Zagazig J.Agric. Res., Vol .31 No.(6) 2004 2757 - 2770

CONSTRUCTING A BIOFILM SYSTEM EFFICIENT IN REMOVING HEAVY METALS FROM POLLUTED WATER

EL'MASRY, M. H.
Department of Biotechnology, Institute of Graduate Studies and Research, University of Alexandria, Alexandria, Egypt.

Accepted 19 / 10 / 2004

ABSTRACT: A study using a biofilm filter system was carried out to determine the efficiency of such method to remove heavy metals from polluted water. Three different supporting materials (sand, gravel and plastic) were primarily fixed with a consortium of four bacterial species; *Bacillus. licheniformis, B. sphaericus, Micrococcus sp.* and *Arthrobacter sp.* Results indicated that sand and plastic materials gave good adhesion with the four bacteria, while only two bacterial species adhered onto gravel. Therefore, sand- and plastic-biofilm filter systems were further investigated to determine their ability to remove heavy metals from polluted water. Sand biofilm showed more ability to accumulate Fe, Cd, and Pb compared to that accumulated by the plastic biofilm, with an increase of 1.2, 1.1, and 1.2 folds, respectively, at 30ml/h flow rate. On the other hand, plastic biofilm accumulated more Fe with an increase of 1.7 and 2.3 folds than sand biofilm at 45 and 60 ml/h flow rates, respectively. Zinc was highly accumulated with 94% removal efficiency, followed by Cd (78%), Pb (75%), and Cu (71%) at 30ml/h flow rate using the sand biofilm filter. That order varied by changing the flow rate except for Cd and Zn that were more accumulated by the sand-biofilm filter than the plastic-biofilm filter.

Key words: Biofilm, heavy metals, sand, plastic, accumulation, efficiency

INTRODUCTION

Various attempts have been made to develop a suitable bioaccumulation system that can efficiently uptake heavy metals and has advantages of high adsorption/ absorption ability, high selectivity, low cost, speed and absence of disposal problems The surfaces of bacteria growing in this environment are a forest of protruding linear macro molecule such as pili, lipopolysaccharides, teichoic acid, and exopoly-sacccharides (Tonn and Gander, 1979; Gujer and Wanner, 1990; Flemming and Wingender 2001).. Thus, bacteria in natural aquatic population have a marked tendency to interact with solid surfaces.

Among the most successful forms of microbial fixation is the immobilization system which is best expressed by the biofilm model. Bacterial biofilm attached to solid surfaces has been recently used to treat polluted water effluents (Yu and Pinder, 1993; Aesoy and Odegaard, 1994; Ribas *et al.*, 1995; Atkinson *et al.*,1996; Watnick, Kolter, 2000).

Therefore, using bacterial strains possessing high efficiency in accumulating metals in a biofilm system will promote the use of such system to remove heavy metals from any polluted aquatic media.

A biofilm system is a biologically active matrix of cells and extracellular products attached to a solid surface. The eventual production of continuous biofilm on the colonized surface is a function of cell division within micro colonies and new requirement of bacteria from the planktonic phase (Costerton *et al.*, 1987).

The advantages of using immobilized microbial cells for wastewater treatment are multiple including that waste and adsorbent are separated from the treated flow in one step with no settling-out time, flow rate/activity relationship can be determined and very large volumes can be treated continuously (Macaskie, 1990).

The aim of this research work was to investigate the efficiency of a biofilm system constructed on different supporting materials with four bacterial species for removing heavy metals from polluted water.

MATERIALS AND METHODS

Microorganisms

Four bacterial species; *Bacillus licheniformis, Bacillus sphaericus, Arthrobacter sp.* and *Micrococcus sp.* were isolated and identified in a previous study (El-Masry *et al.* 1999), where they proved to be potent in accumulating a range of heavy metals.

Constructing the biofilm system

Three cylindrical plastic columns (65x10 cm) were fitted at the bottom by a porous plastic net (d< 1 mm) and supplied with a flow controller (tap) at the outlet. Each cylinder was filled with one of the supporting materials used in this study (sand, gravel and tubular plastic pieces). Particle size of sand was 1mm in diameter; gravel was 0.5- 1.0cm, while the tubular plastic pieces were hollow with surface area about of 50 cm^2 for each.

Sand and gravel were sterilized by autoclaving at 121°C for 2hrs, while the plastic pieces were sterilized in 75% ethyl alcohol over night, then rinsed twice in sterile distilled water, then finally dried in sterile container. Each column was filled with one of the packing materials leaving third of the column-top free. The columns were washed twice with 75% ethanol, and then rinsed twice with sterile H_2O before packing. Each packed column was inoculated with 0.5% (v/v) of each bacterium grown overnight in beef-peptone(BP) broth medium, then packed with sterile BP broth medium (lL for sand, and 2L for plastic). The BP broth medium contained per 1 liter distilled water: 3g beef powder, 5g peptone and 3g NaCl, pH 7, and autoclaved at 121°C for 20 min. Columns were connected with an upflow air supply to provide an aerobic condition for the growing biofilm.

Determination of bacterial population dynamics

The columns were left as a batch culture for 7 days at room temperature (25°C). Afterwards, a sample from each column was collected every 24 hours after inoculation. Serial dilutions (up to 10^{-9}) were made, and then 100 µl of the appropriate dilution was plated under aseptic conditions on BP agar medium (BP broth medium supplemented with 2% agar), and then incubated for 24 hr at 30 °C. Bacterial counts were daily recorded for each species till a constant count was obtained for 3 consecutive days.

The BP culture was replaced with minimal broth medium (MB) for additional 3 days to adapt the bacteria that were fixed on the supporting material to a starvation condition. The minimal broth medium contains per 1 liter distilled water; 1g ammonium sulphate, 3g dihydrogen potassium sulphate, 7g dipotassium hydrogen phosphate, 0.1g magnesium sulphate, and 0.5g sodium citrate, adjusted to pH 7. The medium was then autoclaved at 121 °C for 20 min.

Determination of bacterial biofilm on supporting materials

Sand sample (4g) was washed three times in a sterile plastic bottle with 10 ml of freshly prepared sterile phosphate buffer (pH 7.2). The buffer contained per 1L distilled water: 8g dihydrogen potassium sulphate, 0.34g dipotassium hydrogen phosphate, and 0.34 NaCl. In the first wash, the sample was vigorously shaken for I min and the resulted suspension was plated with the appropriate dilution. The same sample was resuspended in another 10 ml of phosphate buffer, and then centrifuged at 4,000 xg in Heraeus centrifuge (model Labofuge 6000) for 15 minutes, where the resulted suspension was also plated. Finally, the sand samples were resuspended in fresh 10 ml buffer, vortexed for about 5 minutes in order to release the colonized bacteria from the biofilm layer firmly attached to the solid surface, and then the resulted suspension was also plated as previously described (Anwar *et al.*, 1989 and El-Masry *et al.*, 1995).

Ten grams of gravel pieces were similarly treated as sand sample, in order to release and determine its bacterial content. In the case of tubular plastic material, each piece was cut under aseptic condition to about 1 cm^2 pieces and weigh, and then treated as previously described.

Standard plate count (SPC) of bacteria in the biofilm was done by the plate spreading technique using a non-selective BP agar medium. The plates were incubated for 1-2 days (according to bacterial growth) at 30 °C. Bacterial total count as well as counts of each species were determined for each sample. Bacteria attached to sand/gravel/plastic support were enumerated according to their colony characteristics appeared on the agar medium.

Scanning electron microscopy of the biofilm members

Sand particles or plastic pieces (1 cm^2) were processed by air-

drying on SEM stub for 48 hours in a desiccator under vacuum. The stubs were then gold-coated in a sputter-coating machine. Each sample was then examined using scanning electron microscope (Jeol JSM 5300) at x5,000 magnification.

Efficiency of the biofilm system in removing heavy metals from aquatic effluent

In order to test the biofilm consortium to remove heavy metals from aquatic medium, the MB medium was supplemented with of iron (Fe^{+3}), cadmium (Cd^{+2}), zinc (Zn^{+2}, copper (Cu^{+2}), and lead (Pb^{+2}) (1mg each per liter), and then autoclaved at 121 °C for 20 min before use.

MB media supplemented with heavy metals was run over each biofilm system at flow rates of 30, 45 and 60 ml/h for 4 hours continuously with a static pre-incubation for 1 hour before sampling. Samples were taken every 30min to determine heavy metal levels in the samples. The efficiency of the biofilm for removing metals from the aquatic media was calculated in comparison to a control column filled with the supporting material without biofilm.

Measuring heavy metals

Each sample (≈10 ml) was collected in a clean acid-washed, sterile test tube and filtered through sterile cellulose membrane filters of 0.22 µm pore size to retain any biomass. Heavy metals in the collected samples were determined using Atomic Adsorption Spectrophotometer (Perkin-Elmer model 2380), against a control sample taken from the biofilm-free sand or plastic column as appropriate.

RESULTS AND DISCUSSION

Formation of bacterial biofilm

The system used in the present study, consisted of three columns, each of which contained different supporting material (plastic, gravel, and sand). Inoculation of the system was done by a consortium of four bacterial species (*B. licheniformis, B. sphaericus, Micrococcus sp.* and *Arthrobacter sp.*). After packing the column with the bacterial culture, the population dynamics of these bacteria in each system was daily monitored. Table (1) presents the total viable count of bacteria as colony forming units (cfu) in each system. Results showed that bacteria in the three systems achieved their stationary growth phase at day 4. At the end

Table 1: Population dynamics of the bacterial consortium *B. licheniformis*, *B. sphaericus*, *Arthrobacter spp.*, and *Micrococcus sp.* in the growth medium used to construct the biofilm systems I, II, and III.

Day	System I (sand)	System II (Gravel)	System II (plastic)
0	2.6×10^4	2.6×10^4	2.6×10^4
1	2.2×10^6	3.0×10^6	3.2×10^4
2	1.1×10^7	8.3×10^6	2.2×10^6
4	9.0×10^7	1.2×10^8	1.6×10^7
6	1.1×10^8	1.0×10^8	1.0×10^8
7	1.1×10^8	1.1×10^8	1.0×10^8

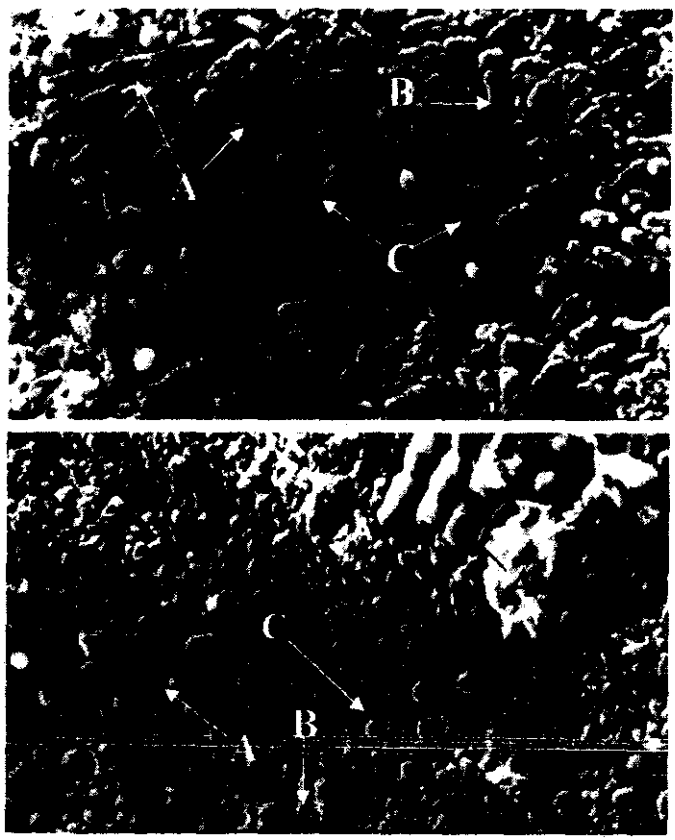

Figure 1: SEM micrograph (x5,000) showing the bacterial biofilm on sand particles (I), and plastic material (II), where A= *Bacillus* spp., B= *Arthrobacter* sp., and C= *Micrococcus* sp.

of log phase, maximum counts of $9.0x\ 10^7$, $1.2x10^8$ and $1.6x10^8$ cfu were obtained for system I (sand), II (gravel) and III (plastic), respectively. At the stationary phase, bacterial growth in the three systems was almost stable.

Bacterial adhesion and stability of the biofilm

Table (2) shows population counts of the bacteria adhered to the three systems (I, II and III) after their stepwise release treatments. Results indicated that sand was adhered with the highest number of bacterial cells ($7x10^4$cfu/g) where the four bacterial species were present. This was followed by plastic, where $5.3x10^2$ cfu/cm^2 ($\approx 1.3x10^4$ cfu/g) were adhered and also contained the four bacterial species, This was also evident in the SEM micrograph of sand (I) and plastic (II) biofilm systems, (Figure 1). Comparing these results with those of gravel particles, it is clearly shown that bacterial adhesion to gravel is very weak and only two species could adhere (*B. sphaericus* & *Arthrobacter sp.*)

Although population counts of those two bacteria recorded $1.1x10^5$ cfu/g, gravel was excluded as supporting material for the biofilm because two of biosorbent agents were absent. According to the previous results, sand and plastic systems were selected as supporting materials for the biofilm filter system used for metal removal.

Removal efficiency achieved by the biofilm filter systems

Figures (2&3) show the remaining levels of heavy metals after treatment by the tested biofilm systems (sand and plastic). Results showed considerable variation among different metals as well as between the biofilm systems. Sand biofilm filter showed more ability to accumulate Fe, Cd, and Pb compared to that accumulated by the plastic biofilm filter, with a fold increase of 1.2, 1.1, and 1.2, respectively, at 30ml/h flow rate.

On the other hand, plastic biofilm filter accumulated more Fe^{+3} with fold increase of 1.7 and 2.3, than sand biofilm at flow rates 45 and 60ml/h, respectively.

Also, the exposure time needed for reaching the maximum metal accumulation was different depending on the type of metal and the biofilm supporting material. Among the tested metals, Zn^{+2} was the highest to be accumulated with 94% removal efficiency, followed by Cd (78%), Pb^{+2} (75%), and Cu^{+2}

Table 2. Population counts of the bacterial species; *B. licheniformis, B. sphaericus, Arthrobacter spp.*, and *Micrococcus sp.* adhered onto the biofilm support materials (sand, gravel, plastic) after release treatments.

Material	Treatment	*B. licheniformis*	*B. sphaericus*	*Arthrobacter sp.*	*Micrococcus sp*
Sand	A	$6.1x10^4$	$15.7x10^4$	$16.5x10^3$	$44.3x10^3$
	B	$4.6x10^2$	$2.1x10^2$	$0.5x10^2$	-
	C	$0.3x10^2$	$0.5x10^2$	$0.7x10^2$	-
	Total	**$6.2x10^4$**	**$15.7x10^4$**	**$1.6x10^4$**	**$4.4x10^4$**
	Grand Total	**($7.0x10^4$ cfu /g)**			
Gravel	A	-	-	-	-
	B	-	$5x10^4$	$6x10^4$	-
	C	-	-	7	-
	Total	-	**5x104**	**$6x10^4$**	-
	Grand Total	**$1.1x\ 10^5$ cfu/g**			
Plastic	A	$1.7x10^2$	$2.3x10^2$	$1.7x10^3$	-
	B	$1.0x10^2$	$9.7x10^2$	$6.0x10^2$	$2.2x10^4$
	C	$1.0x10^2$	$5.6x10^2$	$3.0x10^2$	-
	Total	**$3.7x10^2$**	**$1.7x10^3$**	**$2.6x10^3$**	**$2.0x10^4$**
	Grand Total	**$5.3x\ 10^2$ cfu/cm^2 = $1.3x10^4$ cfu/g**			

A= Vigorous shacking (1 min); B= Centrifugation at 4,000 xg (15 min) ; \C= Vortexing (5 min)

Table 3. Efficiency (%) of the tested biofilm system (sand & plastic) in removing the tested heavy metals at the end of exposure time

Biofilm system	Flow rates (ml/h)														
	30					45					60				
	Fe	Cd	Cu	Pb	Zn	Fe	Cd	Cu	Pb	Zn	Fe	Cd	Cu	Pb	Zn
Sand	47	78	71	75	94	22	46	42	50	80	11	35	40	27	62
Plastic	42	68	70	64	90	38	43	40	50	57	25	30	40	37	40

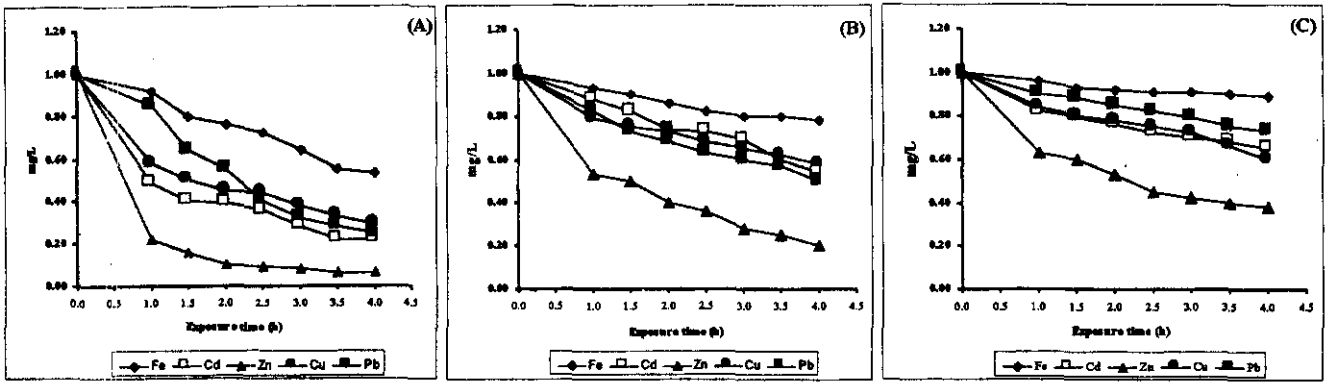

Figure 2: Performance of the sand-biofilm filter at three different flow rates (ml/h) : 30 (A), 45 (B), and 60 (C), and their capacity in removing the tested heavy metals (Fe, Cd, Zn, Cu, Pb) from polluted water.

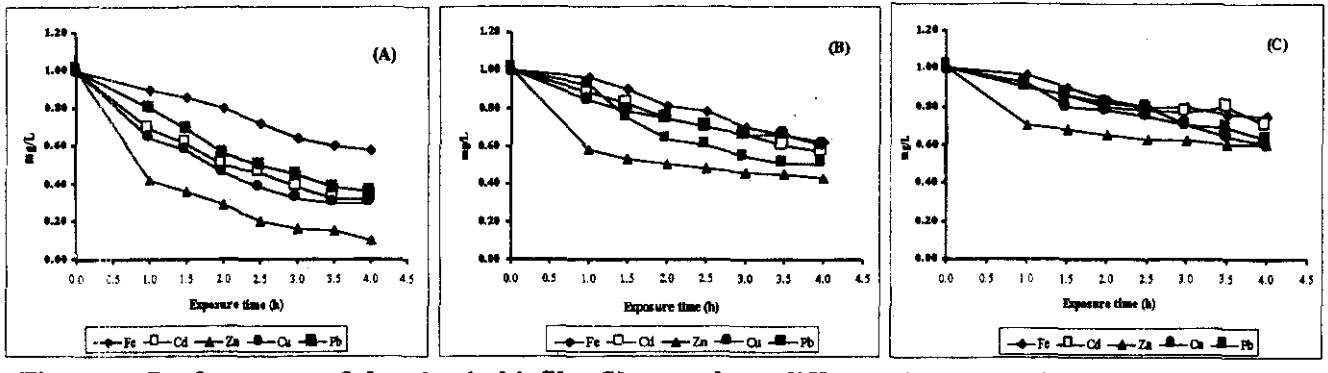

Figure 3: Performance of the plastic-biofilm filter at three different flow rates (ml/h): 30 (A), 45 (B), and 60 (C), and their capacity in removing the tested heavy metals (Fe, Cd, Zn, Cu, Pb) from polluted water.

(71%) at 30ml/h flow rate, as shown Table (3). That order varied by changing the flow rate, except for Zn^{+2} that is an essential metal needed extensively by the micro-organisms for various metabolic and enzymatic processes (Dedyukhina and Eroshin 1991).

In general, the trend of accumulating heavy metals by both tested biofilm systems revealed that sand-biofilm filter is better than plastic-biofilm filter at all the tested flow rates except for Fe^{+2} especially at 45 and 60 ml/h flow rates.

Biosorption of heavy metals by microbial cells has been recognized as a potential alternative to the existing technologies for treating and recovery of heavy metals from industrial waste streams and natural waters since they are considered as potent biosorbent materials (Scott and Karanjkar, 1992,; Cavet *et al.*, 2003). Using these biosorbents is of particular importance especially with large volumes of waste water containing relatively low metal levels.

In most natural environments, association with a surface in a structure known as a biofilm is the prevailing microbial lifestyle. Surface association is an efficient means of lingering in a favorable microenvironment rather than being swept away by the current (Watnick and Kolter, 2000). However, bacteria are rarely to be found as pure cultures in nature because they generally occur in microbial consortia (Mclean *et al.*, 1996). Therefore, a consortium of potent bacteria for accumulating heavy metals was used in this study, where they have been adhered onto a suitable biofilm system.

Generally, microorganisms proved to be effective in accumulating metals, because they are characterized by high metal absorbing ability, selectivity in absorbing metals, and exhibiting metal resistant (Beveridge and Morrag, 1976; Simon and Phung, 1996; Cavet *et al.*,2003). However, using immobilized cells improve the ability to recover and regenerate the biosorbent and facilitate their application in metal removal (Wihelmi and Duncan, 1996).

For an effective metals removal, the system would require a biomass which is readily available, economical, reusable, possessing high uptake capacity, and allowing selective recovery (Macaskie, 1990; Wilhlemi and Duncan, 1995). These requirements promote the

system for potential application both for environmental regulation and economic recovery of heavy metal.

The specific mechanisms by which these metals taken up by the cells are unknown, but in many cases their uptake has been shown to be under genetic control and specifically under plasmid-linked genes (Watnick and Kolter, 2000). Intracellular metal deposition occurs also by non-metabolically mediated processes. It was found that lead (Pb^{+2}) is accumulated intra-cellularly in *Saccharomyces cerevisiae* by diffusion, while Cd^{+2} and Co^{+2} uptake was energy dependent (Wilhlemi and Duncan, 1995).

In a study for removing Cu^{+2} and Zn^{+2} by the filamentous bacterium *Thiothrix* strain Al, the highest removal efficiency (RE) were from 30-75% for Zn^{+2} and from 7-33% for Cu^{+2} (Shuttleworth and Unz, 1993), which are much lower than those obtained in the present study. In another study, RE of Cu^{+2} by a fixed-film reactor reached a maximum of 27% while using a bioreactor system enhanced the RE to 47.6% (Porro et al., 1993).

In conclusion, results of the present study indicated the potential of using biofilm filters for removing heavy metals from contaminated water. Sand biofilm proved to be better than plastic biofilm for removing most of the tested heavy metals. However, the specific surface factor for the supporting material used in constructing the biofilm has to be considered.

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بنـــــاء نظام غشاء حيوى ذوكفاءة فى إزالة المعادن الثقيلة من مياه ملوثة

محمد هشـــام المصـــرى
قسم التكنولوجيا الحيوية – معهدالدراسات العليا والبحوث– جامعةالأسكندرية

أجريت دراسة علىاستخدام نظام غشاء حيوى لتقدير مدى كفاءته فى إزالة المعادن الثقيلة من الماء الملوث. وقد استخدمت ثلاث وسائط مختلفة (الرمل، الزلط ، البلاستيك) لتثبيت مجموعة من أربع بكتريات هى *باسيلس ليشينيفورمس وباسيلس سفيريكس*، وجنس من *أرثروباكتر* ، وجنس من *ميكروكوكس*. وقد أثبتت النتائج الأولية أن الأربع بكتريات المستخدمة يمكن تثبيتها على كل من الرمل والبلاستيك، فىحين أن اثنين فقط أمكن تثبيتها على مادة الزلط، لذلك فقد استخدم نظام الغشاء الحيوى المثبت على الرمل والبلاستيك فى تجارب لاختبار قدرتهما على إزالة المعادن الثقيلة من ماء ملوث بها. وقد أظهر نظام الغشاء الحيوى الرملى قدرة عالية على إزالة الحديد، والكادميوم، والرصاص بمقارنته بنظام الغشاء الحيوى البلاستيكى مع كفاءة أعلى تقدر بـــ ١,٢،١,١،١,٢ مرة على الترتيب وذلك عند معدل تدفق ٣٠مل/ساعة. ولكن من جهة أخرى، كان لنظام الغشاء الحيوى البلاستيكى قدرة أعلى من الرملى على إزالة الحديد بزيادة ١,٧ و ٢,٣ مرة عند معدلى التدفق ٤٥ و ٦٠ مل/ساعة على الترتيب. ويعتبر عنصر الزنك من العناصر التى تراكمت بنسبة عالية تقدر ٩٤ ٪ ، يليه الكادميوم (٧٨٪)، ثم الرصاص (٧٥٪) وأخيرا النحاس (٧١٪) ، وذلك عندمعدل تدفق ٣٠مل/ساعة. إلا أن هذا الترتيب قد اختلف عند معدلات التدفق الأعلى خلاف عنصرى الزنك والكادميوم اللذان تراكما بمعدلات أعلى فى نظام الغشاء الحيوى الرملى بمقارنته بالنظام المستخدم فيه البلاستيك. وقد دلت النتائج المتحصل عليها فى هذه الدراسة أهمية استخدام نظام مرشحات من الأغشية الحيوية للتخلص من المعادن الثقيلة فى المياه الملوثة بها مع الأخذ فى الاعتبار المواصفات المثلى للوسائط التى تتيح تثبيت الميكروبات المستخدمة فى النظام للحصول على أعلى كفاءة إزالة للمعادن الثقيلة من مصادر المياه الملوثة.