Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 20(2008) 1514–1518
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Damage to DNA of effective microorganisms by heavy metals: Impact on wastewater treatment

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Received 14 January 2008; revised 4 April 2008; accepted 5 July 2008

Abstract

The research is to test the damage to DNA of effective microorganisms (EMs) by heavy metal ions As^{3+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}, as well as the effects of EM bacteria on wastewater treatment capability when their DNA is damaged. The approach applied in this study is to test with COMET assay the damage of EM DNA in wastewater with different concentrations of heavy metal ions As^{3+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, as well as the effects of EM treated with As^{3+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+} on COD degrading capability in wastewater. The results showed that the damage of the DNA of EM were negatively correlated with their treatment capability and that EM bacteria maximum tolerant concentrations of these heavy metal ions was at 0.05 mg/L for As^{3+}, 0.2 mg/L for Hg^{2+}, 0.5 mg/L for Cd^{2+}, Cr^{3+}, and Cu^{2+}, and 1 mg/L for Pb^{2+} and Zn^{2+}.

Key words: effective microorganism; heavy metal; DNA damage; wastewater

Introduction

Heavy metals, whether present as metals or as ions, are not broken down by microorganisms in the biological treatment of wastewater. The soluble form is especially toxic as it is generally found in plants and animals and can bioaccumulate in these species, as well as in humans. Likewise, heavy metals are involved in many chemical reactions. They can change forms from a soluble to insoluble state and from an absorbed to a free state. A study of water quality along the Yangtze River – the longest river in China – found that water near major towns was polluted with heavy metal elements. Moreover, their concentrations affected the suitability of water intended for municipal purposes (Zhu and Zang, 2001). Similarly, the Yellow River, the second longest river in China, was also found to be affected by heavy metal pollution, with the total concentration of heavy metals at almost 12.96% of the pollutants found in samples (Ma, 2002). Unfortunately, it was not just the rivers, which were polluted by heavy metals in China, a widespread phenomenon throughout the country. Food is also affected as its production occupies large farmland areas (Wei and Chen, 2001; Sun *et al*., 2003). Heavy metals enter the human food supply from irrigation water or improper land disposal of effluents (Dong and Chen, 1982; Kow *et al*., 1998; Cobbertt, 2002; Tsuj *et al*., 2002). There are several methods to treat wastewater contaminated with heavy metals including physical, chemical, and biological methods. Physical methods widely used include powdered activated carbon and the electrode method (Erokhin *et al*., 2006). Chemical methods include acidification, ion exchange, solubilization, and the use of surfactants and complexing agents (Barrado *et al*., 2001; Sánchez *et al*., 2006). On the other hand, biological methods heavily depend on heavy metal-resistant bacteria screening and domestication (Chelliah *et al*., 2006; Sofia *et al*., 2006). Among the biological processes, effective microorganisms (EMs) have been identified to comprise the most effective application for heavy metal removal. The term "effective microorganism" was coined by Teruo Higa of the University of the Ryukyus in Japan (Teruo and James, 1994). He used the term to encompass those microorganisms that are effective and beneficial in a variety of settings. Initially, Teruo and James (1994) investigated the microorganisms that were useful in crop farming as they were seen as alternatives to the use of pesticides and fertilizers. This idea was later extended to livestock production. Benefits were seen as they improved quality of the effluent from livestock production. Currently, EMs are being used in pig, cattle, dairy, and poultry farming. Teruo and James (1994) also identified more than 80 beneficial species of microorganisms. These are mainly species of phototrophic bacteria, lactic acid bacteria, yeasts, and actinomycetes. Apart from farming, EMs have been applied in cleaning polluted waterways, lakes and lagoons, and septic tanks,

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as well as in wastewater treatment, landfills, and for water purification by Teruo and James (1994). However, these beneficial microorganisms are often affected by the presence of heavy metals. Although the problem has been recognized, a few studies have been carried out on this subject. Specifically, in the traditional activated sludge process, the role of heavy metal concentration was limited to its efficiency in treating activated sludge caused by heavy metal. However, the correlation of EM DNA damage and the efficiency of EM treatment have not yet been studied.

As observed, one form of damage to EM now being considered is genetic damage. One method to investigate this certain type of damage is through the single-cell gel electrophoresis or COMET assay. This approach is considered to be rapid, sensitive, and relatively simple. It combines the simplicity of biochemical techniques for detecting single breaks in DNA strands (strand breaks and incomplete excision repair sites), as well as alkali-labile sites and cross-links, along with the single-cell approach typical of cytogenetic assays (Kumaravel and Awadheshe, 2006; Singh *et al*., 1988). In this study, the susceptibility of EM to genetic damage caused by a range of heavy metals with various concentrations has been investigated. Likewise, the effectiveness of wastewater treatment following exposure to various concentrations of heavy metals has also been investigated further.

1 Materials and methods

1.1 Reagents and chemicals

Wastewater samples were obtained from a sewage pipe located in a dining hall in the South China University of Technology, Guangzhou, China. Reagent-grade dilute solutions of Copper, Zinc, Lead, Cadmium, and Mercury sulphates were used, as well as As_2O_3 and $CrCl_3$. Effective microorganisms were obtained from Guangdong Academy of Agricultural Sciences.

1.2 Determination of the total viable count of bacteria (TVC) under heavy metal stress

In this study, 5,000 ml of sewage water was collected and was settled for 30 min. The supernatant was discarded, retaining only 3,000 ml of the original sample. It was then shaken to resuspend sediment and was equally divided among six bottles (500 ml each). Five samples were dosed with one of the heavy metal solutions, with each bottle containing a different dose. The other was used as a control. Each of the five samples was diluted with 0.5 ml of EM liquid, shaken, and then aerated for 24 h by an AR-6500 pump at a low flow rate. Then, 1 ml of liquid was extracted, diluted 10^8 times, and a sample was spread on LB solid microorganism culture medium. After incubation for 24 h at 28°C, the respective numbers of microorganism colonies were counted. The ratio of the colonies on each dosed sample to the colonies on the control sample was used as an index of the viability of EM in the presence of the heavy metal. This procedure was repeated five times, and the average ratio was calculated for each contaminant. Afterwards, the entire procedure was repeated for each heavy metal. The concentrations added for each are shown in Table 1.

1.3 Determination of COD reduction

The initial chemical oxygen demand (COD) on the raw sewage was measured using the Chinese SEPA standard method (SEPAC, 2000). This process was performed with the remaining supernatant liquid after being settled for 30 min (see above). The COD was also measured for each of the six samples after incubation. The relative reduction in COD was calculated for each treatment, and the average was obtained for the five replications of each treatment.

1.4 Degree of the DNA damage and DNA damage AU (Arbitrary unit)

To evaluate the levels of DNA damage in all cell types, DNA damages were roughly graded as < 5% (no change), 5%–20% (low level damage), 21%–40% (medium level damage), and 41%–100% (high level damage), according to the proportion of cellular tail DNA accounting for the total cellular DNA content (Anderson *et al*., 1994). Fig.1 shows the different levels of DNA damage: no damage (Fig.1d) and low, medium, and high level damage (Fig.1a-c, respectively).

1.5 Comet assay

Alkaline comet assays were undertaken according to the protocols of Tice *et al*. (2000). After the preparation and incubation described above, 1 ml of liquid culture was mixed with 70 μl of 0.7% low melting point (LMP) agarose dissolved in phosphate-buffered saline (PBS) at 37°C. Comet gel was made from this material by pipetting one drop 100 μl on a fully frosted slide, precoated with 0.7% normal melting point (NMP) agarose, and covered with a 22-mm square slide. After the gel had solidified, nuclei were lysed in a strong salt solution for 8 h at 4°C. The solution used comprised 2.5 mol/L NaCl, 10 mmol/L Tris, 100 mmol/L EDTA, and adjusted to pH 10. Fresh 10% dimethylsulfoxide and 1% Triton X-100 were then added. The slide was incubated in fresh electrophoresis buffer (0.3 mol/L NaOH, 5 mmol/L EDTA, at pH 13) for 30 min at room temperature to allow DNA unwinding. The slide was then placed into a horizontal electrophoresis tank and electrophoresed for 30 min at 18 mV, 200 mA

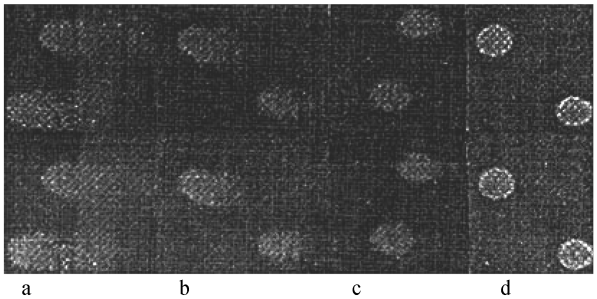

Fig. 1 Typical EM DNA cells with heavy metal treatment. (a) high levels of DNA damage; (b) medium levels of DNA damage; (c) low levels of DNA damage; (d) untreated control.

Table 1 Effects of heavy metals on DNA damage of EMs and their viability

Heavy metal ion	Dose (mg/L)	Control (no damage)	Low damage	Medium damage	High damage	DNA damage cell (%)	AU	EM viability (%)
Cu^{2+}	0	100	1	0	0	1	1	100
	0.10	77	15	6	2	23	48	90.6
	0.50	52	28	14	6	48	74	78.6
	1.00	38	36	18	8	62	96	70.6
	5.00	20	40	32	18	80	158	25.3
	10.00	0	40	35	25	100	185	12.0
Zn^{2+}	0	100	2	0	0	2	2	100
	1.00	96	2	2	0	4	6	98.8
	3.00	60	18	12	10	36	72	84.6
	5.00	45	25	20	10	55	95	78.8
	7.00	19	36	27	18	81	144	45.8
	10.00	9	42	39	19	91	177	18.9
Pb^{2+}	0	100	1	0	0	1	1	100
	0.25	86	8	4	2	14	22	92.5
	0.50	71	15	9	5	29	48	90.6
	1.00	58	23	11	8	42	69	80.2
	2.00	24	36	27	13	76	129	58.6
	4.00	10	42	33	15	90	153	23.0
As^{3+}	0	100	0	0	0	0	0	100
	0.05	79	14	5	2	21	30	93.8
	0.10	46	31	19	4	54	81	76.5
	0.20	34	39	22	5	66	98	72.2
	0.40	13	45	32	10	87	139	66.8
	0.60	6	49	34	11	94	150	31.0
Cr^{3+}	0	100	0	0	0	0	0	100
	0.10	97	2	1	0	3	4	98.6
	0.50	93	4	2	1	7	11	97.8
	1.00	69	16	12	3	31	49	85.6
	5.00	51	28	16	5	49	75	80.6
	10.00	24	42	23	9	76	115	40.0
Cd^{2+}	0	100	1	0	0	1	1	100
	0.05	96	3	1	0	4	5	98.6
	0.10	93	5	1	1	7	10	96.6
	0.50	89	8	2	1	11	15	96.4
	1.00	44	36	14	6	56	82	42.6
	2.00	32	42	17	9	68	103	35.2
Hg^{2+}	0	100	0	0	0	0	0	100
	0.05	82	12	5	1	18	25	93.4
	0.10	80	13	6	1	20	28	92.5
	0.20	70	15	9	6	30	51	90.6
	0.30	22	40	28	10	78	126	73.5
	0.50	16	36	32	16	84	148	51.0

EM: effective microorganism; AU: arbitrary unit. AU $=\sum i \times n_i$, n_i was the number of the No.i grade damage cell.

in an ice-cooled chamber with the same alkaline solution as used to aid unwinding. After electrophoresis, the slide was washed twice in a fresh neutral buffer, 0.4 mol/L Tris adjusted to pH 7.5 with concentrated HCl. Comet cells were observed under a fluorescence microscope (Nikon UFX-II, Tokyo, Japan) after nuclei staining for DNA damage with 15 μl of 5 μg/ml ethidium bromide (EB) in water. The procedure was repeated three times for each treatment and the experiments were carried out in duplicate for comparison.

2 Results and discussion

2.1 Correlation of the levels of EM cell DNA damage with the ability of EM wastewater treatment

As for EMs, the damage to the DNA composition of the cells appears to be the primary mechanism for their reduced ability to biodegrade the wastewater when heavy metals are present (Gindiner and Hickoon, 2000).

Table 1 shows the changes in the number of viable EM cells. Fig.2 indicates the impact of difference of heavy metals on wastewater treatment performance. It suggests that the efficiency of wastewater treatment was affected by the EMs. We found that the levels of EM cell DNA damage were negatively correlated with the ability of EM wastewater treatment. In fact, when Cu^{2+}, Zn^{2+}, Pb^{2+}, As^{3+}, Cr^{3+}, Cd^{2+}, and Hg^{2+} concentrations were at 10, 10, 4, 0.6, 10, 2, and 0.5 mg/L, respectively, the corresponding EM cell DNA damaged cell percentage were at 100%, 91%, 90%, 94%, 76%, 68%, and 84%, respectively (Table 1). At the same time, the ability of EM wastewater treatment decreased to 12%, 18.92%, 23.1%, 31.2%, 40.1%, 35.2%, and 51.2%, respectively (Fig.2).

2.2 Correlation of the number of viable EM cells with the ability of EM wastewater treatment

The community structure can also be affected by pollution. In the wastewater, heavy metals can be chemically combined to enzymes, such as DNA polymerases, which

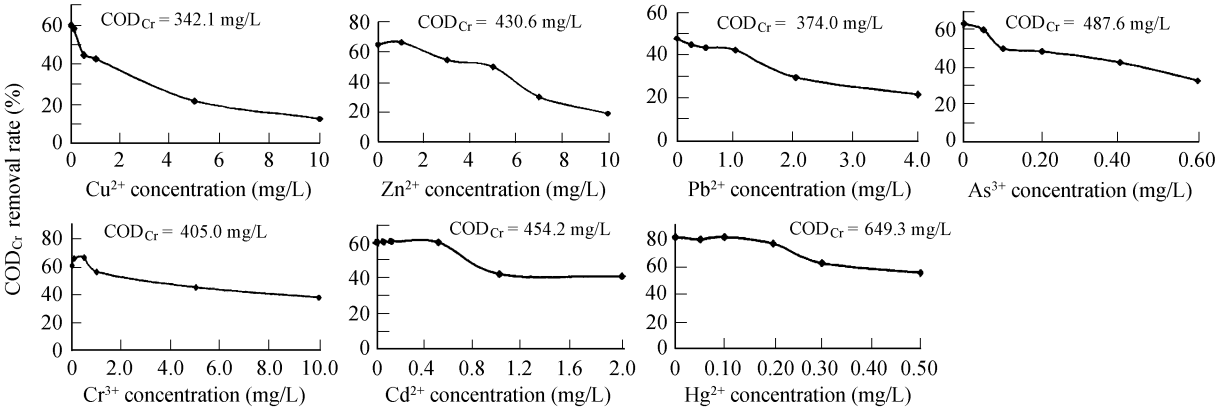

Fig. 2 Effect of heavy metals on EM removal of COD_{Cr} of sewage (pretreatment wastewater).

will lead to the reduction of enzyme activity (Cobbertt, 2002; Tsuj *et al*., 2002; Sun and Zhou, 2003).

Moreover, the number of viable EM cells was positively correlated with the ability of EM wastewater treatment (wastewater COD_{Cr} removal rate). In fact, when Cu^{2+}, Zn^{2+}, Pb^{2+}, As^{3+}, Cr^{3+}, Cd^{2+}, Hg^{2+} concentrations were all at 0, the percentage of EM cell viability were all 100%, and the ability of EM wastewater treatment (wastewater COD_{Cr} removal rate) were maintained at their original percentages of 60%, 65%, 50%, 63.2%, 60%, 60%, and 80%, respectively (Fig.2). However, when Cu^{2+}, Zn^{2+}, Pb^{2+}, As^{3+}, Cr^{3+}, Cd^{2+}, and Hg^{2+}concentrations were at 10, 10, 4, 0.6, 10, 2, and 0.5 mg/L, respectively, the EM cell viability percentage decreased to 12%, 18.9%, 23%, 31%, 40%, 35.2%, and 51% (Table 1). The ability of EM wastewater treatment (wastewater COD_{Cr} removed rate) decreased to 12%, 18.92%, 23.1%, 31.2%, 40.1%, 35.2%, and 51.2%, respectively (Fig.2).

2.3 Correlation of the heavy metal concentration with the EM cell DNA damage

Microorganisms are often sensitive to their environment (Vig *et al*., 2003). Likewise, heavy metals may be toxic to some species as they reduce the rate of biodegradation. Other toxic agents include ultraviolet radiation, ionizing radiation, and genotoxicants. In addition to direct damage to DNA, there may be damage to repair mechanisms, such as cutting of cross-links, adducts, and DNA-protein adducts (Liu *et al*., 2004; Brien *et al*., 2002; Vasant *et al*., 2001; Bagchi *et al*., 2003; Bose *et al*., 1999). However, in EMs, damage to the DNA composition of the cells appears to be the primary mechanism for their reduced ability to biodegrade the wastewater when heavy metals are present (Gindiner and Hickoon, 2000).

We found that the higher the heavy metal concentration was, the more serious the EM cell DNA damage would be. For example, when the Cu^{2+}, Zn^{2+}, Pb^{2+}, As^{3+}, Cr^{3+}, Cd^{2+}, and Hg^{2+} concentrations were at 0, the EM DNA damage AU were at 1, 2, 1, 0, 0, 1, and 0, respectively. However, when the Cu^{2+} , Zn^{2+}, Pb^{2+}, As^{3+}, Cr^{3+}, Cd^{2+}, and Hg^{2+} concentrations in the wastewater reached 10, 10, 4, 0.6, 10, 2, and 0.5 mg/L, respectively, the EM DNA damage AU were observed at 185, 177, 153, 150, 115, 103, and 148, respectively (Table 1). At the same time, the number of cases for the low level DNA damage, the medium level DNA damage, and the high level DNA damage increased (Table 1). Unfortunately, we cannot specify the particular relation of these parameters because the data that we obtained were not enough to speculate their order. Hence, future intensive and detailed research study is required.

Meanwhile, we can see from Fig.2 that the EM tolerance concentration of Cu^{2+}, Zn^{2+}, Pb^{2+}, As^{3+}, Cr^{3+}, Cd^{2+}, and Hg^{2+} are 0.50, 10, 1.0, 0.05, 0.50, 0.50, and 0.10 mg/L, respectively. This indicates that when the heavy metal concentration reached that certain point, the efficiency of the EM wastewater treatment decreased quickly. Perhaps, the uniqueness of the heavy metal is its ability to integrate with the EM cell enzyme or protein.

Currently, EM technology is considered as the most effective method of wastewater treatment. However, if the concentration of heavy metals, such as Cu^{2+}, Zn^{2+}, Pb^{2+}, As^{3+}, Cr^{3+}, Cd^{2+}, and Hg^{2+} in the wastewater reaches the heavy metal tolerance of EMs, its effectiveness for wastewater treatment would decrease. This research study suggests that heavy metals should be removed through a pretreatment process if their presence has been detected in the wastewater. This step would improve the efficiency of EM wastewater treatment. On the other hand, EM could be resistant to heavy metals by inducing it through some chemical and physical processes.

3 Conclusions

This research suggests that the level of EM cell DNA damage is negatively correlated to the performance of EM in wastewater treatment. On the other hand, the number of viable EM cells is positively correlated to the efficiency of wastewater treatment. In our results, EM cell DNA damage became progressively worse along with increasing heavy metal concentration. Likewise, this study suggests that EM DNA is affected by heavy metals. Specifically, As^{3+} has the largest effect among the heavy metals, followed by Hg^{2+}. The EM concentration tolerance of As^{3+} is at 0.05 mg/L, whereas that of Hg^{2+} is 0.20 mg/L. On the other hand, the EM tolerance concentration of Cd^{2+}, Cr^{3+}, and Cu^{2+} are all at 0.50 mg/L, whereas that of Pb^{2+} and Zn^{2+} all are at

1.0 mg/L.

Acknowledgments

This work was supported by the Hi-Tech Research and Development Program (863) of China (No. 2006AA06Z378), the National Natural Science Foundation of China (No. 20777018), and the Scientific Research Item of Guangxi Province Department of Education of China (No. 200608LX109). The authors are grateful to Prof. Elwood Powell (Dr. Elwood Powell, USA, now in SCUT in China) for his critical reading of the manuscript and James Irish for his help in editing the article.

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