *Chlorella* sp. MTF-7 cultures were directly aerated with the flue gas generated from coke oven of a steel plant. The biomass concentration, growth rate and lipid content of *Chlorella* sp. MTF-7 cultured in an outdoor 50-L photobioreactor for 6 days was 2.87 g L^{-1} (with an initial culture biomass concentration of 0.75 g L^{-1}), 0.52 g L^{-1} d^{-1} and 25.2%, respectively. By the operation with intermittent flue gas aeration in a double-set photobioreactor system, average efficiency of CO_2 removal from the flue gas could reach to 60%, and NO and SO_2 removal efficiency was maintained at approximately 70% and 50%, respectively. Our results demonstrate that flue gas from coke oven could be directly introduced into *Chlorella* sp. MTF-7 cultures to potentially produce algal biomass and efficiently capture CO_2, NO and SO_2 from flue gas.

1. Introduction

Global warming, which is induced by an increase in the concentration of greenhouse gases in the atmosphere, is of great concern and has received increasing attention as natural sources of fossil fuels have become exhausted (Favre et al., 2009). Carbon dioxide (CO_2) is one of the main greenhouse gases (GHG) emitted into the atmosphere. Flue gases from power plants are responsible for more than 7% of world CO_2 emissions from energy use (Kadam, 2002), and steel plants are the single largest source of energy-related CO_2 emissions in the world (Gielen, 2003).

The efficient mitigation of GHG emissions is an international issue. Biological methods, particularly microalgal photosynthesis, have several merits, such as higher CO_2 fixation rates than terrestrial plants and no requirement for further disposal of the trapped CO_2. The incorporation of CO_2 into a biomass carbon source, such as carbohydrates and lipids, by microalgal fixation of CO_2 by photosynthesis is the most promising potential method for CO_2 sequestration from flue gas (Lee and Lee, 2003; Doucha et al., 2005; Wang et al., 2008; Brune et al., 2009; Yoo et al., 2010; Ho et al., 2011).

Microalgal biomass can be used for biofuel production by pyrolysis, direct combustion or thermal chemical liquefaction (Mata et al., 2010). The lipid fraction of the microalgal biomass can be extracted and transesterified for biodiesel production (Brennan and Owende, 2010; Lee et al., 2010). Capturing CO_2 from industrial processes using microalgae and the subsequent utilization of the generated biomass for transportation needs would aid in achieving CO_2 sequestration and reducing our overall carbon emissions.

In general, the primary emission in flue gas is CO_2, which is present at concentrations ranging from 3% to 25% depending on the fuel source and the design of the plant (Packer, 2009). This CO_2 is a plentiful carbon source for microalgal cultures. The direct use of the flue gas reduces the cost of pretreatment but imposes extreme conditions on the microalgae, such as the high concentration of CO_2 and the presence of inhibitory compounds such as NO_X and SO_X (Negoro et al., 1999; Lee et al., 2000). Temperature is also an inhibitory growth factor for outdoor microalgal cultivation. The temperature of the microalgal culture broth in the photobioreactors can increase to about 40 °C due to irradiation by sunlight. Feasibility of sequestering CO_2 from flue gas depends on either installing heat exchanger system or using thermophilic species (Kumar et al., 2011). Temperature control must be addressed if microalgae are used for large-scale outdoor cultivation (Béchet et al., 2010).

In the present study, an isolated thermal- and CO_2-tolerant mutant strain of *Chlorella* sp. was used in an on-site outdoor microalgal cultivation with flue gas aeration. The flue gas was generated

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from coke oven of a steel plant. The effects of flue gas aeration on the growth and lipid production of the *Chlorella* sp. mutant strain was investigated. Furthermore, on-site elimination efficiencies of CO_2, NO and SO_2 from the flue gas were evaluated.

2. Methods

2.1. Microalgal cultures, medium and chemicals

The microalga *Chlorella* sp. MTF-7 was originally obtained from the collection of Taiwan Fisheries Research Institute (Tung-Kang, Ping-Tung, Taiwan) and isolated in our laboratory by chemical mutagenesis. The *Chlorella* sp. MTF-7 cells were grown in modified f/2 medium in artificial sea water with 29.23 g L^{-1} NaCl, 1.105 g L^{-1} KCl, 11.09 g L^{-1} $MgSO_4$ $7H_2O$, 1.21 g L^{-1} Tris-base, 1.83 g L^{-1} $CaCl_2$ $2H_2O$ and 0.25 g L^{-1} $NaHCO_3$, with 0.3% (v/v) macro elemental solution and 0.3% trace elemental solution. The macro elemental solution was 75 g L^{-1} $NaNO_3$ and 5 g L^{-1} NaH_2PO_4 H_2O. The trace elemental solution was 4.36 g L^{-1} Na_2 EDTA, 3.16 g L^{-1} $FeCl_3$ $6H_2O$, 180 mg L^{-1} $MnCl_2$ $4H_2O$, 10 mg L^{-1} $CoCl_2$ $6H_2O$, 10 mg L^{-1} $CuSO_4$ $5H_2O$, 23 mg L^{-1} $ZnSO_4$ $7H_2O$, 6 mg L^{-1} Na_2MoO_4 $2H_2O$, 100 mg L^{-1} vitamin B_1, 0.5 mg L^{-1} vitamin B_{12} and 0.5 mg L^{-1} biotin.

2.2. Measurement of microalgal cells, growth rate

Biomass concentration (dry weight per liter) of cultures were measured according to the method reported previously (Chiu et al., 2009a). Regression equations of the relationship between optical density and cell dry weight were established and shown as follows:

$$y = 0.2529x - 0.0153, \quad R^2 = 0.9898$$

The value y is biomass concentration (g L^{-1}). This value was determined according the method previously reported (Chiu et al., 2009a). Microalgal cells were collected, centrifuged and washed with dideionized water. The washed microalgal pellet was dried at 105 °C for 16 h; afterward, the dried cells were for dry weight measurement. The value x_1 is optical density measured by the absorbance at 682 nm (A_{682}) in an Ultrospec 3300 pro UV/Visible spectrophotometer (Amersham Biosciences, Cambridge, UK). Each sample was diluted to give an absorbance in the range of 0.1–1.0 if optical density was greater than 1.0.

The optical density was used to evaluate the biomass concentration of *Chlorella* sp. MTF-7 in each experiment. In the present study, we used biomass concentration (g L^{-1}) for the quantitation of *Chlorella* sp. MTF-7 cell density in the culture. The growth rate was measured and according the equation showed as follows:

$$\text{Growth rate} = \frac{W_f - W_i}{\Delta_t}$$

where W_f and W_i is the final and initial biomass concentration, respectively. Δt is the cultivation time in days.

2.3. Experimental system of indoor photobioreactor

The microalgal cells were cultured in photobioreactors with a working volume of 800 mL (Chiu et al., 2008). The photobioreactors were placed in an incubator at 25 ± 1 °C with a surface light intensity of approximately 300 μmol m^{-2} s^{-1} provided by continuous, cool-white, fluorescent lights. The photobioreactor was made of glass, and the diameter of the photobioreactor was 70 mm. The gas was supplied from the bottom of the photobioreactor. The CO_2-enriched gas was premixed with air and pure CO_2 for the flue gas experiments as a control gas. In the gas airstream, CO_2 concentration was adjusted to 2%, 10% and 25% for cultures as control experiments. The flue gas (approximately 25% CO_2, 4% O_2, 80 ppm NO and 90 ppm SO_2) was collected from coke oven in China Steel Corporation and was directly introduced into microalgal cultures. The gas flow rate was adjusted to 0.05 vvm (volume gas per volume broth per min) using a gas flow meter (Dwyer Instruments, Inc., Michigan city, IN, USA). The evaluation of tolerance to the flue gas in microalgal cultures, initial biomass concentration of *Chlorella* sp. MTF-7 cultures were approximately 0.2 g L^{-1}. The microalgal cells in each treatment were sampled every 24 h for determination of the biomass concentration.

2.4. Experimental system of outdoor photobioreactor

The outdoor photobioreactor was cylindrical and made of acrylic polymer. The column was 300 cm in length and 16 cm in diameter. The working volume of the photobioreactor was 50 L (Ong et al., 2010). The gas flow rate was adjustable using a gas flow meter. The source of flue gas was from a coke oven in China Steel Corporation (Kaohsiung, Taiwan). The concentrations of CO_2, O_2, NO and SO_2 in the flue gas were 23 ± 5%, 4.2 ± 0.5%, 78 ± 4 ppm and 87 ± 9 ppm (October 1–November 15, 2010), respectively. In the intermittent flue gas aeration, culture aeration was controlled by a gas switch, and a gas-switching cycle was performed with a flue gas inlet load for 30 min followed by air inlet load for 30 min (30 min flue gas/30 min air) during the day. The inlet and outlet loads were real-time monitored by a gas analyzer (AMETEK, Inc., Paoli, PA, USA) to determine the concentrations of CO_2, O_2, NO and SO_2.

2.5. Lipid extraction

Lipid extraction was according to the modified method previously reported (Chiu et al., 2009b). The microalgal cells were centrifuged and washed with deionized water twice, and obtained the dry biomass by lyophilization. The dried sample (200 mg) was mixed with methanol/chloroform solution (2/1, v/v) and sonicated for 1 h. The mixture with methanol/chloroform solution was precipitated and added chloroform and 1% NaCl solution to give a ratio of methanol, chloroform, and water of 2:2:1. The mixture was centrifuged and the chloroform phase was recovered. Finally, the lipids were weighted after chloroform was removed under vacuum by a rotary evaporator.

2.6. Microalgal lipid transesterification

The mixture of methanol (1.7 mL), sulfuric acid (0.3 mL) and chloroform (2.0 mL) was added to the microalgal oil, and heated at 90 °C for 40 min with thoroughly mixing during heating. The samples were then cooled to room temperature and mixed with 1 mL dideionized water. Finally, the organic (lower) phase containing fatty acid methyl esters (FAMEs) was collected and the solvent was evaporated.

2.7. Fatty acid profile analysis

The fatty acid composition was determined FOCUS Gas Chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an flame ionization detector (FID) and trace GC capillary column (Thermo Fisher Scientific, Waltham, MA, USA), which was a cyanopropylphenyl based phase specifically designed for the separation of FAMEs. A 30 m long column was used with a diameter of 0.32 mm and a 0.25 μm thick film. The amount of sample injected was 2 μL. The stripping gas was nitrogen at a flow rate of 1.3 mL min^{-1}, and the injector and detector temperatures were

250 and 280 °C, respectively. The initial column temperature was 150 °C where it remained for 1 min, then rising from 150 to 180 °C at 10 °C min^{-1}, remaining at 180 °C for 3 min, then rising from 180 to 220 °C at 1.5 °C min^{-1}, remaining at 220 °C for 1 min, and finally rising from 220 to 260 °C at 30 °C min^{-1}, remaining at 260 °C for 5 min. The fatty acids were identified by comparison of the retention times with those of the standards using the software Chrom-Card Data System (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Chemical analyses

The inlet and outlet loads of airstreams were real-time monitored by a gas analyzer. The concentration of O_2, CO_2, NO and SO_2 in flue gas were measured using Landcom III portable gas analyzer (AMETEK, Inc., Paoli, PA, USA).

2.9. pH and light measurements

The sample pH was directly determined using an ISFET pH meter KS723 (Shindengen Electric, Tokyo, Japan). The pH meter was calibrated daily using standard solutions of pH 4 and 7. Light intensity was measured adjacent to the surface of the photobioreactor using a Basic Quantum Meter (Spectrum Technologies, Plainfield, IL, USA).

3. Results and discussion

3.1. Growth parameters of Chlorella sp. MTF-7 aerated with flue gas

3.1.1. Indoor culture experiments

In our previous study (Chiu et al., 2008), microalgal cell growth was significantly inhibited when wild-type microalgal *Chlorella* sp. cultures were aerated with gas containing a high concentration of CO_2 (>10% CO_2). Given the high concentration of CO_2 in flue gas (about 20–25% CO_2), the growth potential of the isolated microalga, *Chlorella* sp. MTF-7, when aerated directly with flue gas was first evaluated. In indoor culture experiments, batch cultures of *Chlorella* sp. MTF-7 were incubated for 6 days at 25 ± 1 °C under continuous cool white fluorescent light. The light intensity was approximately 300 μmol m^{-2} s^{-1} at the surface of the photobioreactor. The flue gas generated from coke oven of a steel plant was collected in a gas storage bag, and the gas was continuously introduced into the photobioreactor by an air blower.

Fig. 1 shows the growth curves of *Chlorella* sp. (wild-type, WT) and *Chlorella* sp. MTF-7 aerated with flue gas or CO_2-enriched gas (2%, 10%, or 25% CO_2 aeration) for 6 days. The growth potential of *Chlorella* sp. MTF-7 was significantly higher than that of *Chlorella* sp. WT when aerated with flue gas or CO_2. The maximum biomass concentrations in *Chlorella* sp. MTF-7 cultures aerated with 2%, 10% or 25% CO_2 were 1.67, 1.50 and 1.32 g L^{-1}, respectively. The maximum biomass concentration was 2.40 g L^{-1} in the batch cultures of *Chlorella* sp. MTF-7 aerated with flue gas. The average growth rates of the *Chlorella* sp. MTF-7 cultures aerated with flue gas or 2%, 10% or 25% CO_2 were 0.37, 0.25, 0.15 and 0.19 g L^{-1} d^{-1}, respectively. The growth rates of the *Chlorella* sp. MTF-7 cultures aerated with flue gas were approximately 1.5-, 2.5- and 2.0-fold higher than those of the *Chlorella* sp. WT cultures aerated with 2%, 10% or 25% CO_2, respectively. These results indicated that *Chlorella* sp. MTF-7 could be cultured with flue gas aeration; the maximum biomass productivity was 0.64 g L^{-1} d^{-1} in the batch culture aerated with flue gas. The growth potential of *Chlorella* sp. MTF-7 cultures aerated with flue gas from the coke oven of a steel plant, which contained approximately 25% CO_2, 4% O_2, 80 ppm NO and 90 ppm SO_2, was higher than that of the cultures aerated with 2%, 10% or 25% CO_2-enriched gas without pH control. The high growth capacity of microalgae aerated with flue gas has been reported previously (Douskova et al., 2009). The volumetric concentration of CO_2 provided to the control culture was the same as the average concentration in the flue gas (11%). However, the growth rate of *Chlorella vulgaris* cultures aerated by flue gas from an incinerator was 48% higher than that of the control culture. The high concentration of CO_2 in flue gas was a major factor in microalgal growth (Yoo et al., 2010). In our previous study, a high initial density of *Chlorella* sp. could overcome the environmental stress induced by high CO_2 aeration and grow rapidly (Chiu et al., 2008). In this experiment, an initial high-density culture was used, and a gas-switching cycle operation was also introduced. In a high-density culture, the growth inhibition caused by the high CO_2 concentration in the flue gas is reduced, and the pH value of the culture can be stably maintained.

The NO_X present in flue gas inhibits microalgal growth (Lee et al., 2000). However, the toxic effect of NO can also be overcome by high-density cultures, and NO can be a nitrogen source for microalgal cultures. NO absorbed in the medium can be converted to NO_2^- and then oxidized to NO_3^-, which can be utilized as a nitrogen source (Nagase et al., 2001). Gaseous NO can dissolve in the broth of microalgal cultures and can be taken up directly by algal cells through diffusion (Nagase et al., 2001). The flue gas, which contains CO_2 and NO, could provide not only a carbon source for microalgal growth but also an additional nitrogen source.

SO_X in flue gas is also an inhibitor of microalgal growth (Lee et al., 2000). The main form of SO_X in the flue gas generated from a coke oven is SO_2. Lee et al. (2000) reported that the growth of *Chlorella* KR-1 aerated with simulated flue gas containing 150 ppm SO_2 was suppressed because of cellular toxicity when a

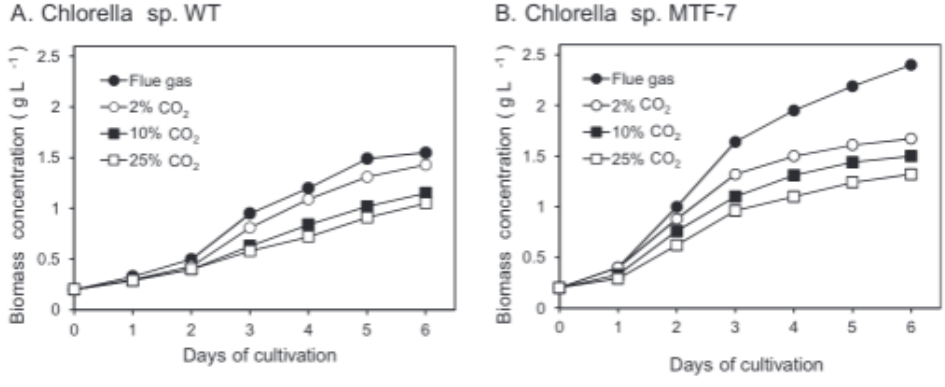

Fig. 1. Growth profiles of *Chlorella* sp. (wild-type, WT) (A) and its mutant, *Chlorella* sp. MTF-7 (B), cultured in an indoor photobioreactor aerated with continuous flue gas or CO_2-enriched gas (2%, 10%, or 25%). The initial biomass concentration was approximately 0.2 g L^{-1}. The microalgal cells were cultivated at ~300 μmol m^{-2} s^{-1}. The flue gas was provided at 0.05 vvm. The cultures were grown for 6 days, and the microalgal cells were sampled every 24 h for growth determinations.

low-density initial biomass concentration (0.1 g L^{-1}) was used, but *Chlorella* KR-1 exhibited good growth when a high-density initial biomass concentration (0.5 g L^{-1}) was used. The toxic effect of SO_2 could be overcome by acidophilic microalgal isolation (Kurano et al., 1995; Lee et al., 2002). Hauck et al. (1996) reported that an acidophilic microalga, *Cyanidium caladrium*, grew well in the presence of 200 ppm SO_2 in simulated flue gas aeration. Considering that growth of most algal strains reported was completely inhibited, when the cultures aerated with flue gas which contained SO_2 concentration higher than 50 ppm (Kurano et al., 1995; Hauck et al., 1996). In our study, the isolated mutant strain, *Chlorella* sp. MTF-7, showed remarkably excellent tolerances to SO_2 and grew well in cultures supplied with gas containing approximately 90 ppm SO_2 when an initial biomass concentration of at least 0.5 g L^{-1} *Chlorella* sp. MTF-7 was used.

The satisfactory growth of *Chlorella* sp. MTF-7 in cultures supplied with gas containing approximately 90 ppm SO_2 may be due to its ability to tolerate highly oxidative molecular species. Bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) are microalgal growth inhibitors that are formed in water from SO_2 (Yang et al., 2004). As SO_2 dissolves in the culture broth, HSO_3^- is formed: HSO_3^- can be converted to SO_3^{2-} and SO_4^{2-} at appropriate pH values. As HSO_3^- is converted to SO_4^{2-}, highly oxidative molecular species are formed, such as superoxide anions, hydrogen peroxide and hydroxyl radical. These highly oxidative molecular species can cause the peroxidation of membrane lipids and the bleaching of chlorophyll; thus, microalgal growth is inhibited by the processing of HSO_3^- to SO_4^{2-} (Ranieri et al., 1999; Noji et al., 2001). The inhibitory effect of SO_2 on *Chlorella* sp. MTF-7 growth might be eliminated by screening specific mutant strains and using a high concentration of inoculum.

To assess the potential of *Chlorella* sp. MTF-7 to be cultured by the side of the stack of a coke oven for the on-site bioremediation of flue gas without a cooling system, the growth of *Chlorella* sp. WT and *Chlorella* sp. MTF-7 when aerated with flue gas at different culture temperatures was also evaluated. Fig. 2 shows the growth curves of *Chlorella* sp. WT and *Chlorella* sp. MTF-7 when aerated with flue gas at 25, 30, 35 or 40 °C. The average growth rates of the *Chlorella* sp. WT cultures that were aerated with flue gas at 25, 30, 35 or 40 °C were 0.23, 0.21, 0.14 and 0.11 g L^{-1} d^{-1}, respectively. The average growth rates of the *Chlorella* sp. MTF-7 cultures that were aerated with flue gas at 25, 30, 35 or 40 °C were 0.37, 0.39, 0.32 and 0.24 g L^{-1} d^{-1}, respectively. The optimal growth temperature for *Chlorella* sp. MTF-7 was 30 °C, and the maximum biomass productivity of *Chlorella* sp. MTF-7 cultured at 30 °C and aerated with flue gas was 0.70 g L^{-1} d^{-1}. However, the growth rate and biomass productivity of *Chlorella* sp. MTF-7 that was cultured at higher temperatures (35 and 40 °C) remained high and were significantly greater than those of *Chlorella* sp. WT, even when the wild-type microalgal cells cultured at 25 and 30 °C.

3.1.2. Outdoor culture experiments

To evaluate microalgal growth performance during on-site flue gas aeration, a *Chlorella* sp. MTF-7 culture system was installed next to the smokestack of a coke oven at the China Steel Corporation in southern Taiwan (Supplementary Fig. S1). The flue gas from the coke oven was introduced into the microalgal cultures by suction pump, and air was supplied by an air pump. The gas was provided with either continuous flue gas aeration or intermittent flue gas aeration controlled by a gas-switching cycle operation (Supplementary Fig. S2). For continuous flue gas aeration, the flue gas was supplied continuously for 9 h during the day. For intermittent flue gas aeration, the flue gas was supplied in 30-min intervals every hour from 07:30 to 16:30; a gas-switching cycle was performed with a flue gas inlet load for 30 min followed by an air inlet load for 30 min (30 min flue gas/30 min air) for 9 h during the day.

Fig. 3 shows the growth curves that resulted when different initial biomass concentrations (0.5, 0.75, 1.0 and 1.25 g L^{-1}) of the *Chlorella* sp. MTF-7 inoculum were aerated with continuous (Fig. 3A) and intermittent flue gas (Fig. 3B) at 0.05 vvm. The growth profiles of *Chlorella* sp. MTF-7 aerated with flue gas were stable and linear with respect to the initial biomass concentration of the inoculum, whether the flue gas supply was continuous or intermittent. The average growth rates of *Chlorella* sp. MTF-7 when the initial biomass inoculum was 0.5, 0.75, 1.0 or 1.25 g L^{-1} were 0.13, 0.11, 0.11 and 0.05 g L^{-1} d^{-1} with continuous flue gas aeration, and 0.30, 0.36, 0.29 and 0.28 g L^{-1} d^{-1} with intermittent flue gas aeration, respectively. The growth rates of the cultures aerated with intermittent flue gas were 2.3-, 3.1-, 2.6- and 5.2-fold higher than those of the cultures aerated with continuous flue gas when initial biomass concentrations of 0.5, 0.75, 1.0 or 1.25 g L^{-1} were used, respectively. During a 6-day cultivation in which the initial biomass concentration of *Chlorella* sp. MTF-7 was 0.75 g L^{-1}, the maximum biomass growth rate was 0.52 g L^{-1} d^{-1}, and the average biomass growth rate was 0.36 g L^{-1} d^{-1}. The growth potential of *Chlorella* sp. MTF-7 cultures aerated with intermittent flue gas was significantly higher than that of *Chlorella* sp. MTF-7 cultures continuously aerated with flue gas. The intermittent flue gas aeration strategy for cultivation could enhance microalgal growth and also increase the utilization of the CO_2 in the flue gas. These results demonstrate that *Chlorella* sp. MTF-7 can grow well in an outdoor photobioreactor aerated directly with flue gas from a coke oven.

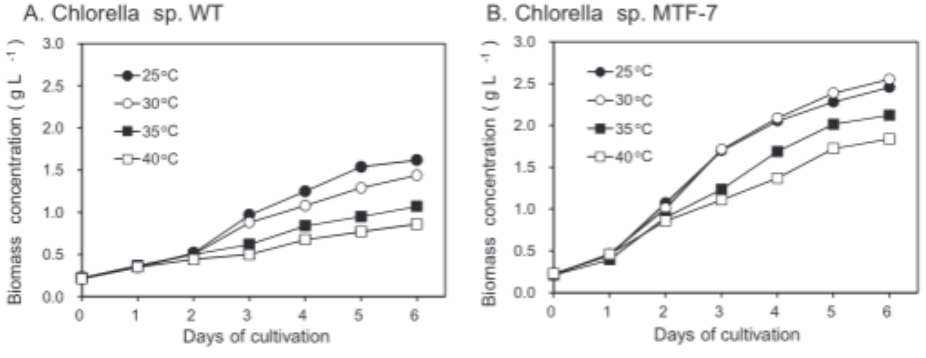

Fig. 2. Growth profiles of *Chlorella* sp. (wild-type, WT) (A) and its mutant, *Chlorella* sp. MTF-7 (B), cultured in an indoor photobioreactor aerated with continuous flue gas and operated at different temperatures (25, 30, 35 or 40 °C). The initial biomass concentration was approximately 0.2 g L^{-1}. The microalgal cells were cultivated at ~300 μmol m^{-2} s^{-1}. The flue gas was provided at 0.05 vvm. The cultures were grown for 6 days, and the microalgal cells were sampled every 24 h for growth determinations.

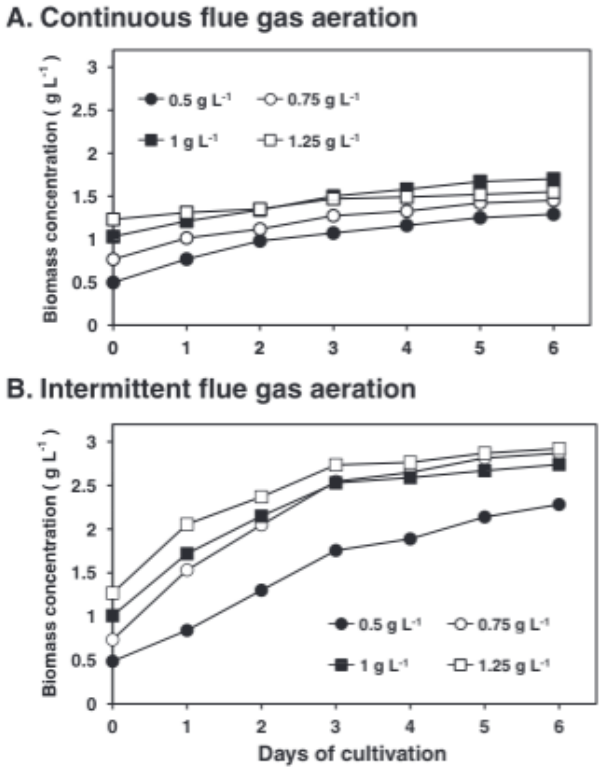

Fig. 3. Growth profiles of *Chlorella* sp. MTF-7 cultured in an outdoor photobioreactor aerated with continuous (A) or intermittent flue gas (B). The initial biomass concentrations were 0.5, 0.75, 1.0 and 1.25 g L^{-1}. The microalgal cells were cultivated during the day (1000 to 1800 μmol m^{-2} s^{-1}). The flue gas was provided at 0.05 vvm. The cultures were grown for 6 days, and the microalgal cells were sampled every 24 h for growth determinations.

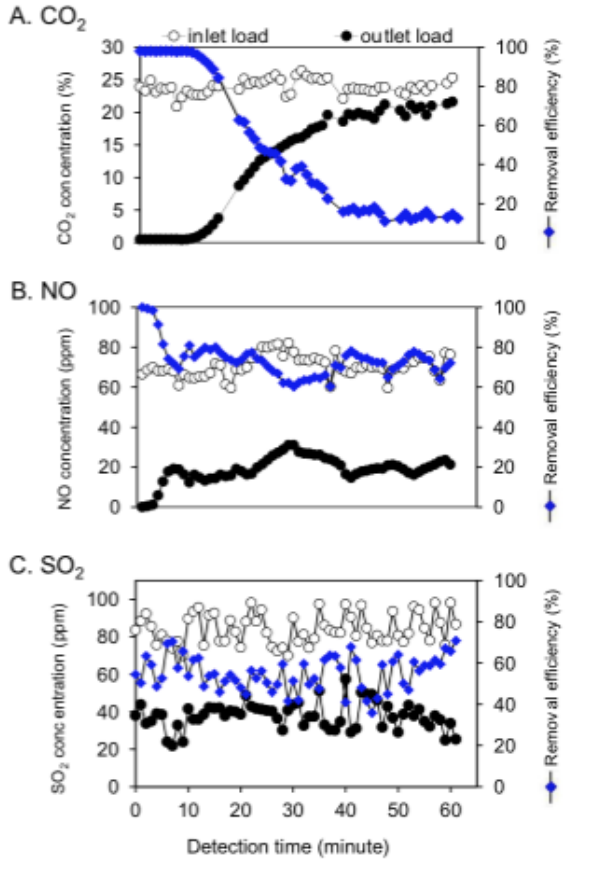

Fig. 4. The efficiency of CO_2 (A), NO (B) and SO_2 (C) removal from flue gas by *Chlorella* sp. MTF-7 cultures under continuous flue gas aeration. The biomass concentration of the microalgal culture was approximately 2 g L^{-1}, and it was cultivated during the day (1000 to 1800 μmol m^{-2} s^{-1}). The flue gas was provided under continuous operation at 0.05 vvm. The CO_2, NO and SO_2 concentration in the inlet load (open circle) and the outlet load (solid circle) of the flue gas was monitored in real time once every minute with a flue gas analyzer. The CO_2, NO and SO_2 removal efficiency (blue diamond) was calculated from the difference in the CO_2, NO and SO_2 concentrations of the inlet and outlet loads. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

3.2. Flue gas bioremediation by continuous flue gas aeration

3.2.1. CO_2 removal

For the on-site bioremediation of CO_2 in flue gas from a coke oven, the time course of the CO_2 removal efficiency of the microalgal cultures that were aerated with flue gas was determined. The CO_2 concentrations in the inlet and outlet loads of the flue gas used to aerate the microalgal cultures were monitored in real time by CO_2 gas sensors. Fig. 4A illustrates the inlet load, outlet load and CO_2 removal efficiency of *Chlorella* sp. MTF-7 under flue gas aeration. The CO_2 removal efficiency was 95% at 10 min after flue gas introduction and 50% after 25 min of flue gas aeration. The decrease in the CO_2 removal efficiency of the microalgal culture was due to the continuous inlet load of the flue gas. After aeration for 40 min, a constant 13% CO_2 removal efficiency was reached. Li et al. (2011) recently reported that the screened and isolated mutant *Scenedesmus obliquus* WUST4 could remove about 64% of the CO_2 from flue gas (18% v/v) generated from a coke oven. When a water pump was used to counter-circulate the water and increase the gas retention time, the efficiency of CO_2 removal from the flue gas by a microalgal culture reached 82.3 ± 12.5% (Vunjak-Novakovic et al., 2005). The result is also confirmed that according the regression lines of the relation between biomass concentration and the CO_2 removal efficiency, the CO_2 removal efficiency was approximate 20% (Chiu et al., 2009b). Moreover, this phenomenon is also confirmed in previous study that the CO_2 removal efficiency was remarkably consistent and showed a stable pattern from outlet CO_2 measurements (Chiu et al., 2008).

3.2.2. NO and SO_2 removal

The time courses of the NO and SO_2 removal efficiencies of the microalgal cultures that were aerated with flue gas were also determined. Fig. 4B illustrates the inlet load, outlet load and NO removal efficiency of *Chlorella* sp. MTF-7 under flue gas aeration. The average NO concentrations of the inlet and outlet loads of the flue gas were 78 ± 4 and 20 ± 5 ppm. The NO removal efficiency of the *Chlorella* sp. MTF-7 cultures was approximately 73%. The SO_2 concentrations in the inlet and outlet loads of the flue gas were 87 ± 9 and 37 ± 7 ppm, respectively, and the SO_2 removal efficiency of the *Chlorella* sp. MTF-7 cultures under flue gas aeration was approximately 55% (Fig. 4C). Nagase et al. (2001) have reported that, in addition to reducing CO_2 in flue gas, microalgal cultures are effective continuous flue gas bioscrubbers for NO (>60% removal efficiency). Since most power stations should equipped with the flue gas desulfurization for SO_X emission control below 100 ppm, *Chlorella* sp. MTF-7 may be used for the direct CO_2, NO and SO_2 capture from the flue gases. To make the biological CO_2, NO and SO_2 capture process to be economically feasible, the utilization of the produced microalgal biomass is a critically important issue.

3.3. Flue gas bioremediation by a gas-switching cycle operation

3.3.1. CO_2 removal

To improve the efficiency of CO_2 removal from flue gas, a gas (flue-gas/air) -switching cycle operation was intermittently performed to introduce the flue gas into the microalgal cultures.

First, the CO_2 removal efficiency at different time intervals of intermittent flue gas aeration was evaluated. In the intermittent flue gas aeration, the culture aeration was controlled by a gas switch, and a gas-switching cycle was performed with a flue gas inlet load for 20, 30 or 40 min followed by an air inlet load for 30 min (i.e., 20, 30 or 40 min flue-gas/30 min air) during the day. The CO_2 concentrations of the inlet and outlet loads when *Chlorella* sp. MTF-7 was aerated with intermittent flue gas at 20, 30 or 40 min time intervals was illustrated (Supplementary Fig. S3). The average CO_2 removal efficiencies at the 20, 30 or 40 min time intervals were 70, 63 and 45%, respectively.

To evaluate the system stability for CO_2 removal during intermittent flue gas aeration, the intermittent flue gas aeration was operated with a 30-min time interval. CO_2 removal in the intermittent flue gas aeration of the *Chlorella* sp. MTF-7 culture was stable, and the inlet and outlet load patterns were also stable and similar to each other during the three gas-switching cycles (Fig. 5A). The pattern of the changes in the pH of the *Chlorella* sp. MTF-7 culture was stable during intermittent flue gas aeration (Fig. 5B). The pH value decreased from 7.9–8.2 to 6.3–6.5 after 30 min of flue gas aeration. After the gas was switched to air aeration, the pH value returned to 8.0 ± 0.2 after 30 min of air introduction. The fluctuations in the dissolved CO_2 in the microalgal culture broth also followed a repetitive pattern during the gas-switching aeration cycles (Fig. 5C). During flue gas aeration, the pH decreased, and the dissolved inorganic carbon (DIC) concentration increased because of CO_2 absorption. During air aeration, the pH increased, and the DIC concentration decreased, implying that the increase in the outlet load of CO_2 contributed to the pH decrease. Our system was operated for 9 cycles during the day, and the patterns of the fluctuations in the values of pH, DIC and CO_2 removal efficiency were stable throughout (data not shown).

3.3.2. NO and SO_2 removal

The NO and SO_2 removal efficiencies were also evaluated during the intermittent flue gas application. Fig. 6A illustrates the patterns of the NO concentrations of the inlet and outlet loads when *Chlorella* sp. MTF-7 was aerated with intermittent flue gas. The outlet load of NO was efficiently removed by the *Chlorella* sp. MTF-7 culture aerated with intermittent flue gas. The average NO removal efficiency reached approximately 70%. The patterns of the NO concentrations of the inlet and outlet loads were stable for each cycle of flue-gas/air switching. Fig. 6B illustrates the patterns of the SO_2 concentrations of the inlet and outlet loads when *Chlorella* sp. MTF-7 was aerated with intermittent flue gas. The SO_2 removal efficiency was also stable, and the average SO_2 removal efficiency reached approximately 50%.

To determine whether CO_2, NO and SO_2 in the flue gas could be continuously bioremediated with intermittent flue gas aeration, a double-set of photobioreactor system that alternately aerated with flue gas was established and operated (Supplementary Fig. S2). The flue gas aeration on/off time was controlled by a gas switch. There were two sets of gas switches. When the A gas switch was on for 30 min to allow flue gas aeration into system A, the B gas switch was off for 30 min to allow air aeration into system B. Therefore, flue gas was introduced into system A for 30 min and subsequently into system B for 30 min to permit continuous flue gas bioremediation. The inlet and outlet loads of the flue gas were monitored with a portable gas analyzer. According to calculations that were based on the CO_2, NO and SO_2 concentrations of the inlet and outlet loads, the average CO_2, NO and SO_2 removal efficiencies were maintained at 61, 68 and 51%, respectively. The gas-switching cycle operation was also extended to the double-set of photobioreactor system. This double-set of photobioreactor system was alternately aerated with flue gas. Via the gas-switching cycle operation, CO_2, NO and SO_2 could be constantly removed from the flue gas. The CO_2, NO and SO_2 removal efficiencies, respectively, were maintained at approximately 60%, 70% and 50% in the constant removal gas-switching cycle operation (data not shown). As mentioned above, *Chlorella* sp. MTF-7 cultures could be aerated directly with intermittent flue gas, and a strategy of gas-switching cycle without a pH control is a promising approach for continuous flue gas bioremediation. To achieve the desired flue gas bioremediation capacity, the photobioreactors could be multiplied and connected in parallel.

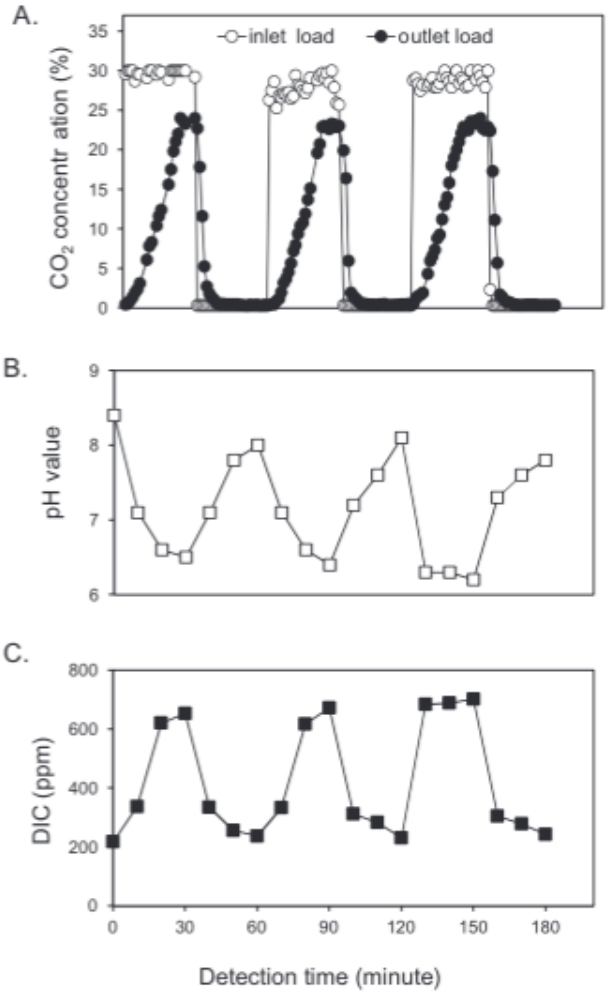

Fig. 5. The patterns of the inlet and outlet load CO_2 concentrations (A), pH value (B), and dissolved inorganic carbon (DIC) concentrations (C) of *Chlorella* sp. MTF-7 cultures aerated with intermittent flue gas at 0.05 vvm. The flue gas was controlled by a gas-switching cycle operation. A gas cycling switch was used with a flue gas inlet load for 30 min followed by an air inlet load for 30 min (30 min flue gas/30 min air). The CO_2 concentration was monitored at the inlet and outlet loads with a flue gas analyzer. The inlet and outlet loads of the gases were monitored once every minute. The culture broth was sampled every 10 min for the pH and DIC measurements.

3.4. The effect of flue gas on lipid production in microalgal cells

To investigate the effects of flue gas aeration on lipid production in *Chlorella* sp. MTF-7, microalgal cells were harvested after 6 days

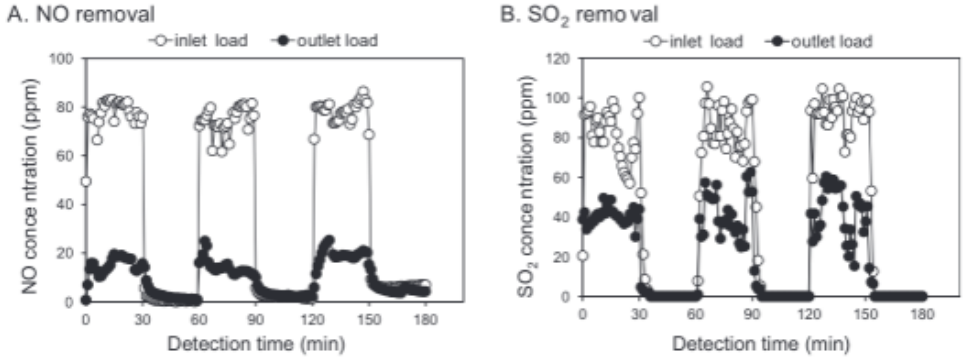

Fig. 6. The patterns of the inlet and outlet load NO (A) and SO_2 (B) concentrations of *Chlorella* sp. MTF-7 cultures aerated with intermittent flue gas at 0.05 vvm. The NO and SO_2 concentrations were monitored in real time at the inlet load (open circle) and the outlet load (solid circle) gas with a flue gas analyzer once every minute.

of flue gas and CO_2-enriched gas (25%) aeration. The lipid content of *Chlorella* sp. MTF-7 was 25.2 ± 1.2% (n = 3) when aerated with flue gas and 26.4 ± 2.3% (n = 3) when aerated with CO_2-enriched gas (25%). The lipid content was no significant difference between that of *Chlorella* sp. MTF-7 aerated with flue gas and CO_2-enriched gas. The fatty acid composition of the microalgal lipids was analyzed by GC. Supplementary Table 1 shows the main fatty acid compositions of *Chlorella* sp. MTF-7 aerated with CO_2-enriched gas or flue gas. Palmitic acid (C16:0) was the predominant fatty acid. Compared to the *Chlorella* sp. MTF-7 culture aerated with CO_2-enriched gas (25%), the *Chlorella* sp. MTF-7 culture aerated with flue gas had more palmitic acid (C16:0) (55.2 ± 4.5% *vs.* 42.0 ± 2.3%). In addition, the relative saturated fatty acid content of the *Chlorella* sp. MTF-7 culture aerated with the flue gas was also higher than that of the *Chlorella* sp. MTF-7 culture aerated with CO_2-enriched gas (62.3 ± 4.1% *vs.* 48.6 ± 2.7%).

Although the lipid content of *Chlorella* sp. MTF-7 aerated with flue gas was slightly lower than that of *Chlorella* sp. MTF-7 aerated with CO_2-enriched gas, the lipid productivity of the *Chlorella* sp. MTF-7 cultures aerated with flue gas was higher due to their higher growth rate. For the purpose of biodiesel production from algal biomass, lipid productivity should be a selection parameter and a critical variable for the evaluation of algal species and culture operation (Chiu et al., 2009a; Griffiths and Harrison, 2009; Chen et al., 2011). When compared with aeration with 25% CO_2-enriched gas, aeration with flue gas had no significant effect on the lipid content of *Chlorella* sp. MTF-7 cultures. Our results suggest that a *Chlorella* sp. MTF-7 culture aerated with flue gas is a suitable candidate for microalgal lipid production. The most common fatty esters in biodiesel are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) (Knothe, 2008). The fatty acid profile of *Chlorella* sp. MTF-7 consists mainly of C_{16} and C_{18} fatty acids, and the major composition of the fatty acids in the microalgal strain was C16:0. C16:0 is the major saturated fatty acid in 12 microalgal strains that represent 8 classes, including *Chlorophyceae* (Patil et al., 2007). The fatty acids of *Chlorella* sp. MTF-7 are mainly saturated. The saturated fatty acid content in *Chlorella* sp. MTF-7 aerated with flue gas was higher than that of *Chlorella* sp. MTF-7 aerated with enriched CO_2. The properties of a biodiesel fuel, including its ignition quality, combustion heat, cold filter plugging point (CFPP), oxidative stability, viscosity, and lubricity, are determined by the structure of its component fatty esters. High levels of saturated fatty acids tend to increase the stability of biodiesel because unsaturated fatty acids result in poor oxidative stability. The effect of flue gas on fatty acid compositions was not clear and would be further investigated.

4. Conclusion

A thermal- and CO_2-tolerant mutant strain, *Chlorella* sp. MTF-7, can act as a bioscrubber to directly and efficiently remove CO_2, NO and SO_2 from flue gas. The microalgal cultures operated by intermittent flue gas aeration, growth potential of the microalgal strain could be enhanced, and CO_2, NO and SO_2 in the flue gas were efficiently removed. We have also demonstrated a practical operation in which the flue gas from coke oven of steel plant directly supplied the carbon source for biomass production of the microalgal strain.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.06.091.

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Resource 5:

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Biohybrid nanofibers containing manganese oxide–forming fungi for heavy metal removal from water

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Abstract

Manganese-oxidizing fungi support bioremediation through the conversion of manganese ions into manganese oxide deposits that in turn adsorb manganese and other heavy metal ions from the environment. Manganese-oxidizing fungi were immobilized onto nanofiber surfaces to assist remediation of heavy metal–contaminated water. Two fungal isolates, *Coniothyrium* sp. and *Coprinellus* sp., from a Superfund site (Lot 86, Farm Unit #1) water treatment system were incubated in the presence of nanofibers. Fungal hyphae had strong association with nanofiber surfaces. Upon fungal attachment to manganese chloride–seeded nanofibers, *Coniothyrium* sp. catalyzed the conformal deposition of manganese oxide along hyphae and nanofibers, but *Coprinellus* sp. catalyzed manganese oxide only along its hyphae. Fungi–nanofiber hybrids removed various heavy metals from the water. Heavy metal ions were adsorbed into manganese oxide crystalline structure, possibly by ion exchange with manganese within the manganese oxide. Hybrid materials of fungal hyphae and manganese oxides confined to nanofiber-adsorbed heavy metal ions from water.

Keywords

Fungi, heavy metal, nanofibers, manganese oxide, water purification

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Introduction

The release of heavy metal contaminants into groundwater poses a serious threat to human and environmental health.[1,2] Techniques used for heavy metal removal from water include chemical precipitation reactions, ion exchange, and adsorption.[3] Chemical precipitation is most widely used industrially because the reactions are simple and its cost is relatively low. Heavy metal cations react with hydroxide or sulfide anions to form water-insoluble salts. However, toxic by-products, such as low-density sludge and hydrogen sulfide (H_2S) fumes, can persist upon reaction.[3] Ion exchange is a highly efficient, high-capacity heavy metal removal technique. Heavy metal ions attach to polymeric beads or fibrous filter media as they replace protons belonging to sulfonic acid ($–SO_3H$) or carboxylic acid (–COOH) groups on the surface.[4] Adsorption has many advantages over other technologies due to its effectiveness, flexible design, and low cost.[2,3]

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Biogenic manganese oxide (Mn(III/IV)O)[5] and fungal cells[6] are well-known bioadsorbents for heavy metals. Manganese (Mn(II))-oxidizing fungi are found throughout aquatic and terrestrial environments; biogenic Mn(III/IV)O naturally participates in the environmental remediation.[7–10] These ascomycetes were found to oxidize Mn(II)[11]—*Acremonium, Alternaria, Cladosporium, Coniothyrium, Curvularia, Penicillium, Phoma, Verticillium*—along with wood-rotting basidiomycetes (e.g. *Coprinellus*).[12] The exact mechanism of Mn(III/IV)O formation by fungi is not clear; fungi may produce multicopper oxidases to aid Mn(II) oxidation.[11]

In general, biogenic Mn(III/IV)O has superior adsorption properties compared to synthetically produced Mn oxides. Unit cell forms of MnO_6 octahedra reassemble layered (e.g. birnessite) or tunneled structures (e.g. todorokite).[8] Most biogenic birnessite contains a significant amount of Mn(III) in the octahedral layers.[8] Mn(III/IV)O has an abundance of vacancies, which lends to its highly adsorptive nature.[7,13] Dissolved salts are often found throughout the interstices of layered Mn(III/IV)O.[8]

Heavy metal removal is influenced by the surface area of adsorbents. Thus, nanoscale particles are expected to have greater adsorption efficiency per mass than microscale adsorbents that are 100–10,000 times their size.[2] Nanoparticle separation and contamination is a major challenge to its use in water purification.[14] Therefore, high surface area nanofibers are presumed the best platforms for particle confinement and next-generation hybrid materials.[15]

Electrospinning technique produces nanofibers from polymeric solutions that are placed under high voltage. Nanofibrous adsorbents are formed by electrospinning dopes containing nanosize particles[16] or dopes containing sol–gel precursors for their synthesis.[17,18] Also, inorganic particles are synthesized at the surface of nanofibers hydrothermally[19] or by the sol–gel approach for particle synthesis along nanofibers.[20] For example, Park et al.[21] seeded electrospinning dopes with polymerized micelles to nucleate the hydrothermal growth of calcium carbonate ($CaCO_3$) spheres—which threaded the nanofibers. The $CaCO_3$-coated nanofibers adsorbed anthraquinone, acid blue dye from water. Wu et al.[18] fabricated composite nanofibers of silicon dioxide (SiO_2), functionalized with thiol groups, that were capable of >400 mg/g of copper ion (Cu(II)) adsorption.

Thus, hybrids of Mn(III/IV)O-yielding fungi and nanofibers are posed as environmentally sound approaches for heavy metal removal from water. This novel approach immobilizes bioadsorbents along the surface of nanofibers. The implications of this innovative approach are to support the scalable use of nanotechnologies toward groundwater treatment. Mn(III/IV)O morphologies, as catalyzed by *Coprinellus* species (sp.) and *Coniothyrium* sp., were investigated. *Coprinellus* sp. and *Coniothyrium* sp. are plant pathogens, but neither of them poses serious threats to human health.[22] The adsorptive capacity of *Coniothyrium* sp. hybrids was tested against Mn(II), Cu(II), and lead (Pb(II)). The US Environmental Protection Agency's (US EPA) secondary maximum contaminant levels for Mn(II) is 0.05 mg/L.[23] US EPA action levels are 1.3 mg/L Cu(II) and 0.015 mg/L Pb(II) in drinking water. If one-tenth of tap water exceeds either action level, then additional treatments must be used to amend water systems.[24]

In this study, heavy metal adsorbents were fashioned from hybrids of electrospun nanofibers and Mn(II)-oxidizing fungi. Mn(II) oxidation and deposition along the surface of immobilized fungi and nanofibers were tested against growth conditions for *Coprinellus* sp. and *Coniothyrium* sp. Afterward, the efficacy of nanofibrous hybrids was tested for heavy metal adsorption in water charged with high concentrations of Mn(II), Cu(II), and Pb (II).

Materials

Strains designated C1CAP-d1AYA and ASB2-d1KA, classified as *Coprinellus* sp. and *Coniothyrium* sp., respectively, were isolated from the activated carbon column (CC) and air stripper (AS) of Superfund site, Lot 86, Farm Unit #1 remediation system in Raleigh, NC.[25,26] Dextrose, yeast extract, casamino acids, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic) acid (HEPES), agar, calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), manganese(II) chloride ($MnCl_2$), iron(III) chloride ($FeCl_3$), copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$), $CoCl_2{\cdot}6H_2O$, $Na_2MoO_4{\cdot}2H_2O$, and 88% hydrolyzed poly(vinyl alcohol) (PVA, having a molecular weight of ~130 kDa), 70% glutaraldehyde (GA) in water, and 1 mol/L sulfuric acid (H_2SO_4) were purchased from Sigma-Aldrich. Groundwater samples were donated by Wake County government in North Carolina.

Methods

Nanofiber preparation

Neat PVA and PVA/$MnCl_2$ nanofibers were electrospun from aqueous dopes. Therefore, 11 wt% of PVA was dissolved in 100 mL of water under constant stirring for 1 h at 60°C. PVA/$MnCl_2$ was prepared from a 100 mL solution of 11 wt% PVA and 0.1 g of $MnCl_2$.

PVA and PVA/$MnCl_2$ nanofibers were electrospun at 15 kV, horizontal tip to collector distance of 8 cm, and feed rate of 0.8 mL/h using an 18-gauge needle. Nanofibers were collected onto aluminum mesh at room temperature and 40%–50% relative humidity.

Crosslinking nanofibers

PVA and PVA/$MnCl_2$ nanofibers were crosslinked in GA solution. A 300 mmol/L GA in water/acetone was prepared

Table 1. Summary of nanofibers with and without fungal treatment.

Sample name	Description
PVA	Neat electrospun PVA nanofibers
PVA/$MnCl_2$	PVA nanofibers having 0.1 wt% $MnCl_2$
Mn(III/IV)O-#17	*Coniothyrium* sp. incubated in Mn(II)-containing media and have Mn(III/IV)O
–	*Coniothyrium* sp. incubated in Mn(II)-containing media with PVA nanofibers
Mn(III/IV)O-#17-PVA/$MnCl_2$	*Coniothyrium* sp. incubated in Mn(II)-containing media with PVA/$MnCl_2$ nanofibers
Mn(III/IV)O-PVA/$MnCl_2$ (-#17)	Mn(III/IV)O-PVA/$MnCl_2$ after removing *Coniothyrium* sp. hyphae with 2 h of ethanol sonication
–	Mn(III/IV)O-PVA after removing *Coprinellus* sp. hyphae with 2 h of ethanol sonication
–	Mn(III/IV)O-PVA/$MnCl_2$ after removing *Coprinellus* sp. hyphae with 2 h of ethanol sonication

PVA: poly(vinyl alcohol).

from 70% aqueous GA stock and 50 mL of acetone. One drop (~50 µL) of H_2SO_4 catalyst was added. Nanofibers of $4 \times 5\,cm^2$ were immersed into GA solution for 24 h at room temperature. Afterward, nanofibers were air-dried at room temperature for at least 24 h before use.

Media preparation

Liquid Leptothrix media, with and without Mn(II) ions, were prepared in three steps. In Step 1, 1 g of dextrose, 0.5 g of yeast extract, 0.5 g of casamino acids, and 2.38 g of HEPES acid were added to 1 L of distilled water. Afterward, in Step 2, this solution was autoclaved for 30 min and cooled to 60°C using a room-temperature water bath. Finally, in Step 3, 0.5 mmol/L $CaCl_2$, 0.83 mmol/L $MgSO_4$, 1 mmol/L $MnCl_2$, 3.7 mmol/L $FeCl_3$, and 1 mL of trace metal mix were added to prepare Leptothrix media having Mn(II) ions. Leptothrix media without Mn(II) did not have 1 mmol/L $MnCl_2$ added in Step 3.

For solid leptothrix media preparation, 15 g agar was added in Step 1 with the same steps. Trace metal mix was prepared by adding 10 mg $CuSO_4 \cdot 5H_2O$, 44 mg $ZnSO_4 \cdot 7H_2O$, 20 mg $CoCl_2 \cdot 6H_2O$, and 13 mg $Na_2MoO_4 \cdot 2H_2O$ in solution. Afterward, the trace metal mix was filtered through sterile 0.2 µm porous filters.

Fungal treatment of nanofibers

Leptothrix culture media were inoculated with *Coprinellus* sp. and *Coniothyrium* sp. in the presence of nanofibrous sheets (of $1 \times 1\,cm^2$ or $4 \times 5\,cm^2$). Nanofibers and fungal plugs were simultaneously added to liquid media. On solid plates, fungal plugs were placed on top of the nanofibers, under sterile conditions. PVA and PVA/$MnCl_2$ nanofibers were immersed in 20–30 mL of Leptothrix culture media in 75 mL Erlenmeyer flasks. Fungal growth over 4 weeks occurred at room temperature in the dark.

The fungi were removed from the fungi-nanofiber hybrids by immersion in 20 mL of deionized water, stirring at 500 r/min for 30 min, or sonication in 20 mL of water or ethanol for 2 h. Table 1 summarizes the samples used throughout this study and their descriptions.

Microscopic analysis

Confocal micrographs of fungi incubated on Leptothrix solid plates and in the presence of nanofibers were imaged on the LEXT OSL4000 3D. The morphology of fungi-nanofibers and nanofibers were observed using field emission scanning electron microscopy (FE-SEM, FEI, Verios 460L) at an operating voltage of 2 kV. Specimens were sputter-coated with gold and palladium before SEM analysis. Image analysis of SEM micrographs was used to calculate the average fiber diameter from at least 50 nanofibers.

Spectroscopic analysis of Mn(III/IV)O

The surface chemistry of nanofibers and their mycogenic hybrids were characterized by energy dispersive spectroscopy (EDS, attached to the Verios 460L). The operating voltage was 20 kV. EDS spectra were used to confirm the locale of metal oxides and absorbed heavy metals.

The Nomadic™ Raman Microscope by BaySpec was used to analyze Mn(III/IV)O nanofibers. Raman spectra were collected using the 532 nm laser at 150 mW power, 10X objective lens, 20 scans, and 1 s exposure time.

Heavy metal adsorption

Heavy metal sorption by PVA/$MnCl_2$, Mn(III/IV)O-#17, Mn(III/IV)O-#17-PVA/$MnCl_2$, and Mn(III/IV)O-PVA/$MnCl_2$(-#17) were measured. In total, 3 mg of each sample was added to 20 mL of the heavy metal ion solution.

Specimens were immersed in heavy metal solutions (of 0.55 mg/L Mn(II), 2.63 mg/L Cu(II), and 0.029 mg/L Pb(II)) at pH 7 for 24 h at room temperature. Inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin Elmer 8000) was used to measure unabsorbed concentrations of heavy metal ions.

Adsorption q (mg/L) was determined by equation (1)

$$q = V\frac{(C_i - C)}{S} \quad (1)$$

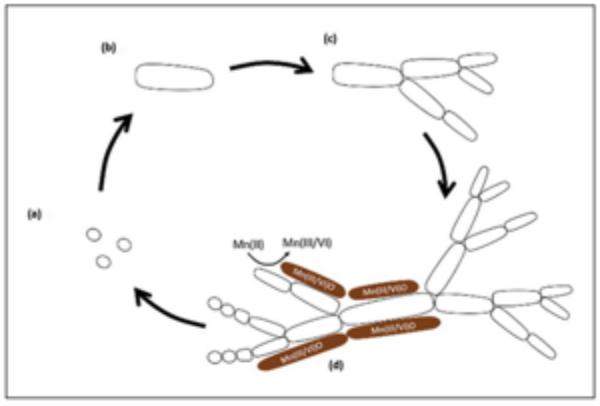

Figure 1. Fungus grows from (a) spores that germinate into (b) fungal cells; fungal cell grows into elongated cells of (c) hypha; (d) then hyphae aggregate to form mycelium that can reproduce fungi through the release of spores. Mn(II)-oxidizing fungi secrete enzymes that catalyze the conversion of Mn(II) into Mn(III/IV), as observed by dark brown mineral deposits of Mn(III/IV)O.

V is the volume (l) of solution in contact with sorbent; C_i and C are initial and final concentrations of the sorbate (mg/L), respectively; S is the dry weight of sorbent (g). Mn(II) release into solution was determined by the same way.

Results and discussion

Fungal growth and Mn(III/IV)O deposition

Figure 1 summarizes the processes of fungal growth and Mn(III/IV)O formation. Photographs of fungi grown in Leptothrix media revealed white, cotton ball-like mycelia (Figure 2(a) and (b)). Dark brown deposits, indicative of Mn(III/IV)O,[9,27] formed after 1 week of *Coprinellus* sp. and *Coniothyrium* sp. incubation in Mn(II)-containing media (Figure 2(a) and (b)). Adding PVA/$MnCl_2$ nanofibers into media without Mn(II) did not yield the Mn(III/IV)O deposits (Figure 2(a) and (b)). The average diameter of PVA nanofibers without $MnCl_2$ was 186 ± 116 nm, and PVA/$MnCl_2$ nanofibers was 126 ± 46 nm (Figure 2(c) and (d), respectively).

Fungal growth in the presence of nanofibers and Mn(II) was monitored over 4 weeks (Figure 2(e)). Incubation beyond 2 weeks did not enhance the spread of Mn(III/IV)O deposits along nanofibers. Nevertheless, nanofibers were maintained in culture media for more than 4 weeks to obtain fully grown fungi.[9] PVA/$MnCl_2$ nanofibers were more densely covered with mycelia.

PVA/$MnCl_2$ nanofibers yielded more mycelia growth, and hyphal structures tended to adhere more strongly to nanofibers in comparison to neat PVA nanofibers that were immersed in Mn(II)-containing media (Figure 2(a) and (b)). The role of Mn(II) for Mn(III/IV)O was evident from fungi grown on the solid plates which are placed in supporting information. Likewise, more mycelia and Mn(III/IV)O grew on PVA/$MnCl_2$ nanofibers when grown on Leptothrix solid plates (see Supplemental Figure S1). Confocal microscopy images (see Supplemental Figure S2) showed Mn(III/IV)O deposits only on PVA/$MnCl_2$ fibers cultured in Mn(II)-containing media.

The attachment of *Coprinellus* sp. and *Coniothyrium* sp. mycelia to nanofibers was confirmed by inspection of SEM images (Figure 3). Mn(III/IV)O morphology can differ between each fungal species.[9] *Coprinellus* sp. catalyzed the growth of spherical Mn(III/IV)O particles when cultured in Mn(II)-containing media. Spherical Mn(III/IV)O particles were more prominent along the hyphae of PVA/$MnCl_2$ nanofibers than on neat PVA nanofibers. *Coniothyrium* sp. catalyzed the growth of sheet-like Mn(III/IV)O in Mn(II)-containing media. Extracellular matrix appeared at the interface of nanofibers and *Coniothyrium* sp. hyphae. Round spores were observed as seen in Figure 3. Thus, nanofibers did not inhibit fungal reproduction by spores.

Fungi can secrete various oxidative enzymes as their hyphae continue to lengthen. This results in the cycling of nutrients throughout the environment.[28] Mn(II)-oxidizing fungi express extracellular oxidative enzymes, such as Mn(II) peroxidase. This group of proteins is capable of oxidizing Mn(II) to Mn(IV) at accelerated rates of Mn biomineralization: several orders of magnitude faster than either abiotic catalysis on mineral surfaces or homogeneous oxygenation in aqueous solution.[12,29] *Coprinellus* sp. and *Coniothyrium* sp. apparently have different modes of Mn(II) oxidation. Mn(II)-oxidizing enzymes associated with the hyphae cells of *Coprinellus* sp. In contrast, enzymes were secreted from *Coniothyrium* sp. hyphae. Oxidizing enzymes were captured by the extracellular matrix, which exuded from *Coniothyrium* sp. and onto the surrounding nanofibers (Figure 3(b)). Santelli et al.[9] reported that locale of Mn(III/IV)O deposition (i.e. on hyphal surfaces or extracellular polymer adjacent to cells) varies among different ascomycete fungi probably due to primary placement of enzymes.

Elemental analysis of fungal Mn(III/IV)O hybrids

Mn(II) within PVA/$MnCl_2$ fibers was not detected by EDS because Mn(II) at the surface of PVA/$MnCl_2$ nanofibers was below the EDS detection limit, that is, 0.1 wt%. Therefore, any Mn(II) detection must result from the oxidation of Mn(II) in solution. With both fungal types, Mn(II) content as measured by EDS was qualitatively higher along PVA/$MnCl_2$ than neat PVA nanofibers (Figure 4). EDS spectra showed impurities of Fe, S, Mg, and Ca within Mn(III/IV)O. As expected, Mn(III/IV)O can adsorb other metal ions from Leptothrix media. Ivarsson et al.[27] detected non-Mn(II) metal ions within the vacancies of biogenic Mn(III/IV)O by

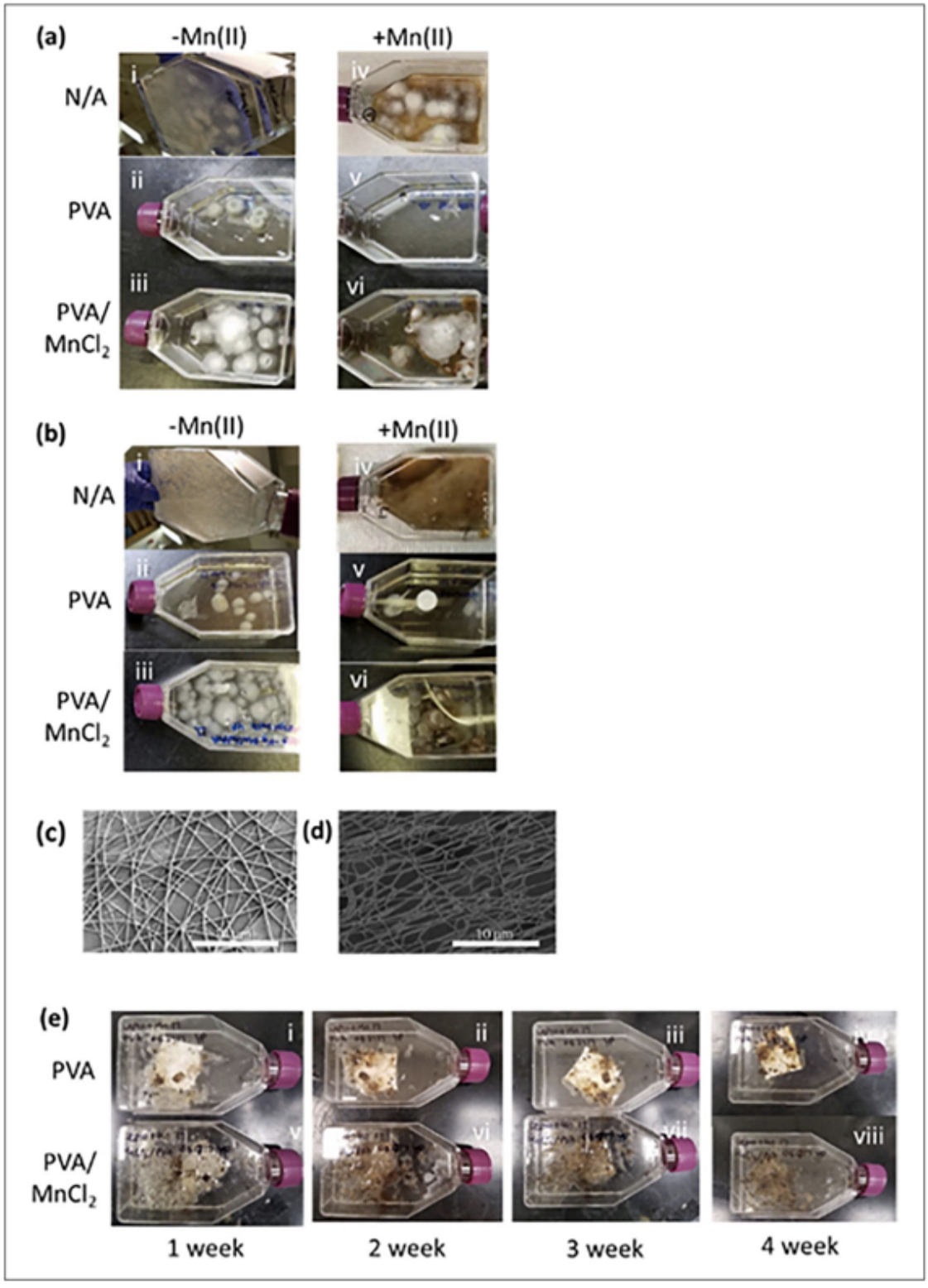

Figure 2. Fungi (a) *Coprinellus* sp. and (b) *Coniothyrium* sp. were grown for 1 week in Leptothrix media (a(i)–(iii), b(i)–(iii)) without Mn(II) or (a(iv)–(vi), b(iv)–(vi)) with Mn(II). Fungi were incubated (i, iv) without nanofibers or (ii, v) with PVA or (iii, vi) with PVA/$MnCl_2$ nanofibers (of $1 \times 1\,cm^2$). SEM images of (c) PVA and (d) PVA/$MnCl_2$ nanofibers are shown. Photographs show (e) *Coniothyrium* sp. grown for up to 4 weeks in Mn(II)-containing media the $4 \times 5\,cm^2$ sheet of nanofiber (either (i)–(iv) PVA or (v)–(viii) PVA/$MnCl_2$). This growth pattern was also observed for *Coprinellus* sp.

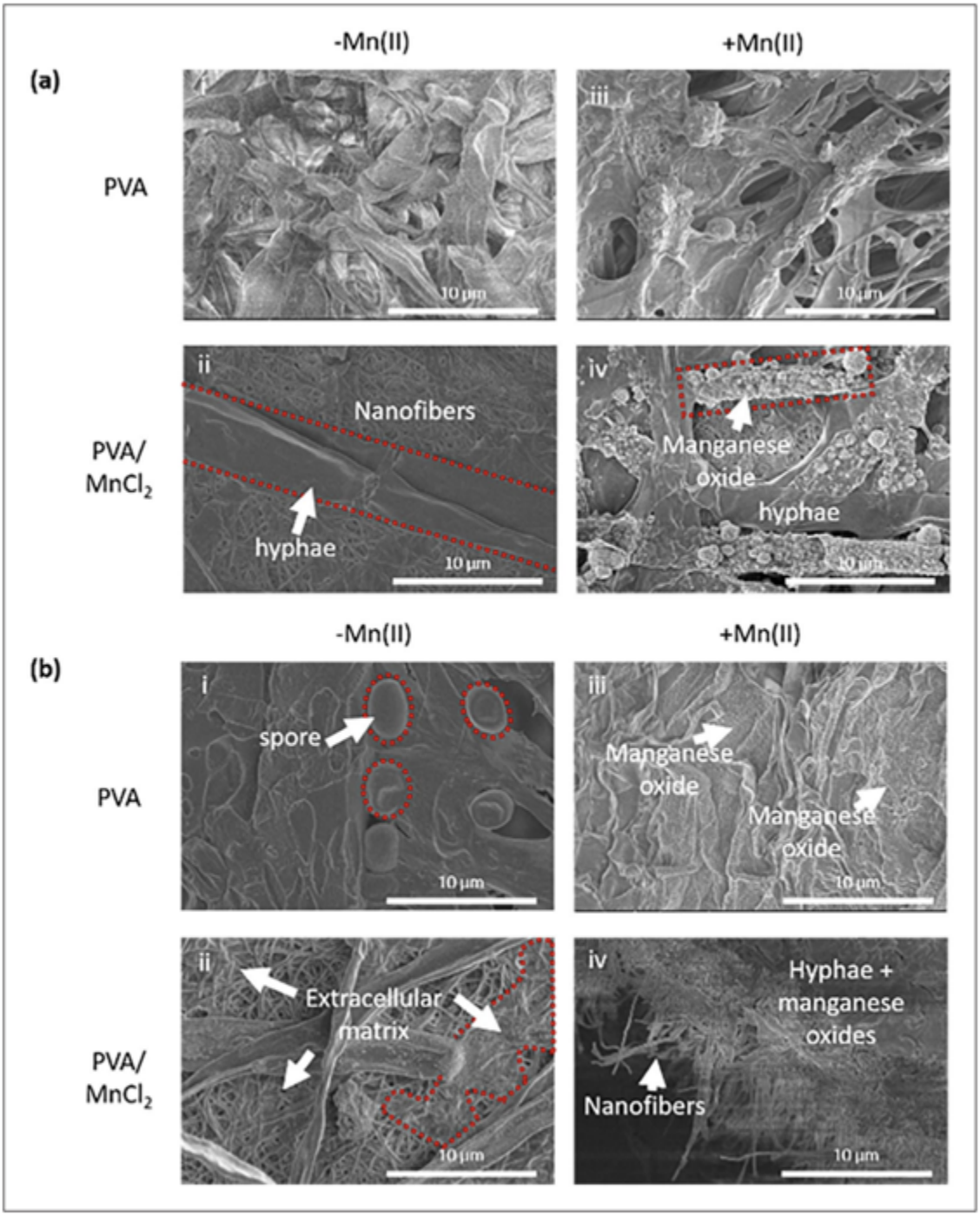

Figure 3. Micrographs show (a) *Coprinellus* sp. (b) *Coniothyrium* sp. grown on (i, iii) PVA and (ii, iv) PVA/$MnCl_2$ nanofibers immersed in liquid media (i, ii) without Mn(II) (−Mn(II)) or (iii, iv) with Mn(II) (+Mn(II).

EDS. *Coniothyrium* sp. can release enzymes coupled with P, S, K, and Ca.

Mn(III/IV)O deposition on nanofibers

A layer of fungal hyphae intermeshed with nanofibers is illustrated in Figure 5. As hyphae are grown on the surfaces of nanofibers, their filament-like structures are entangled with each other to form a layer. However, cracks formed in the topical layer of *Coniothyrium* sp. after stirring hybrids immersed in water (Figure 5(a)). The cross-sectional view shows hyphae attached to both sides of nanofibers in a sandwich structure (Figure 5(b)). After sonicating the sample for 1 h in water, some fungal hyphae remained on the

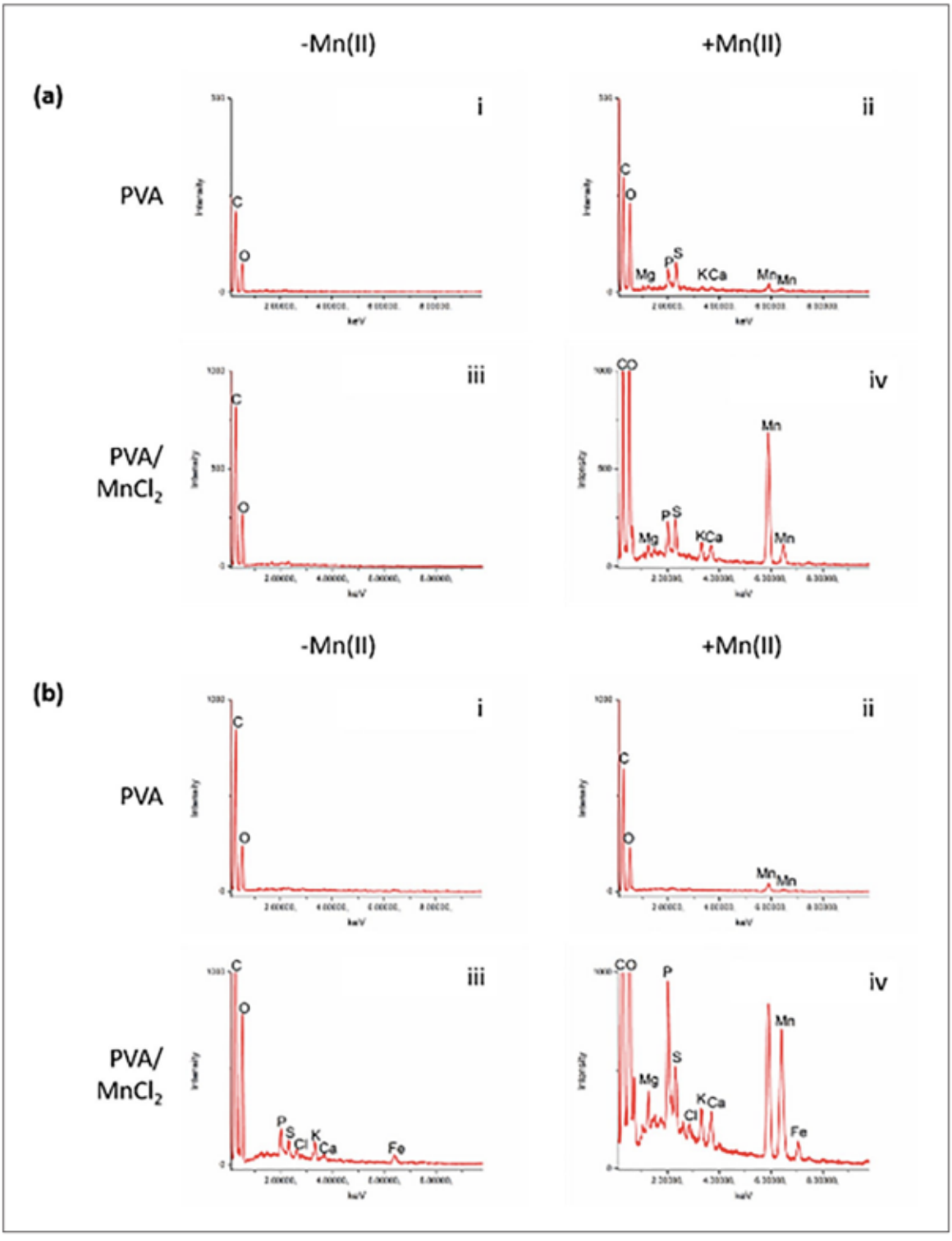

Figure 4. EDS graphs of (a) *Coprinellus* sp. and (b) *Coniothyrium* sp. on PVA (i, ii) or PVA/$MnCl_2$ nanofibers (iii, iv) in liquid media (i, iii) without Mn(II) (−Mn(II)) or (ii, iv) with Mn(II) (+Mn(II)).

nanofibers (Figure 5(c)). But sonicating the fungi-nanofiber hybrids for 2 h in ethanol removed most of the fungal hyphae (Figure 5(d)).

This latter technique was used to remove fungus from hybrids that were incubated in Mn(II)-containing media. A conformal Mn(III/IV)O coating was found on the fiber

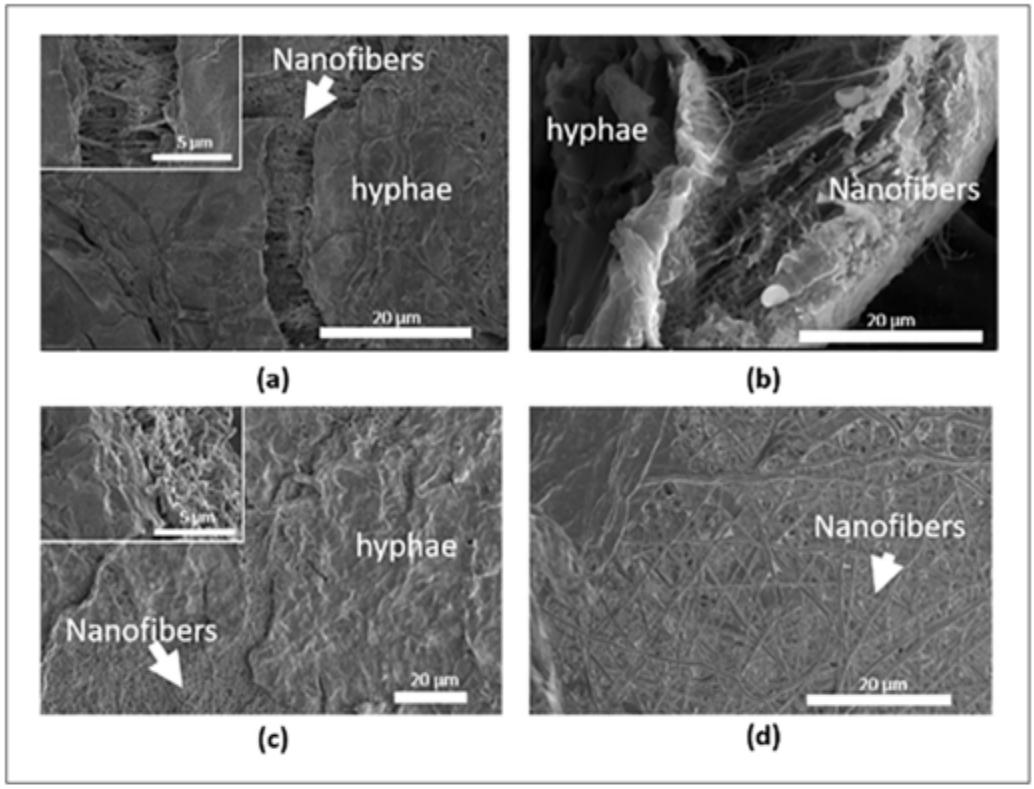

Figure 5. SEM images of Mn(III/IV)O-#17-PVA/$MnCl_2$ after water immersion and magnetic stirring for 30 min at 500 r/min: both (a) longitudinal and (b) cross-sectional views are shown. Hybrids were further treated with (c) 1 h of sonication in water or (d) 2 h of sonication in ethanol.

surface after removing hyphae by 2 h of ethanol sonication (Figure 6(d)). Mn was not detected on the surface of PVA and PVA/$MnCl_2$ nanofibers that were incubated with *Coprinellus* sp. (Figure 6(a) and (b)). Also, Mn was not identified on the surface of neat PVA nanofibers that were incubated with *Coniothyrium* sp. (Figure 6(c)).

Heavy metal ion removal by Coniothyrium sp.-nanofiber hybrids

Coniothyrium sp.-nanofiber hybrids with and without fungal hyphae were compared with Mn(III/IV)O-coated fungi and PVA/$MnCl_2$ nanofibers in terms of heavy metal removal in laboratory charged water. Water samples were charged with 0.55 mg/L Mn(II), 2.63 mg/L Cu(II), and 0.029 mg/L Pb(II) at levels twice as high as the US EPA's "secondary maximum contaminant levels" for drinking water. Each nanofiber sample was capable of removing Mn(II), Cu(II), and Pb(II) from laboratory charged solutions (Table 2). The lowest amounts of heavy metal adsorption occurred by PVA/$MnCl_2$ nanofibers. It is noteworthy that hyphae-nanofiber derivatives removed heavy metals as well if not better than Mn(III/IV)O-coated hyphae alone (Table 2). Both Mn(III/IV)O-#17-PVA/$MnCl_2$ and Mn(III/IV)O-PVA/$MnCl_2$(-#17) removed more than 90% of the Mn(II) from the water (i.e. > 150 mg/L) in 24 h, then charged water was well below the US EPA limit. The nanoscale dimensions of Mn(III/IV)O-coated nanofibers appear to enhance Mn(II) sorption capacities. Taffarel and Rubio[30] experimented with hybrid nanotechnologies and reported Mn(III/IV)O-coated zeolite at 4 g/L of water removed 90% of the Mn(II) from water within 2 h.

Hybrid nanofibers were capable of removing the largest amount (mg/g) of Mn(II) from water but were least capable of removing Pb(II) from water. Molecular size could potentially exclude larger heavy metal ions from absorption by Mn(III/IV)O. Laus and De Fávere[31] also noted the importance of molecular size because Cu(II) was adsorbed by chitosan better than Cd(II). Nelson et al.[32] reported Pb(II) adsorption by bacteriogenic Mn(III/IV)O and synthetic β-MnO_2 were 1344.67 mg/g and 0.078 mg/g, respectively. Droz et al.[12] noted that bacteriogenic Mn(III/IV)O (from *Pseudomonas putida* GB-1) had more structural defects than mycogenic Mn(III/IV)O (from *Coprinellus* sp.). However, extended X-ray absorption fine structure (EXAFS) spectra examinations reported by Duckworth et al.[26] indicated mycogenic manganese oxides from Lot 86 biofilm (containing these particular isolates) were structurally comparable to bacteriogenic Mn(III/IV)O and with similar binding of Ba(II), Co(II), and Zn(II).

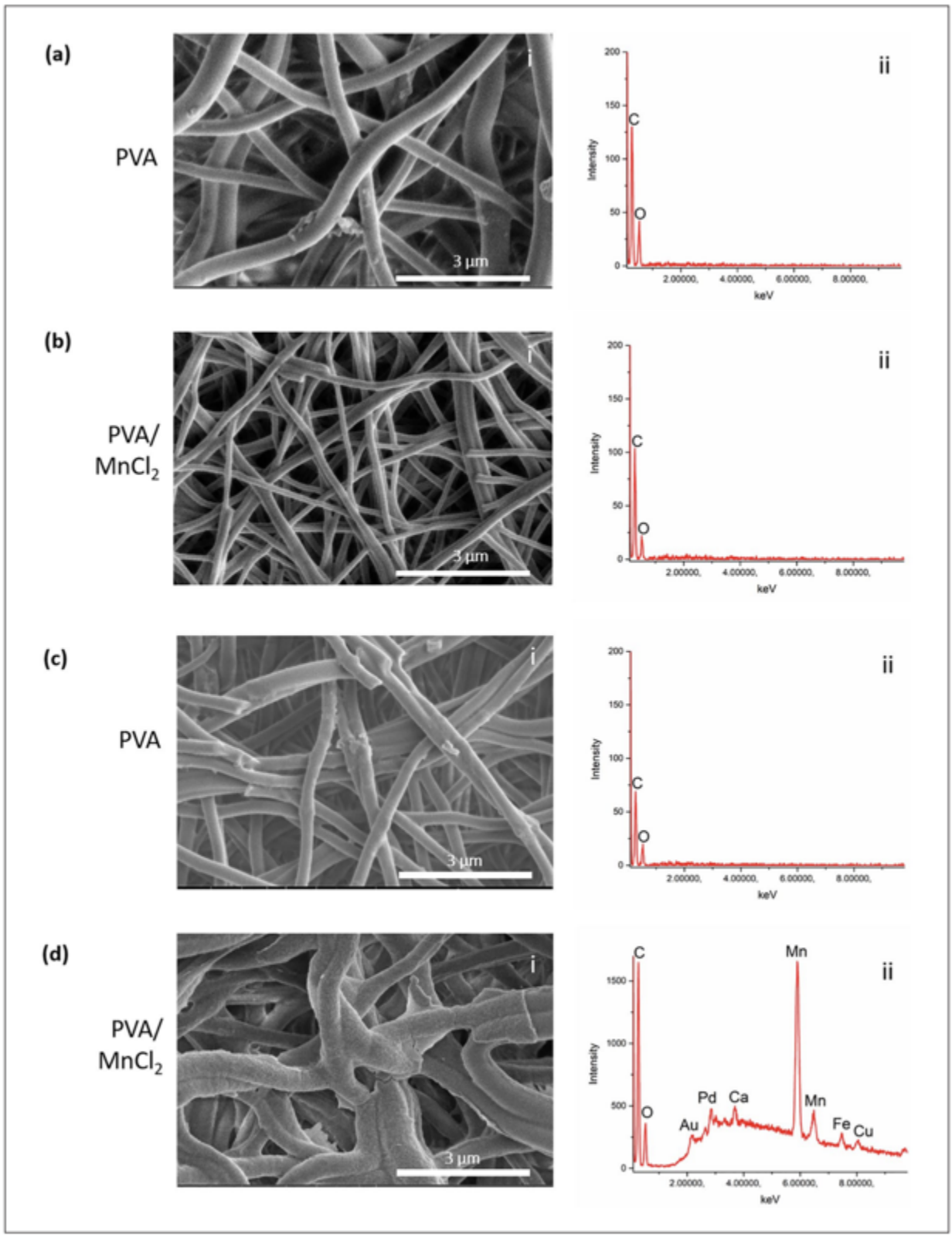

Figure 6. Fiber surface after removing fungal hyphae by 2 h of sonication in ethanol was observed by (i) SEM and (ii) EDS spectra. *Coprinellus* sp. incubated with (a) PVA and (b) PVA/$MnCl_2$ nanofibers are shown. *Coniothyrium* sp. incubated with (c) PVA and (d) PVA/$MnCl_2$ nanofibers are shown.

Table 2. Comparing aqueous heavy metal adsorption *Coniothyrium* sp. derived Mn(III/IV)O coatings and nanofibers after 24 h.

	Mn(III/IV)O-#17	$PVA/MnCl_2$	Mn(III/IV)O-#17-$PVA/MnCl_2$	Mn(III/IV)O-$PVA/MnCl_2$ (-#17)
Mn(II) (mg/g)	2.69 ± 0.01	0.66 ± 0.01	3.41 ± 0.01	3.65 ± 0.01
Cu(II) (mg/g)	10.22 ± 0.08	6.62 ± 0.04	11.64 ± 0.03	10.04 ± 0.03
Pb(II) (mg/g)	0.10 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.04 ± 0.01

PVA: poly(vinyl alcohol).

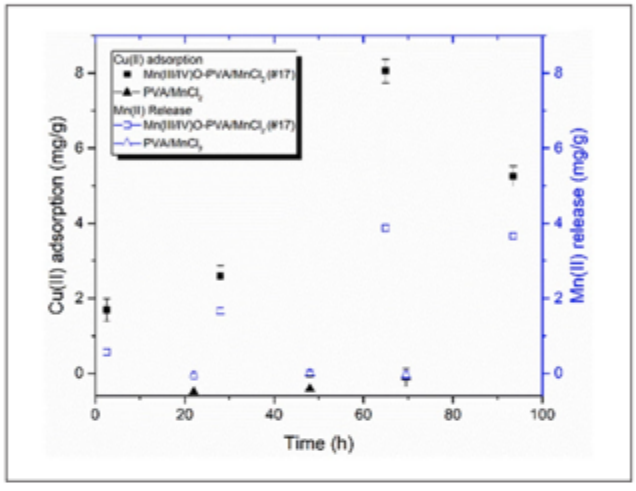

Figure 7. Cu(II) adsorption and Mn(II) release into Cu(II) solution (at 2.6 mg/L) by Mn(III/IV)O-$PVA/MnCl_2$ (-#17) and $PVA/MnCl_2$ nanofibers over time.

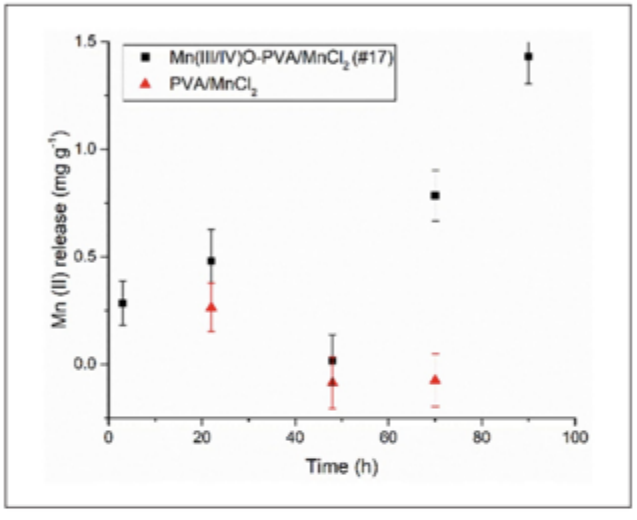

Figure 8. Mn(II) release to deionized (DI) water with nanofibers.

In the interest of having nanofibers scaffold Mn(II)-oxidizing fungi in nature, Cu(II) adsorption by hybrid Mn(III/IV)O-#17-$PVA/MnCl_2$ was tested in the laboratory. Over time, Cu(II) concentration decreased as Mn(II) concentration increased (Figure 7). However, the diffusion of Mn(II) from samples of nanofiber was not a significant source of Mn(II) leaching. Figure 8 shows lower levels of Mn(II) released from samples immersed in deionized water over time. Other heavy metal ions appear to replace Mn(II) among the mineral deposits. But in their absence, Mn(II) is readily adsorbed by Mn(III/IV)O (Table 2).

Groundwater is a natural example of water having multiple types of heavy metal ions. Composition of groundwater is shown in Supplemental Table S1 (see supporting information). Therefore, *Coniothyrium* sp.-nanofibrous hybrids were immersed in groundwater. The concentration of Zn(II), Fe(II), and Pb(II) decreased in water due to Mn(III/IV)O adsorption. But, Mn(II) increased in the presence of other heavy metal ions (Table 3). Thus, it is important to understand the limitations of Mn(II) removal. Biogenic Mn(III/IV)O formation reduces Mn(II) from the environment; nevertheless, ion exchange between heavy metal ions and Mn(II) within the metal oxides can in turn release Mn(II) into the environment. Therefore, Mn(II)-oxidizing microbials must remain active to continually convert Mn(II) to metal oxide adsorbents.

Table 3. Comparing heavy metal adsorption over 24 h from groundwater between the control and hybrid *Coniothyrium* sp. nanofiber.

	$PVA/MnCl_2$	Mn(III/IV)O-#17-$PVA/MnCl_2$
Fe(II) *removal* (%)	17 ± 1	16 ± 1
Mn(II) *removal* (%)	17 ± 1	−642 ± 3

Note: Original concentrations of groundwater heavy metal ions at pH 7 were below the detection limits of Pb(II) (< 0.003 mg/L), Zn(II) (< 0.5 mg/L), Fe(II) (1.21 mg/L), and Mn(II) (0.11 mg/L).
PVA: poly(vinyl alcohol).

Conclusion

Having the nanofibers scaffold Mn(III/IV)O mitigates contamination from unconfined fungus and inorganic particulates in the water. Oxidative enzymes secreted from *Coniothyrium* sp. and onto the surface of nanofibers. As a result, Mn(II)-oxidizing enzymes coated the surface of nanofibers and catalyzed the nucleation and growth of

Mn(III/IV)O along them. For synthetic mineralization, nanofibers are typically seeded with polyelectrolytes to nucleate the growth of inorganic minerals.[21,33] Mn(III/IV)O-coated nanofibers and fungi-nanofiber hybrids effectively reduced Mn(II) contamination from laboratory charged water. However, Mn(II) leaching was noticed when Mn(III/IV)O nanofibers were exposed to groundwater containing various heavy metal contaminants.

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Supplemental material

Supplemental material for this article is available online.

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Resource 6:

Community Guide to Bioremediation

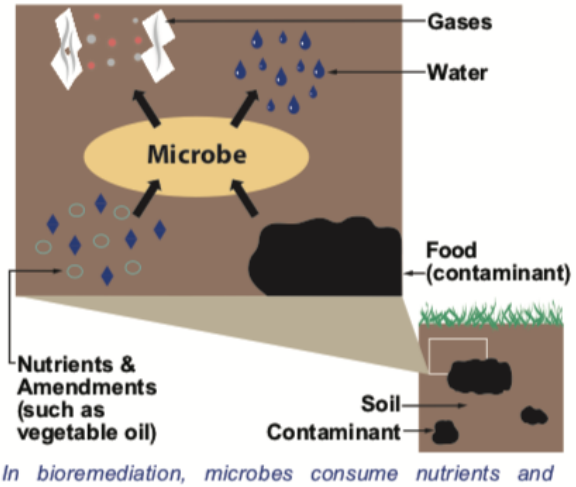
What Is Bioremediation?

Bioremediation is the use of microbes to clean up contaminated soil and groundwater. Microbes are very small organisms, such as bacteria, that live naturally in the environment. Bioremediation stimulates the growth of certain microbes that use contaminants as a source of food and energy. Contaminants treated using bioremediation include oil and other petroleum products, solvents and pesticides.

How Does It Work?

Some types of microbes eat and digest contaminants, usually changing them into small amounts of water and gases like carbon dioxide and ethene. When soil and groundwater do not have enough of the right microbes, microbes are added. This process is called "bioaugmentation."

For bioremediation to be effective, the right temperature, nutrients and food also must be present. Proper conditions allow the right microbes to grow and multiply—and eat more contaminants. If conditions are not right, microbes grow too slowly or die. Adding "amendments" may improve conditions. Amendments range from household items like molasses and vegetable oil, to air and chemicals that produce oxygen. Amendments are often pumped underground through wells to treat soil and groundwater "in situ" (in place).



In bioremediation, microbes consume nutrients and contaminants, and release gases and water.

The conditions necessary for bioremediation in soil cannot always be achieved in situ. The climate may be too cold for microbes to be active, or the soil might be too dense to allow amendments to spread evenly underground. At such sites, EPA might dig up the soil to clean it "ex situ" (aboveground) on a pad or in tanks. The soil may be heated, stirred or mixed with amendments to improve conditions.

Sometimes mixing soil causes contaminants to evaporate before the microbes can eat them. To prevent the vapors from contaminating the air, the soil can be mixed inside a special tank or building where vapors from chemicals that evaporate are collected and treated.

Is Oxygen Always Needed?

Some contaminants can be bioremediated only in an aerobic environment — one that contains oxygen — because the microbes need oxygen to grow. Other contaminants can be bioremediated only in an anaerobic (without oxygen) environment. Anaerobic microbes do not need oxygen to grow.

To clean up contaminated groundwater in situ, wells are drilled to pump some of the groundwater into aboveground tanks. The water is mixed with amendments before it is pumped back into the ground. An alternative is to pump the amendments directly underground. The amendment-enriched groundwater allows microbes to bioremediate the rest of the contaminated groundwater underground. Groundwater also can be pumped into an aboveground "bioreactor" for ex situ treatment as part of a "pump and treat" system. (See *Community Guide to Pump and Treat*.) Bioreactors are tanks in which groundwater is mixed with microbes and amendments for treatment. Depending on the site, the treated water may be pumped back underground or discharged to surface water or to the public sewer system.

How Long Will It Take?

It may take a few months or even years to bioremediate a site, depending on several factors that vary from site to site. For example, bioremediation will take longer where:

- Contaminant concentrations are high, or contaminants are trapped in hard-to-reach areas, like rock fractures or dense soil.
- The contaminated area is large or deep.
- Conditions such as temperature, nutrients and microbe population must be modified.
- Cleanup occurs ex situ.

Is Bioremediation Safe?

Bioremediation relies on microbes that live naturally in soil and groundwater. These microbes pose no threat to your community. Microbes added for bioaugmentation typically die off once contaminants and proper conditions are gone. The chemicals added to stimulate bioremediation also are safe. For example, the nutrients added to make microbes grow are commonly used on lawns and gardens. To ensure that bioremediation is working and to measure progress, samples of soil and groundwater are tested regularly.

How Might It Affect Me?

Bioremediation often occurs underground and does not cause much disruption to your community or the site. You may notice increased truck traffic as equipment comes to the site. You also might hear the operation of pumps, mixers and other construction equipment used to add amendments or improve site conditions to begin the bioremediation process.

Why Use Bioremediation?

Bioremediation has the advantage of using natural processes to clean up sites. Because it may not require as much equipment, labor or energy as some cleanup methods, it can be cheaper. Another advantage is that contaminated soil and groundwater are treated onsite without having to transport them elsewhere for treatment. Because microbes change the harmful chemicals into small amounts of water and gases, few if any waste by-products are created.

Injection of vegetable oil underground to improve conditions for bioremediation.

Bioremediation has been selected for use at hundreds of Superfund sites and other cleanup sites across the country.

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Example

Anaerobic bioremediation is being used to clean up groundwater at a portion of Dover Air Force Base in Delaware. Previous industrial activity and storage of hazardous waste contaminated an area approximately 2,800 feet long and 650 feet wide with solvents. Remediation began in 2006 with the injection of more than 100,000 gallons of a solution of vegetable oil and sodium lactate. The solution was injected in 49 locations to depths of 5 to 30 feet underground. In 2012, a second source of contamination several hundred feet from the original source area was detected. An additional 140,000 gallons of solution were injected in 20 new locations. Routine groundwater monitoring shows that concentrations have decreased to below cleanup levels in all monitoring wells, with occasional small fluctuations above cleanup levels in a few wells.

For More Information

- About this and other technologies in the Community Guide Series, visit: https://clu-in.org/cguides or https://clu-in.org/remediation/
- About use of cleanup technologies at a Superfund site in your community, contact the site's community involvement coordinator or remedial project manager. Select the site name from the list or map at http://www.epa.gov/superfund/sites to view their contact information.

Resource 7:

How Microbes Help Us Reclaim Our Wastewater

April 9, 2020 SHARE THIS

A key challenge to setting up a civilization is the plumbing. This is one reason why major cities, particularly those with a long history, are conspicuously placed along rivers; pressurized water and the kind of plumbing we have today would not be unfamiliar thousands of years ago to civilizations like the Mayans. Despite its long and pivotal role in our success, once wastewater is done swirling down our toilet bowls, most of us are blissfully unaware of what happens next. It may not come as a surprise that microbes are heroes of this untold story.

Separating the Waste from the Water

Before we examine these little heroes of sanitation, let's establish the big picture of wastewater management. The ultimate goal is to take water rendered unusable by waste and purify it sufficiently to restore it to the environment. Waste removed during the process is digested by microbes, and what remains is dried and disposed of in landfills, incinerators or applied to soil as a conditioner, depending on the source and process. Large-scale operations manage the bulk of our wastewater and follow a process called activated sludge. Invented a little over 100 years ago, this process incorporates the following basic steps: filtration, activation (aeration), clarification (settling) and disinfection.

Sludge is the euphemistic term used to describe the brown, viscous liquid that results after raw sewage has been filtered to remove grit. The sludge itself is inhabited by a diverse community of microbes, including bacteria, protozoans and even some eukaryotes like tardigrades, that have hitched a ride (perhaps through us) along the sewers connecting our homes to the waste management facility. Sludge comprises an incredibly rich medium, full of organic matter that we find unappetizing, but bacteria find delicious. Once this sludge has been processed by bacteria, it is called activated sludge, which can refer to both the material itself and the waste management process.

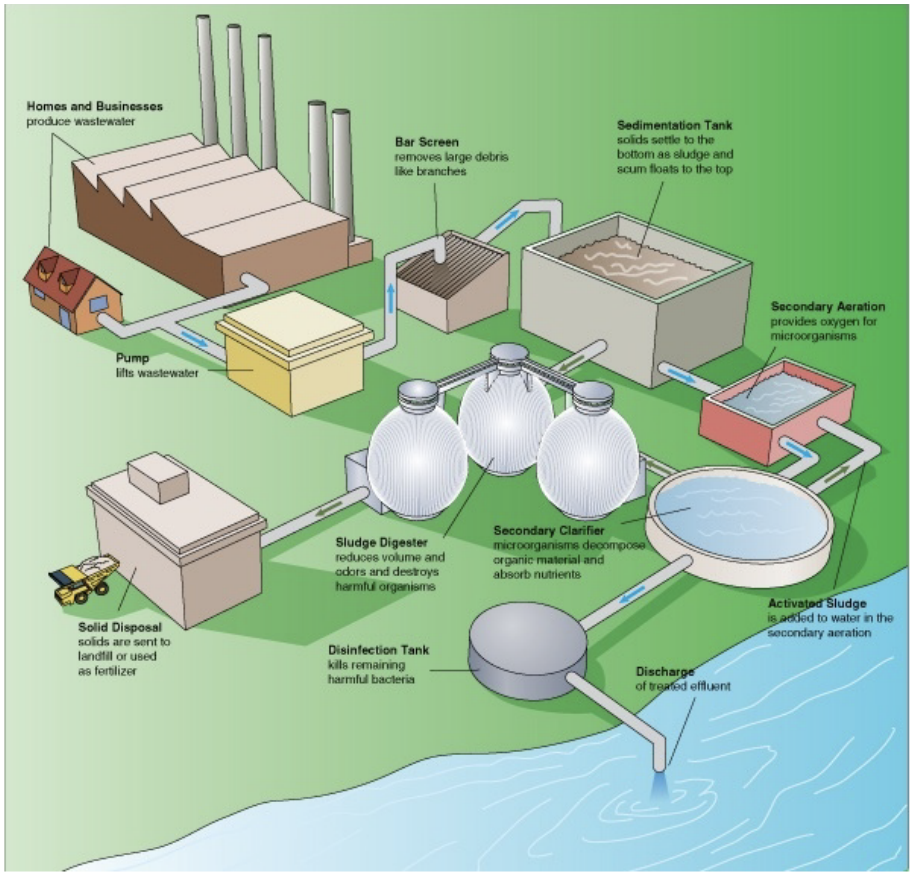


Illustration showing the main steps of the activated sludge process used by large-scale wastewater management facilities.
Source: https://commons.wikimedia.org/wiki/File:Steps_in_a_typical_wastewater_treatment_process.png

The cast of characters varies in each waste management facility, but a recent global survey of the microbiome of wastewater activated sludge found that there are 28 core bacterial members of healthy activated sludge. The most abundant of these are *Dokdonella kunshanensis*, *Zoogloea* species and *Nitrospira* species. These are all aerobic, gram-negative bacteria. We know little about *Dokdonella kunshanensis,* other than it can be readily isolated from activated sludge. We know more about the other two species. The name *Zooglea* means “living glue” because of the species' proclivity to form sticky biofilms. *Nitrospira* species help oxidize nitrite to nitrate, and are important for cycling aquaria because nitrate is much less toxic to fish than the ammonia they excrete. As we shall see, these traits facilitate the activated sludge process. However, these most abundant bacteria still represent only a small percentage (~3% of total abundance) of the diverse bacteria present in activated sludge microbiomes.

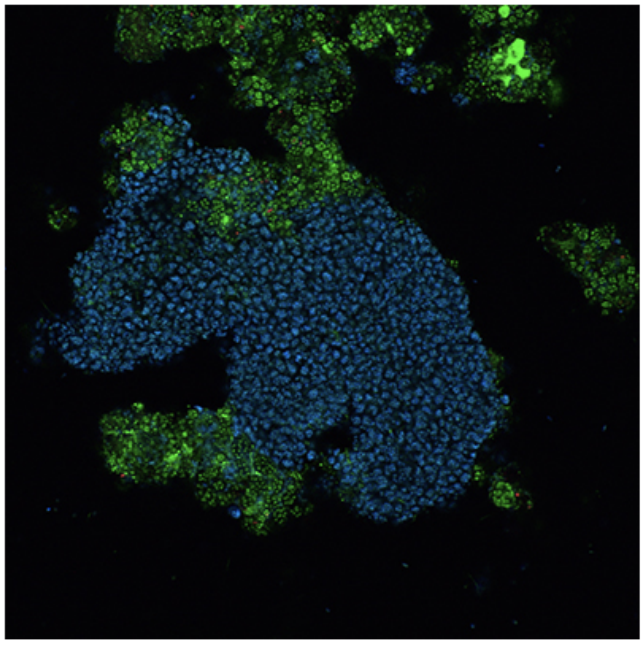
Aeration

After passing through filtration, key bacteria are alive, but not thriving like we need them to, so we 'activate' them through aeration. Stirring or bubbling the sludge introduces oxygen throughout, which encourages air-loving microbes to begin to actively grow and reproduce, while discouraging the growth of other kinds of microbes. This simple selection, inherent to the process, is similar to how home microbiologists cultivate a specific, useful subset of microbes for composting or for a sourdough starter. In fact, facilities sometimes prime incoming sludge with activated sludge to ensure bacterial communities from healthy batches are present from the beginning.

The aerobic bacteria in the sludge digest the organic material around them in order to reproduce and grow, and change the chemical makeup of the sludge, oxidizing ammonia into nitrate and nitrite in a process called nitrification. The process follows a progression that will be familiar to anyone who has studied microbiology: there is a lag period where these bacteria initially begin to grow, followed by an exponential growth phase, a stationary phase and finally a senescent phase where starving bacteria begin to die off. In their bubbly, sludgy new home, these bacteria are doing most of the work for us, turning sludge into more bacterial cells.

Most of the role that we play in this process is trying our best to keep the microbes on track. This involves taking samples of the sludge to track its progress. Metrics, like the amount of dissolved oxygen and organic matter, the amount and types of bacteria, such as culturable indicator species (e.g., coliform bacteria), and other indicators, are used to identify various stages of the process. Waste management facilities also use biological oxygen demand (a measure of the amount of oxygen being consumed by microbes) to calculate the food to mass (or microbe) ratio. These values allow scientists to chart their course to the stationary phase when the sludge no longer needs to be aerated.

When things are going well, it's easy to see. Clumps of bacteria, called flocs, form in the sludge as these microbes help us reclaim the water within. Similar to its homophone, flocculation is a process where these aerobic bacteria produce biofilms composed of extracellular polymeric substances that allow them to stick together. These biofilms help microbiologists monitor when a healthy consortium of bacteria are actively working to digest waste, while signs like excessive foam point to microbes that aren't team players.



A floc of bacteria at 400X mangification removing phosophorous from medium in the lab. All bacteria were stained green, and Candidatus Accumulibacter Phosphatis, which accumulates phosphorus, were stained in blue. Courtesy of Connor Skennerton.
Source: https://commons.wikimedia.org/wiki/File:EBPR_FISH_Floc.jpg

If the wrong microbes show up or the process goes off track, then we intervene chemically or remove excess sludge. Filamentous bacteria can become the "wrong microbes" if they don't cooperate with the biofilm consortia of bacteria and produce excessive filaments. This "filament bulking" prevents sludge from settling. In particular, *Nocardia* species and *Microthrix parvicella* convert oil and grease into a brown foam by increasing hydrophobicity in the system, which stabilizes the foam.

By the end of the process, a mature food chain of diverse microorganisms capable of transforming the sludge biochemically emerges. Bacteria feed on sludge, amoebae and ciliates (such as peritrichs) feed on the bacteria and tardigrades (and the occasional nematode) comprise the apex predators. These higher order members of the food web become more prominent during the exponential phase of bacterial growth, but if they become too common, it's a sign something has gone wrong (such as aerating the sludge for too long).

Clarification and Disinfection

When it's time to stop bubbling, the activated sludge enters its next phase: clarification. As it says on the back of bottles of an unnamed juice brand "settling is natural," and so we wait while the flocs and remaining sludge settle out of a now watery solution. Once the water has clarified to the satisfaction of the facility, the activated sludge, which has concentrated at the bottom, is sent off for further processing.

Remaining sludge goes through a second bacterial digestion without oxygen. Anaerobic bacteria further break down the sludge and reduce nitrate and nitrite into nitrogen gas through a process called denitrification. Biogas (primarily methane and carbon dioxide) produced during this anaerobic digestion is burned off or further purified for sale to energy companies. Such anaerobic digestion can occur at various stages of the process.

The very last of the activated sludge that survives primary and secondary microbial digestion is then dried. This "waste activated sludge" is ready to leave the facility as fertilizer or smoke, depending on its composition. Here, many weeks and microbial assists later, is the final destination of our modern sewage.

The supernatant (Latin for "great swimmers") on the top after clarification is disinfected with chemicals, like chlorine, or with ultraviolet (UV) radiation. This final step effectively kills any remaining organisms, pathogenic or otherwise, that have made it this far. If pathogens are a concern, checks are made throughout the process to ensure they are eliminated as the water is reclaimed. Finally, the water that may have once passed through your kidneys is ready to rejoin the ecosystem, often by way of local lakes and rivers.

For most of us, our contribution to this process is pressing the toilet handle. As is often the case, we can thank microbes for doing the hard work and making it look easy.

RESEARCHER ARTICLE NUTRIENT CYCLING WASTE WATER BIOREMEDIATION MICROBIOME

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