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Evaluation of ozone for preventing fungal influenced corrosion of reinforced concrete bridges over the River Nile, Egypt

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Abstract Fungal influenced corrosion (FIC) of some corroded sites in three selected bridges [Embaba bridge (E-bridge), Kasr al-Nile-bridge (K-bridge) and University bridge (U-bridge)] located over the River Nile in Egypt were investigated. Six fungal species, belong to 12 fungal genera, were isolated from the corroded reinforced concrete of the three tested bridges. Fourier transform infrared spectroscopy (FTIR) was screened for the most dominant fungal species (*Fusarium oxysporium)* which showed in all tested bridges that indicated the presence of amine group accompanied with polysaccharides contents. FIC of the most deteriorated bridge (K-bridge) was documented with FTIR. The association of fungal spores with corrosion products was recorded with scanning electron microscope (SEM). Evaluation of ozone for preventing FIC of the K-bridge was carried out by recording the corrosion rate and the corresponding inhibition efficiency (IE%). No mycelial growth with 100% IE was observed at 3 ppm ozone concentration after 120 min exposure time. With longer duration of ozone exposure, the membrane permeability of *F. oxysporium* was compromised as indicated by protein and nucleic acid leakages accompanied with lipid and tryptophan oxidation. The total intracellular and extracellular proteins of *F. oxysporium* were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) indicated the increasing of the supernatant protein on the expense of the cellular protein bands with extending ozone exposure time (0–80 min).

Keywords Fungi · Corrosion · Ozone · Bridges

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Introduction

Bridges located in the river environments suffer many corrosion problems, where microbial contamination of concrete causes corrosion of the embedded reinforcement. This leads to cracking of the concrete from the expansive forces of the corrosion product (Cramer et al. 2002). Corrosion is a long term process that effectively weakens structural elements and increases their vulnerability to extreme loads. Resin coated concretes are susceptible to microbial deterioration (Milde et al. 1983). Corrosion of concrete has an enormous economic impact, especially when replacement or repair of corroded municipal systems or highway infrastructures is required (Gu et al. 1998). Reinforced concrete bridges subject to corrosion was tested by Choe et al. (2009).

The role of microorganisms in concrete corrosion processes has been linked to the generation of acids (Sand et al. 1994). Microbial involvement in the biodegradation of concrete has been studied extensively (Jozsa et al. 1996; Mansch and Bock 1994).

Fungal influenced corrosion (FIC), can be defined as an electrochemical process where the participation of the fungi is able to initiate, facilitate or accelerate the corrosion reaction (Aviam et al. 2004). Fungi modify the environment, creating crevices, differential aeration zones or a more aggressive environment with the presence of metabolites (Ibars et al. 1992).

Corrosion control of fungal colonies with biocides is very difficult because of their potential hazard and also some of fungal enzymes are capable of breaking down toxic biocides and converting them to nutrients for the cells (Costerton et al. 1995).

Ozone is a powerful oxidizing agent which finds increasing application in many different fields to kill microbes (Azarpazhooh 2008). The use of ozone for sterilizing water bridge was necessary, where the presence of water often accelerates ozone reactions with organic substances (Shizaki et al. 2008). Ozone has inhibitory effect on a variety of microorganisms (Yamazaki et al. 2004). Successful use of ozone as a disinfectant for freshwater aquaculture systems has been recognized, where ozonation of seawater has also received considerable attention (Sugita et al. 1992). Ozonated water has a rapid antimicrobial effect on microorganisms (Nagayoshi et al. 2004).

The unique well stated experimental fact reported is that ozone attacks the unsaturated fatty acids present in cell membranes with the possible consequent lipid peroxidation (Bocci et al. 2002). Microbial cell damaged and inactivated because the oxidizing action of ozone is directed toward the nucleic acids of these organisms (Flyunt et al. 2002). The initial ozone attack is directed against the cell walls with subsequent leakage of cellular content and destruction of the bound DNA, however, ozone is also mutagen and this implies the direct DNA damage (Theruvathu et al. 2001). Ozone react with various amino acids but sulphur containing amino acids like trypotophan are most susceptable (Huth et al. 2007). The deleterious effect of ozone on life is an advantageous to human where, the toxicity for small animals is 3–12 ppm (Ali 2006).

In this paper, particular attention is devoted to bridges subject to a microbial hazard. Isolation and identification of fungal species on three tested bridges over the River Nile in Egypt were carried out. The fungal deteriorated bridge samples were showed by SEM. Investigation of the corrosion products on the most corroded bridge was carried out by FTIR spectroscopy. The present study summarizes recent progress with novel strategy for fungal corrosion control using ozone exposure. The effect of ozone exposure on lipid, tryptophan oxidation and protein, nucleic acid leakage were recorded. SDS-PAGE protein analysis of the most dominant fungal species was recorded in absence and presence of ozone.

Experimental method

Tested bridges

Fungal influenced corrosion (FIC) of three bridges [Embaba bridge (E-bridge), Kasr al-Nile-bridge (K-bridge) and University bridge (U-bridge)] over the River Nile in Cairo, Egypt were tested. E-bridge was built in 1913 (490 m length) at the north of Cairo, while K-bridge was built in 1872 (406 m length) and it was the first bridge built on Nile river, it was originally named the Khedive Ismail bridge, after that it was named Kasr al-Nile or Al-Tahrer bridge. The U-bridge was built in 1958 (484 m length) on the western branch of River Nile. The three tested bridges suffer different grades of corrosion (thick and flaky rust with some visible fungal colonies has been found by inspection) and this can be seen in the field at lower deteriorated damped concrete parts over River Nile.

Isolation and identification of fungal species on the three tested bridges

Microorganisms were isolated directly from corroding surface for the three tested bridges using a sterile metal loop. The microscopic fungi were inoculated on solid glucose-Czapek's agar medium (5 plates to each sample) supplemented with bacterial antibiotics to suppress bacterial growth. The microorganisms were grown at 28°C for 7 days. The grown colonies of microorganisms were counted and identified according to various manuals (Gilman 1957; Barnett and Hunter 1972; Samson and Reenen-Koekstra 1988; Moubasher 1993; Kern and Blevins 1997).

Investigation of the corrosion products on the most corroded bridge by FTIR spectroscopy

Fourier transform infrared spectroscopy (FTIR) investigations were recorded using spectrophotometric

analysis using UV/Visible FTIR in Micro Analytical Center-Faculty of Science-Cairo University. The corrosion products of the most deteriorated bridge samples and the reference spectra of the most dominant fungal species on the three tested bridges were examined (Derrick et al. 1999).

Scanning electron microscope (SEM)

Studies were carried out by using SEM Model Phillips XL30 with accelerating voltage 25 kV, ×420 and resolution for 50 μm was used to study the fungal deteriorated bridge samples (Goldstein et al. 1992).

Ozone treatment

Ozone was generated via a controlled flow of oxygen through a corona discharge in the ozone generator (OZO-2000). Pure discs (1 cm) of the most dominant fungal species was grown on Dox medium plates. Plates were partially opened and were held in ozone chamber and exposed at 1, 2, 3 and 4 ppm and the exposure times were 0 (control), 30, 60, 120 and 180 min. Linear growth (cm) were measured after 7 days incubation at 28°C. Five plates were used for each treatment and control.

Weight loss measurement

The ozonated selected dominant fungal species (1 cm disc) was incubated with the most deteriorated bridge samples in Dox liquid media at 28°C for 7 days and the values of inhibition efficiency (IE%) obtained from weight loss method at different concentrations of ozone inhibitors (1, 2, 3 and 4 ppm) at different exposure times (0 (control), 30, 60, 120 and 180 min.). IE% was calculated using the following equation (Hosseini et al. 2010):

$$IE\% = \frac{w_0 - w_1}{w_0} \times 100 \quad (1)$$

where w_0 and w_1 are the weight loss in the absence and presence of ozonated fungal species, respectively. Mass loss was measured with an electronic balance.

Determination of lipid and tryptophan oxidation

In this experiment, five sterile Erlenmeyer flasks containing 100 ml of Dox media inoculated with the ozone treated mycelial dics (1 cm diameter) of the most dominant fungal species at different exposure doses. Five flasks inoculated with untreated fungal discs were used as control. All flasks were incubated at 28°C for 10 days. The filtrate collected for malondialdehyde determination (MDA) by thiobarbituric fluorometric assay (Badcock et al. 1997). The amount of tryptophan present in the filtrate was measured by fluorescence reading at 336 nm. The increase in fluorescence indicates increased amounts of unoxidized tryptophan and a decrease in fluorescence correlates with the amounts of tryptophan residues being oxidized (Komanapalli and Lau 1996).

Determination of protein and nucleic acid leakage and SDS-PAGE protein analysis

Protein concentration was determined spectrophotometrically at 260 nm by the method of Segel (1968). Determination of DNA was carried out quantitavely according to the method of Burton (1968) by measuring the colour developed after treating the extracted DNA with diphenylamine reagent and the absorbance was measured at 600 nm. The colorimetric analysis of ribose sugar using orcinol reaction (Ashwell 1957) was applied for quantitative determination of RNA. Sodium dodecyl-sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12.5% acrylamide gel according to the method of Laemmli (1970). Proteins was visualized by silver staining.

Results and disscusion

Isolation and identification of fungal species

Six fungal species belong to 12 terrestrial fungal genera were isolated from the three tested bridges [Embaba bridge (E-bridge), Kasr al-Nile-bridge (K-bridge) and University bridge (U-bridge)] (Table 1). *Fusarium* was the leading genus, where the most dominant species in all tested bridges was *F. oxysporium*. Our data demonstrate that the presence of *Fusarium* sp. capable of degrading concrete and may cause much more damage than other fungal species. Nasar and Munshi (1980) reported that the fungal population in freshwater, is mainly composed of *Fusarium*. The domination of *F. oxysporum* can be

Table 1 : Total count (TC), number of cases of isolation (NCI) and occurrence remarks (OR) of fungi isolated from the three tested bridges [Kasr al-Nile-bridge (K-bridge), Embaba bridge (E-bridge) and University bridge (U-bridge)] on glucose-Czapek's agar medium

Fungal genera and species	K-bridge			E-bridge			U-bridge		
	TC	NCI	OR	TC	NCI	OR	TC	NCI	OR
Aspergillus	105			34			20		
A. flavus	38	3	M	12	2	L	20	2	L
A. fumigatus	21	2	L						
A. niger	32	4	H	22	3	M			
A. terreus	14	2	L						
Curvularia	5								
C. Lunata	5	1	R						
Fusarium	111			38			17		
F. moniliforme	35	4	M						
F. oxysporum	54	5	H	38	5	H	17	4	H
F. solani	22	4	M						
Mucor	3								
M. racemosus	3	1	R						
Rhizopus	3								
R. stolonifer	3	1	R						
Scopulariopsis	11								
S. brevicaulis	7	1	R						
S. brumptii	4	1	R						
Total count	233			60			37		
Number of species	12			3			2		

1–2 Rare, *2–3* Low, *3–4* Medium, *4–5* High

explained by the access of the river water and run off from the surrounding soils (Ortoneda et al. 2004). *Fusarium* sp. appears to act by penetrating the material responsible for the deterioration through organic acid production followed by chelation to form insoluble complexes (Gu et al. 1998). On the other hand, K-bridge was the most deteriorated tested bridge (233 colony/gm), where 12 fungal species (*A. flavus, A. fumigatus*, *A. niger, A. terreus, Curvularia lunata, Fusarium moniliforme, F. oxysporum, F. solani, Mucor racemosus, Rhizopus stolonifer, Scopulariopsis brevicaulis* and *S. brumptii*) were isolated. The bulk of the isolated fungal species were found in the K-bridge may be refer to being the oldest bridge that built on Nile river. Most of the isolated species were previously isolated from Egyptian water areas and from other water areas in the world (EL-Hissy et al. 1990). Langor and Sweeney (2008) mentioned that the more obvious types of origin of these terrestrial fungi in fresh water would be animal or plant, the whole or part, living or dead. The next experiment was carried out on the high frequent fungal species (*F. oxysporium*).

Investigation of the fungal corrosion products by FTIR spectroscopy

The FTIR spectra of the most dominant fungal species (*F. oxysporium*) showed that the amines absorption spectral region between 3500 and 3400 cm $^{-1}$ and polysaccharides contents were from 1200 to 900 cm $^{-1}$, where these absorption bands indicate the presence of proteins and are indicative of fungi (Little et al. 2001) (Fig. 1a). Fischer et al. (2006) stated that there are strong indications that fungal metabolite production can be reliably distinguished by FTIR spectroscopy.

The tested degradation spectra are arranged in order of increasing degradation with prominent of the amines and polysaccharides groups in the deteriorated samples of K-bridge. As the K-bridge is oxidized, the carboxylate contribution (1560 wave numbers) decreases and the carbonyl contribution (1750 wave numbers) increases (Fig 1b). The degradation process was more pronounced in Fig. 1c, where the carbonyl contribution increases with the absence of carboxylate. One of the most striking spectral features that develops during microbial corrosion is the intense of carbonyl-stretching band (1750 cm^{-1}) (Uzarski 2006). On the basis of carbonyl intensity considerably above 1750 cm^{-1}, as observed in the broad C=O mode, it is postulated that strained carbonyl species, such as carbonates, lactones, peroxides, and anhydrides may be involved at the late stages of oxidation (Mawhinney and Yates 2001). Analyses of the corrosion products was able to reveal the types of compounds formed on the corroded surfaces (Parshutin et al. 2009). FTIR spectra of the tested K-bridge deteriorated samples provide evidence for the presence of fungal mycelia (protein and polysaccharides contents) and the degradation of the tested K-bridge with the production of organic acids, including carboxylic and hydroxyl acids. The first product of microbial oxidation of hydrocarbons are alcohols, aldehydes and aliphatic acids (Atlas 1981). When fungi are involved in concrete degradation, their metabolic acids would probably be responsible on degradation, however, they

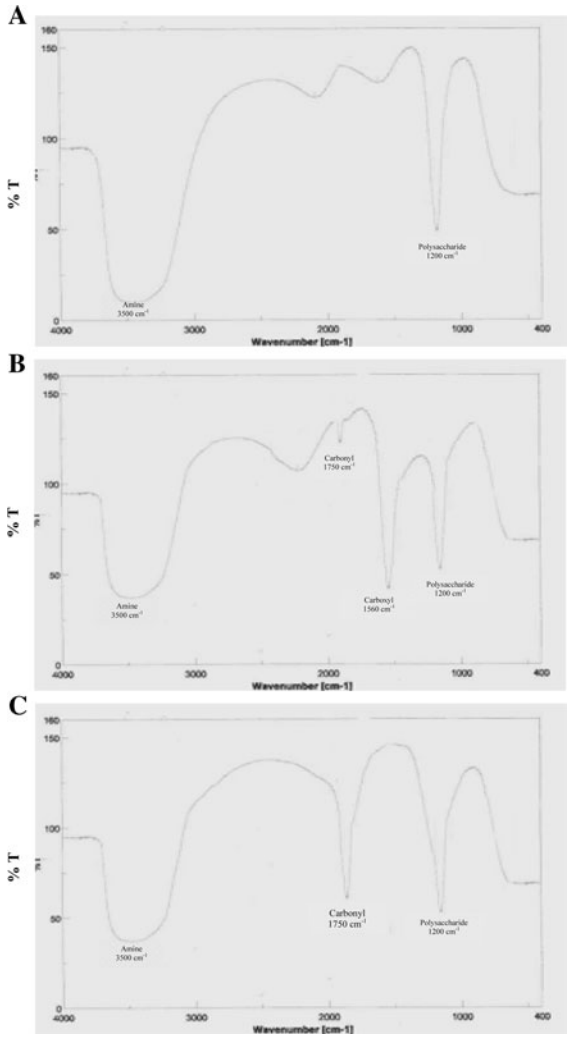

Fig. 1 Reference spectra of *F. oxysporium* (**a**). Some characteristic spectral ranges collected from fungal deteriorated K-bridge samples in order of increasing corrosion products that are dominated by certain chemical structures (**b, c**)

Fig. 2 SEM micrograph of fungal spores associated with corroded K-bridge sample. *scale bars* = 20 μm

would rapidly form calcium complexes, so that free acids would not be detected (Dutton and Evans 1996). The fungal corrosion in the tested K-bridge samples section was expected and confirmed the existing problem.

Scanning electron microscope (SEM) of the fungal corroded K-bridge

The observations of corroded K-bridge samples documented extensive spores of *F. oxysporium* in association with corrosion (Fig. 2). Videlal and Herrera (2005) found that photographic and SEM observations of deteriorated objects documented extensive fungal growth in association with localized corrosion, where mycelia are capable of indefinite growth in the presence of adequate moisture and nutrients so that fungi often reach macroscopic dimensions. Fungi are capable of etching and extending the fungal hyphae into the interior of the concrete resulting in enlargement of the damaged area and an increase in porosity (Gu et al. 1998). Microbial colonization may affect the sites of local corrosion attack (defects, micro-pores, micro-cracks) causing formation of concentration cells in localized corrosion (Juzeliunas et al. 2007).

Fungal corrosion inhibition evaluation by ozone

The inhibition efficiency (IE%) for the inoculated K-bridge sample in the absence and presence of different doses of ozonated *F. oxysporium* cells was obtained. Table 2 indicates that any increases in the exposure time at any concentration of gaseous ozone resulted in a significant gradual suppression in linear growth of *F. oxysporium* and consequently the inhibition efficiency increases with the increase in ozone dose. No mycelial growth with 100% IE were observed at 4 ppm at all exposure times. Labjar et al. (2010) stated that the corrosion inhibition efficiency increases with increasing of the inhibitor concentration. The spores most sensitive to ozone were, in general, relatively small and hyaline (Ali 2006). Two major mechanisms have been identified in ozone destruction of the target organisms (Guzel-Seydim et al. 2004): the first mechanism is that ozone oxidize sulfhydryl groups and amino acids of enzymes, peptides and proteins to shorter peptides. The second mechanism is that ozone oxidizes polyunsaturated fatty acids to acid peroxides. The differential activity of ozone

Table 2 Effect of different concentrations of gaseous ozone (1, 2, 3 and 4 ppm) on the radial growth (RG) (cm) of *F. oxysporium* and inhibition efficiency (IE%) at different exposure times (0, 30, 60, 120 and 180 min)

Fungal species	Ozone Concentration (ppm)	Exposure time (min)									
		0		30		60		120		180	
		(RG) (cm)	IE %	(RG) (cm)	IE %	(RG) (cm)	IE %	(RG) (cm)	IE %	(RG) (cm)	IE (%)
F. oxysporium	1	–	–	7.5	38.25	5.3	51.75	2.1	92.82	0.0	100.0
	2	–	–	6.2	77.54	4.5	84.41	1.42	97.0	0.0	100.0
	3	–	–	3.5	87.41	1.7	96.02	0.0	100.0	0.0	100.0
	4	–	–	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
LSD ($P = 0.05$)		–	–	1.2	9.3	0.5	11.5	0.4	2.1	–	–

against the test fungi might be due to the variation in their organic matter content which may accelerate or reduce the toxicity of ozone. This suggestion is recommended by Ali (2006) which found that the specific interaction of sucrose or exopolysaccharides with ozone affects the ozone activity.

Photolysis of ozone to oxygen atoms could lead to the generation of the hydroxyl radical (OH), a key reactive species during the decomposition process (Jans and Hiogne 1998). The inhibition of mycelial growth and sporulation of fungal cell due to oxidizing action of ozone was achieved by Liew and Prange (1994). Corrosion rates were assayed by weight loss and corrosion products (Zelinka et al. 2009). Due to the failure of the tested fungal growth at 4 ppm at all exposure time, so the other experiments will be held at the significant critical concentration of ozone (3 ppm).

Lipid and tryptophan oxidation of *F. oxysporium*

There was a significant increase of malondialdehyde, a product of lipid oxidation by *F. oxysporium* with increase of exposure time to ozone reaching to the maximum value (150 pmol/mg) after 80 min. exposure time (Table 3). Valacchi et al. (2004) stated that lipids are likely targets for attack by ozone. Ozone exposure results in the production of lipid peroxidation products (Foucaud et al. 2006). The position(s) of carbon–carbon double bonds within lipids can dramatically affect their structure and reactivity and thus has a direct bearing on biological function using ozone-induced fragmentation (Thomas et al. 2007). The accumulation of lipid peroxidation products upon ozone treatment of microbial cells was recorded only after the lethal damage (Pressman 2007). Ozone-induced oxidation of endogenous lipids upon exposure to 0 and 100 ppb (Kelly 2010). Ozone cause lipid peroxidation as a major cause of membrane deterioration (Pauls and Thompson 1980). The amount of malondialdehyde was twice after 40 min ozone exposure (Komanapalli and Lau 1996).

Ozone causes oxidative damage to the proteins of the tested *F. oxysporium*, as assessed by the oxidation of tryptophan residues reaching to the maximum

Table 3 Effect of the critical concentration of ozone (3 ppm) on lipid (pmol/mg), tryptophan oxidation (fluorescence at 336 nm) and protein (ug/ml), nucleic acid (DNA, RNA mg/ml) leakage of the most dominant fungal species (*F. oxysporium)* at different exposure times (0 (control), 20, 40, 60 and 80 min)

Exposure time	MDA (pmol/mg)	Fluorescence (336 nm)	Protein (μg/ml)	DNA (mg/ml)	RNA (mg/ml)
0 min (Control)	70	4500	5	0.12	0.16
20 min	88	3500	8	0.13	0.20
40 min	110	3000	12	0.14	0.23
60 min	120	2100	18	0.19	0.30
80 min	150	1000	20	0.25	0.35
LSD at 0.05	10	500	4	0.02	0.05

value after 80 min ozone exposure time (Table 3). Knight and Mudd (2004) stated that the aromatic amino acids tryptophan, are oxidized by ozone causing the degradation of the relative moiety. The most sensitive amino acid to ozone is tryptophan which is degraded (Cataldo 2006). The oxidation of lipid and proteins have structural and functional roles in biomembranes, could alter the ability to regulate permeability (Komanapalli and Lau 1996). Ozone is highly reactive with many organic substances including various amino acids (Ishizaki et al. 2008). Oxidation of tryptophan by ozone was tested by Herbert et al. (2010).

Leakage of *F. oxysporium* protein and nucleic acid

A statistically significant increase of the tested extracellular protein content of *F. oxysporium* was observed from 0–80 min There are time dependant increase in nucleic acids leakages with extending ozone exposure times reaching to the maximum significant accumulation (0.25 and 0.35 mg/ml) of DNA and RNA after 80 min exposure to ozone, respectively. Non significant increase in nucleic acids contents was observed at the lowest ozone exposure times (0–40 min) (Table 3). The obtained result was in accordance with Gupta et al. (2010) who stated that there are a significant increases in the levels of protein, RNA following ozone exposure. Also Poli et al. (2007) found that with longer duration of ozone exposure (up to 30 min) cell viability decreased and membrane permeability was compromised as indicated by more significant increase in protein and nucleic acid leakage and lipid oxidation. Proteins were significantly induced after 2 days exposure to elevated ozone and gradually increased during exposure period (Feng et al. 2008). A group of low molecular weight, acidic proteins accumulated during three days of ozone fumigation (Kaltenbach et al. 2004). The accumulation of the proteins increased as the duration of the ozone treatment was lengthened (Chen and Gallie 2004). The effects of ozone on nucleic acid are probably compounded of the effects of ozone on the constituent purines and pyrimidines, each of which appears to be individually affected (Christensen and Giese 2004). Ozone penetrates cell membrane and reacts with cytoplasmic substances, therefore, chromosomal DNA may be one of targets of the ozone degradation, and its damage may be one of the factors responsible for the cell killing (Todar 2009). Ozone concentrations at ground levels modulate oxidative DNA damage (Palli et al. 2009). Degradation of nucleic acids by ozone was recorded by Shinriki et al. (2003). DNA and RNA are targets of ozone (Ishizaki et al. 2008). Ozone reacts predominantly with guanine or thiamine moieties in nucleic acids (Komanapalli and Lau 1996). The action of ozone occurs by cell lysing or rupture of cell walls with interaction of ozone with key biopolymers (proteins and nucleic acids) present in biological environments, where Tryptophan was the most reactive amino acid towards ozone followed by tyrosine while phenylalanine appears much less reactive (Cataldo 2006).

SDS-PAGE protein analysis of *F. oxysporium*

The SDS-PAGE analysis of the supernatant protein of *F. oxysporium* shows increasing amounts of proteins with extending ozone exposure time, where the maximum increasing amounts of proteins at 80 min of ozone exposure (Fig. 3a). The pattern of intercellular protein of *F. oxysporium* exposed to ozone are also observed on SDS-PAGE (Fig. 3b), where the intensity of the cellular protein bands on the gel decreased with time, indicating a gradual decrease for

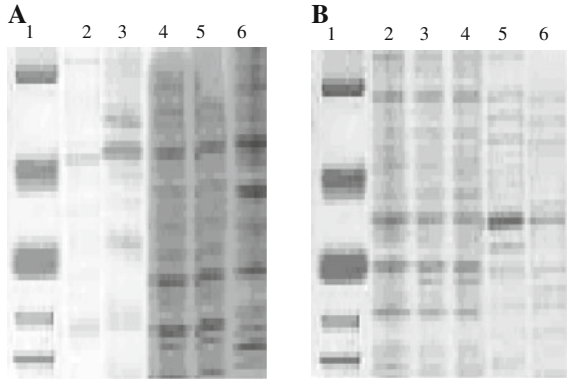

Fig. 3 **a** Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of supernatant proteins of *F. oxysporium* exposed to ozone (3 ppm) for 0–80 min *Lane 1* Protein marker, *Lane 2* for 0 min (unexposed cells), *Lane 3* for 20 min, *Lane 4* for 40 min, *Lane 5* for 60 min, and *Lane 6* for 80 min. **b** Cellular protein extracted from *F. oxysporium* exposed to ozone (3 ppm) for 0–80 min *Lane 1* Protein marker, *Lane 2* for 0 min. (unexposed cells), *Lane 3* for 20 min., *Lane 4* for 40 min, *Lane 5* for 60 min and *Lane 6* for 80 min

cellular proteins, noticeable at 60–80 min This results correlates with the study of Bocci et al. (2009) suggesting that a substantial fraction or all of the ozone reacts within a cell membrane, where prolonged ozone exposure is intracellular proteins. Two dimensional gel electrophoresis demonstrated the differential, exposure-dependent accumulation of a small group (two to four) of ozone-responsive proteins (Tang et al. 2000). Ozone cause widespread oxidation of internal cellular proteins causing rapid cell death (Guzel-Seydim et al. 2004).

Conclusion

The author highlight the serious fungal corrosion problems of contamination of the river bridges and then explain a decontaminating methodology developed to resolve it. Chemical and mechanical sterilization methods adversely affect public health as well as the environment. Ozonation can be successfully used for reducing the microbial loads. The high oxidizing power and spontaneous decomposition also make safety, for this reason ozone may replace chemical sanitizer as a common sanitizing agent. The present data demonstrate that the initial target of ozone fungal toxicity is the membrane, affecting both lipid and protein components.

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