Aim and Scope

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CONTENTS

Dry Anaerobic Digestion of Agro-Food Waste in a Batch System

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ABSTRACT: Dry anaerobic digestion systems work with wastes that have a dry matter content above 20%. This technology has been used above all to treat the organic fraction of municipal solid waste making its application to the agro-food industry waste a novel approach. Co-digestion of vegetable and MBM by means of a batch system shows a good efficiency in spite of the presence of certain compounds at inhibitory levels ($N\text{-}NH_3$ and VFA) which are detected in the process leachate.

INTRODUCTION

PRODUCTION of organic waste is growing worldwide, while agricultural soils are witnessing a progressive reduction in their organic content. The new concern regarding the productivity and sustainability of agro-ecosystems thus leads to the formulation of a new approach in soil management that stresses the recycling of waste organic matter into soil. Nevertheless, the use of waste organic matter as a soil conditioner is facing difficulties because of the very peculiar nature of this refuse. These products could be harmful for the environment and human health because they contain xenobiotic compounds and microbial pathogens. There are some sanitation treatments to eliminate this kind of pollution, but the best way to avoid this problem is processing really "clean" organic waste such as those generated in the agro-food industry.

Anaerobic digestion is a reliable process when treating this type of waste. Due to their high moisture and low energy content, energy recovery by incineration is not a good option. With respect to sustainability, the anaerobic digestion of bio-waste is more favourable than composting or other technologies, since fossil fuels can be substituted, the CO_2 emissions can be reduced and a better energy balance is achieved thanks to the production of methane-rich biogas [1, 2, 3]. Moreover, anaerobic digestion in combination with composting can be considered a bio-waste management option which contributes to carbon sequestration by means of soil application of the end-product (compost) as a soil amendment or fertilizer and with other purposes such as land reclamation [4]. In addition, sequestration of CO_2 through land application of organic waste may have a significant effect in meeting the emission reduction targets of the Kyoto Protocol [5, 6, 7, 8].

An interesting option for improving this biological process is co-digestion, i.e. the use of a co-substrate which in most cases increases the biogas yields due to positive synergies occurring in the digestion medium [9, 10, 11]. Anaerobic co-digestion can also be beneficial in terms of dilution of potential toxic compounds, nutrient balance or maximum organic loading rate [12, 13]. However, anaerobic digestates are not generally suitable for direct application on soil because of the presence of phytotoxic substances and the high moisture level in the digested product. If digestion has not occurred within the thermophilic range of temperatures the end product will not be hygienised to the recommended levels [14]. Thus, it is accepted that an aerobic thermophilic post-treatment is needed to obtain a high quality soil amendment [15].

Anaerobic batch systems are not as efficient as continuous flow ones and as a result they are less commonly used at full scale, except in developing countries due to the lower investment needed. However, this kind of experiments are useful because they can be performed in a relatively short period of time and the equipment required is simple as well as inexpensive [16]. Therefore, this technology can be an appropriate option for making a detailed study of the whole anaerobic process at laboratory scale.

The overall aim of this paper is the assessment of the dry anaerobic digestion efficiency for treating "clean" co-substrates from the agro-food industry.

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MATERIALS AND METHODS

A small vertical cylinder shaped pilot reactor (8 l useful volume) was used in this study (Figure 1). This digester works as a batch process at a mesophilic range of temperatures (37°C) and 30% dry matter. There is a leachate recycling system to get a good distribution of nutrients and biomass inside the reactor. Biogas production is measured by means of a gas meter by liquid displacement. The feedstock was composed of vegetable waste (*Cynara cardunculus* specie) and MBM (Meat and Bone Meal). These waste were mixed with an appropriate innoculum, organic amendment (compost) and wood shaving. The latter compounds are needed to get a balanced anaerobic process, and the different proportions (in dry matter) were selected according to Brummeler [17] as shown in Table 1.

Table 1. Properties of Each Component in Input Mixture and Their Proportion.

Components	TS (%)	VS (%)	VS (%TS)	Component Fraction (%TS)
Organic amendment	80.9	29.7	36.7	40
Innoculum	32.0	15.9	41.8	25
Vegetables	5.7	5.1	88.0	10
MBM	94.9	69.4	73.1	10
Wood shaving	89.8	86.4	96.3	15
Mixture	**30.1**	**16.8**	**55.8**	**100**

Analytical Methods

Several chemical analyses were done to check the performance of the process, according to *Standard Methods 20th* ed. [18]: Total Solids -TS-, Volatile Solids -VS-, soluble Chemical Oxygen Demand -COD- (1.5 µm filter), pH, Total Ammonia Nitrogen -TAN-, Alkalinity (Intermediate, Bicarbonate and Total). VFA composition was measured by using a GC system (Agilent GC-6890), equipped with a FID detector and a DB-FFAP capillary column. Biogas composition was determined by means of other GC system (HP6890 GC-TCD).

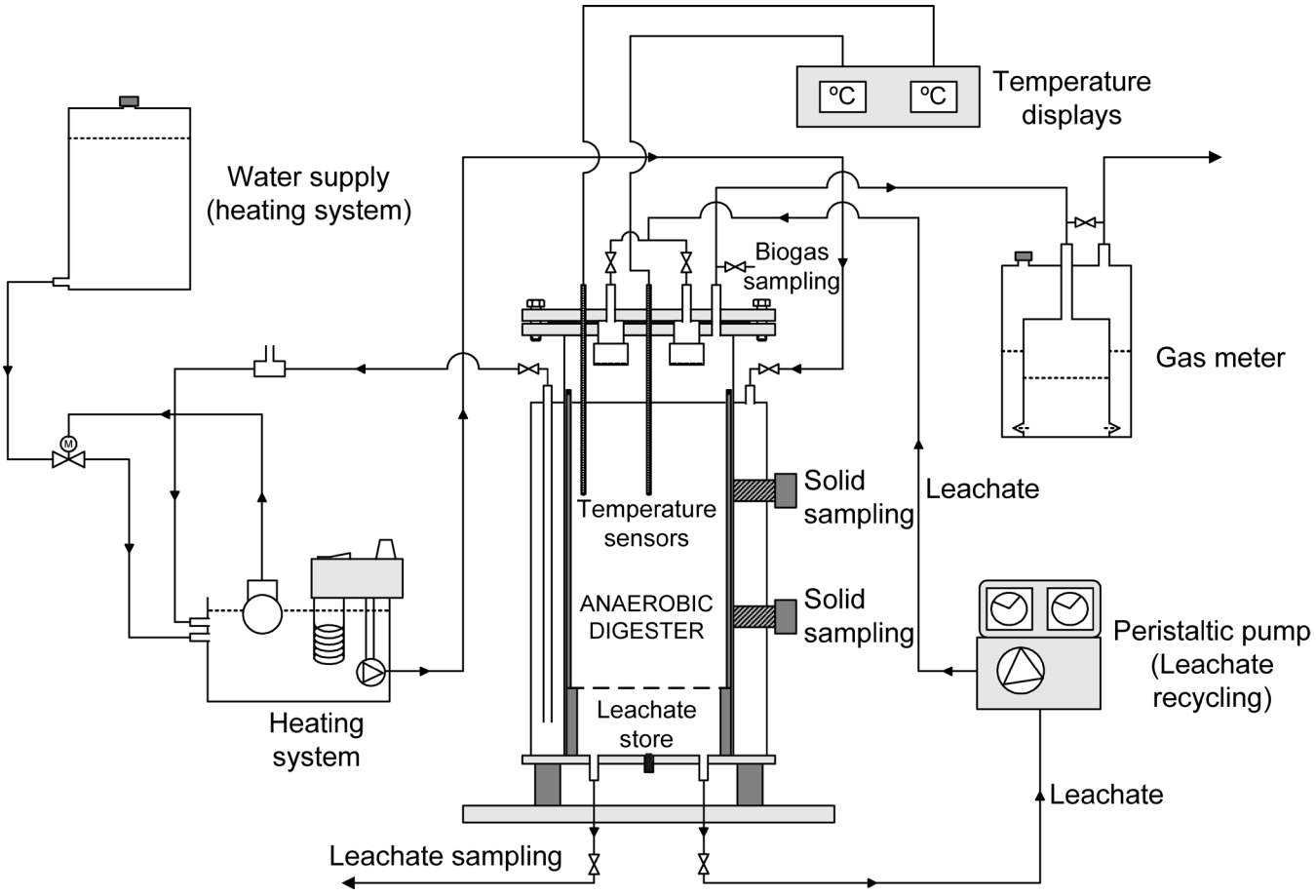

Figure 1. *Pilot plant design.*

RESULTS AND DISCUSSION

The reactor performed in a batch mode for around 30–40 days of useful work at an OLR of 5.1 kg $VSm^{-3}_{reactor}day^{-1}$ (a typical value in a non-continuous system). In this period four stages can be distinguished:

A. Start up and acclimatisation
B. Transient state where most of the organic matter is removed
C. The most stable stage where the remaining organic matter is degraded
D. Exhausting organic matter removal and start of the endogenous phase

The study of the leachate generated during the batch was critical as this is necessary to follow the reactor evolution as the fermentation and methanogenesis take place in the liquid phase within the solid waste matrix. As the analysis of the leachate composition is easier than in the liquid fraction within the reactor, a comparison of the composition in the soluble fraction in the reactor matrix and in the leachate was carried out. The results are shown graphically in Figures 4, 5 and 6. Similar soluble COD values are found in both liquids except in stage A where products form a rapid hydrolysis of organic matter were perhaps not washed by the leachate produced. A clear parallel evolution was seen in the pH values as well as for the TAN. These measurements of soluble compounds in the leachate can show the reactor situation also regarding particulate organic matter solubilization.

The leachate production is high at stage A, with a clear appearance but an unpleasant odour. In stages B, C and D leachate production is not as high because the matrix grows more viscous and because of the filter at the bottom of digester being blocked up. The liquid in this period gets dark and has a less aggressive smell as a result of the disappearance of VFA.

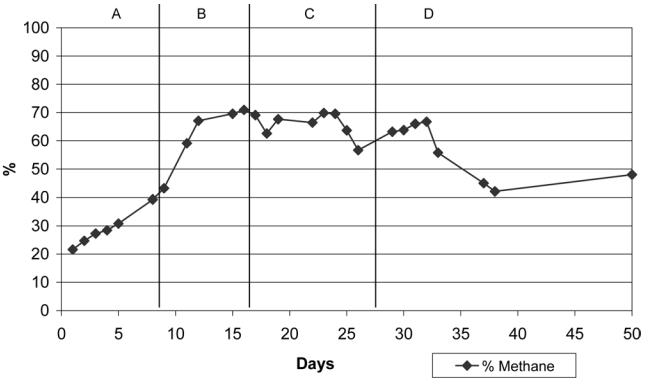

Figure 3. *Evolution of methane proportion.*

Stage A

The soluble COD (Figure 4) reaches its highest values in the cycle (near 60 g/l). The main compounds causing this rise are the VFA (Figure 8) which is also reflected in the high value of the intermediate alkalinity (Figure 7). The low value of bicarbonate alkalinity would lead to a drop in pH but the huge amount of TAN (near 4 g/l) means that the pH stays at neutral values (Figure 5). The high level of the VFA makes one think that there is some inhibition acting on the methanogenic bacteria, which together with the short time in stage A, makes the reactor show a small gas production although the methane percentage in the biogas increases up to 40% (Figures 2 and 3). In other words, the stage is interesting as a start up and biomass acclimatisation period. The rapid start of hydrolysis plus fermentation is clear, TAN coming from the degradation of proteins and VFA being the most indicative parameters of this.

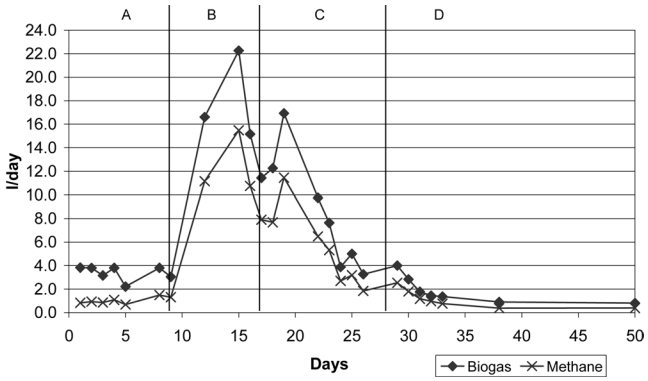

Figure 2. *Biogas and methane production.*

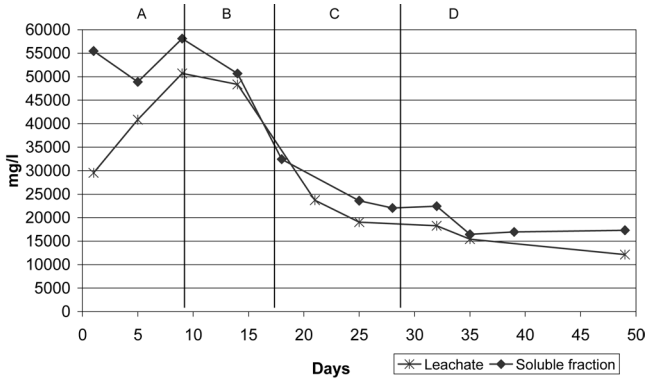

Figure 4. *Soluble COD evolution.*

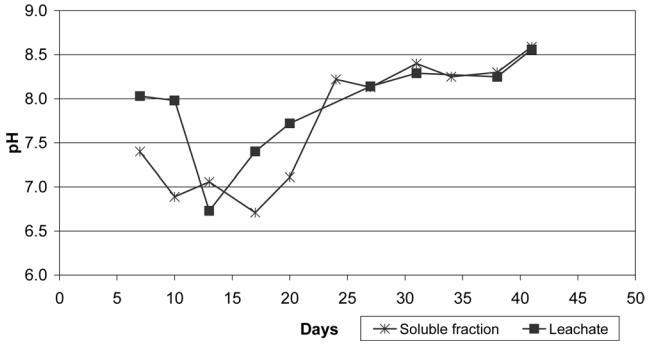

Figure 5. *Evolution of pH.*

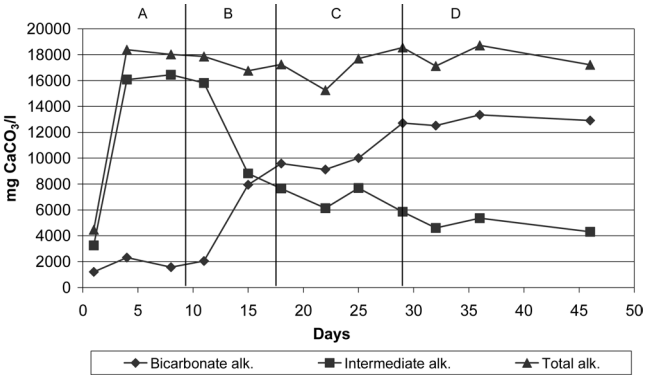

Figure 7. *Evolution of alkalinity (sol. fraction).*

Stage B

The most remarkable point here is the rapid increase in the methane production rate, reaching values over 20 l/day, raising the methane content of biogas up to a value of 70%. This is the consequence of the rapid degradation of soluble COD and VFA during this stage.

The intermediate alkalinity decreases sharply and so reflects the VFA elimination. The bicarbonate alkalinity (Figure 7) increases as a result of the methanogenic activity that produces CO_2. The change in both alkalinities in opposite directions, together with the maintenance of TAN at high values makes the pH increase up to values that are slightly alkaline (around 8).

Stage C

This stage could be regarded as the "most stable" in the cycle although in a batch system no steady state is reached. The methane production rate shows a sharp drop, with a stable methane percentage in the gas (60%). These methane production values are the consequence of a decrease in the soluble COD removal rate (slope in Figure 4) which is partially reflected in the reduction of VFA. The increase of propionate may explain the abrupt peak in biogas at this stage. The pH value rises as the effect of three buffer systems: the dissociated-undissociated VFA one decreases the pH, while the bicarbonate-carbon dioxide and ammonium-ammonia systems increase it.

Bicarbonate and intermediate alkalinity tend to stabilize although the former continues to increase and the latter decreases. However, the ratios between intermediate alkalinity and bicarbonate alkalinity -IA/BA- (around 0.8) and intermediate alkalinity and total alkalinity -IA/TA- (about 0.4) can be considered as high values, and therefore show unstable conditions rigorously speaking [19]. Moreover, the concentration of dissolved free ammonia nitrogen ($N\text{-}NH_3$) is very high in terms of inhibitor effect and is increasing (500–700 mg/l, Figure 6). This form is widely regarded as the really inhibitory one and several authors propose two different mechanisms for the inhibition of methanogenesis by free ammonia. The first mechanism is direct inhibition of methane synthesizing enzyme, whereas the second mechanism involves passive diffusion of hydrophobic free ammonia through the cell membrane and its immediate conversion to ammonium which causes variations of the intracellular pH [20, 21, 22]. The methane percentage, the degradation of VFA and soluble COD, and the almost constant values of

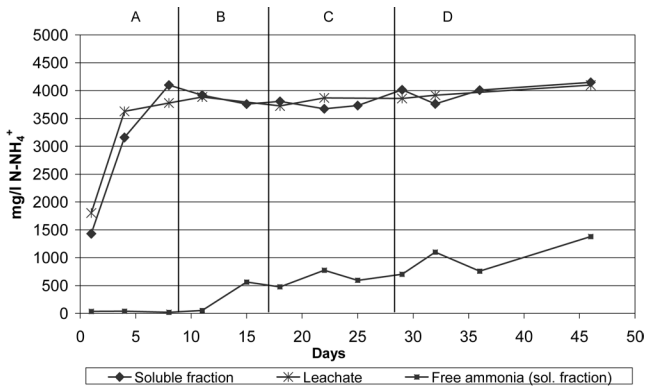

Figure 6. *TAN and free ammonia evolution.*

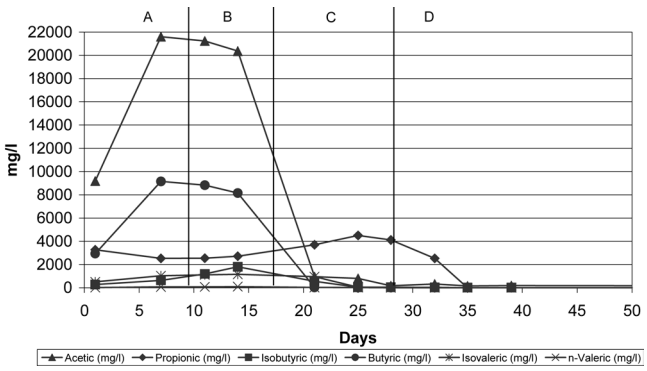

Figure 8. *Evolution of VFA (sol. fraction).*

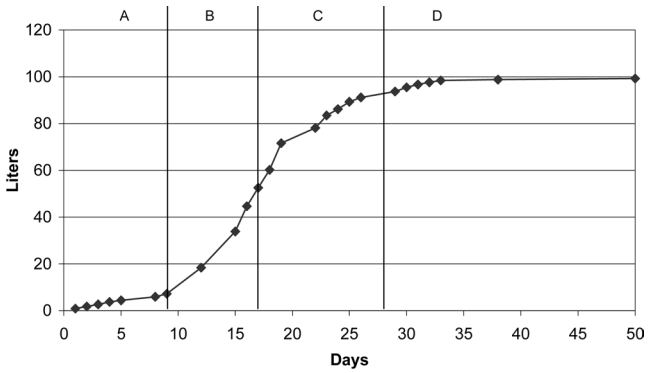

Figure 9. *Cumulative methane production.*

alkalinities and pH make one look for an explanation for this apparent inconsistency. It can be found in two facts. First, the innoculum which the reactor started with had been working during three similar batch cycles, which leads to think of some degree of acclimatisation to ammonia inhibition, as previously reported by several authors [23, 24, 25]. Secondly, the "dry digesters" may have some methanogenic niches within the solid matrix, whose presence is favoured by the formation of preferential paths during leachate recycling. The level of the inhibitor compounds here may probably be smaller than in the leachate [26]. In spite of the maintenance of the digestion in balanced conditions, it is logical to think that the process is partially inhibited, as occurs in some cases of swine manure treatments. In this case it would be better to adjust the C/N ratio to between 20 and 30, as reported by Stroot *et al.* [27] for anaerobic systems. The value measured at the beginning of the process was around 15, which is a bit lower than desired. This issue might prevent the ammonia nitrogen inhibition.

Stage D

At this stage, the soluble COD decreases until it stays at a stable value of 15 g/l with the VFA concentration being negligible (100 mg/l). The biogas production goes down to values under 1 l/day with a stable but low methane percentage (50%).

The pH rises and is maintained at a value of 8.5 and the TAN concentration rises to over 4 g/l. As a consequence, the free ammonia value reaches 1000 mg/l. The bicarbonate and intermediate alkalinities have constant values, which rend a ratio of AI/AB close to 0.3.

Process Overview

Looking at the cycle as a whole, the parameter evolution during the batch period is the one expected in such an inherently transient system. The stage D exhibits the stability of a finished cycle in terms of organic matter degradation (Figure 9). The initial increase in the VFA concentration (stage A) together with the drop in pH and the further growth in the free ammonia concentration, points to the importance of a proper selection of the raw waste proportion in the input mixture so as to prevent an unbalanced process start-up. The correlation between soluble COD and VFA concentrations (Figure 10) indicates that the latter is one of the main constituents of the former. However, the figure shows a COD fraction (around 15 g/l) that has does not correspond to any VFA, which makes one think that the process leaves a high amount of other soluble organic compounds that cannot be degraded under current experimental conditions. Moreover, there is a good correlation between intermediate alkalinity and VFA concentration (Figure 11) although there is a group of compounds that reflect a high intermediate alkalinity (about 4.5 g/l) which does not correspond to any VFA but to other compounds containing carboxylic or similar groups. Both figures make one think that the soluble fraction of the final content in the reactor has compounds such as long chain fatty acids polyphenols or other non-biodegradable constituents such as fulvic or humic acids. It would be desirable to characterize and measure the concentration of the soluble biodegradable residual compounds, in order to asses the need for a further aerobic composting before spreading the digested product on soil as an organic amendment. Another question regarding the application of the end product to soil is the hygienization. The process temperature was around 37°C (mesophilic range). Referring to this point, it can be considered that a treatment within the thermophilic range would be desirable. However, in that case the free ammonia concentration would reach values close to 2 g/l, and

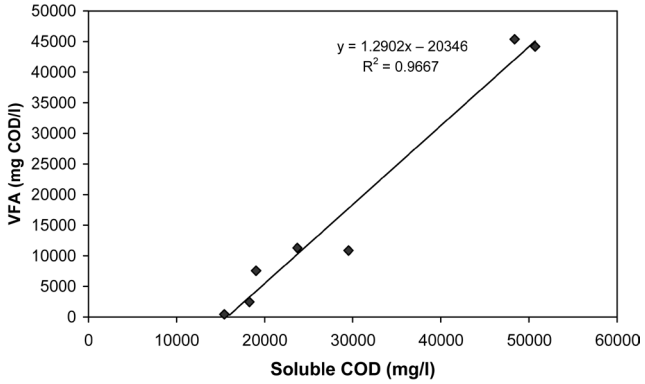

Figure 10. *VFA vs COD correlation.*

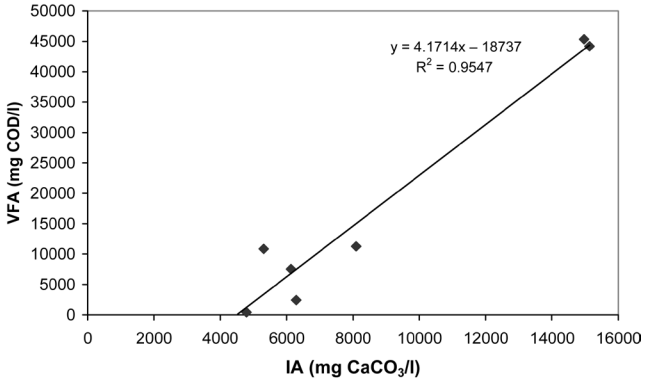

Figure 11. *VFA vs Intermediate Alk. Correlation.*

this would cause an important negative effect on the digestion. The hygienization should be done by means of a post-composting process, where thermophilic temperatures are reached.

The efficiency of volatile solids removal in the process is difficult to express, due to the high content of non-biodegradable organic matter (70% VS) compared to the biodegradable fraction (30% VS). This fact means that only 10–15% (in dry matter) of the input material can be really transformed (i.e. the biodegradable volatile solids, BVS). Another factor to be noted is the heterogeneity of the samples. Therefore, the VS measurement is not the best choice to evaluate the digester efficiency. As a result of this, a mass balance as well as methane production are the best way to estimate the process efficiency. Thus, the estimated BVS removal of the waste was around 60–70%. The specific methane production (according to VS feed) was 0.24 $m^3CH_4kg^{-1}VS_{fed}$, working at an OLR of 5.1 $kgVSm^{-3}{}_{reactor}$ day^{-1} (a typical rate in batch systems). Other authors have reported comparable values treating similar waste [28, 29, 30]. In a study carried out by Wu *et al.* [31], the specific methane production (according to BVS added) was 0.48 $m^3CH_4kg^{-1}BVS_{fed}$, whereas the rate described by Cho *et al.* [32] using MBM as feedstock was within the range 0.35–0.38 $m^3CH_4kg^{-1}BVS_{fed}$. However, it must be stressed that there may be some differences regarding the input mixture composition. Finally, the methane production rate reached a value of 0.33 $m^3CH_4m^{-3}{}_{reactor}day^{-1}$.

CONCLUSIONS

The dry anaerobic co-digestion of organic waste from the agro-food industry is quite an attractive technology for research purposes and for being used in developing countries. The batch system at pilot scale allowed the study of different stages in the cycle, enabling the observation of some interesting phenomena. The role played by the leachate produced during the batch cycle was fundamental, both in the reactor performance and in the study of the process. Its composition is similar to that of the soluble fraction in the reactor solid matrix. The process works reasonably stable in spite of the high levels of free ammonium and volatile fatty acids that are supposed inhibitors at such concentrations. Two reasons could explain this behaviour. One is that the innoculum biomass was already acclimatised in three previous similar cycles. The other is that in dry digestion there are some peculiar phenomena regarding the reactor matrix rheology, such as the presence of methanogenic niches where the inhibitors are in lower concentrations than those found in the leachate. It is difficult to see the evolution of VS due to the low content of organic matter that is really transformed (10–15% in dry matter). However, the digester performance can be assessed by measuring the leachate composition and the methane production. The end product from the treatment had a high concentration of ammonia and soluble organic compounds measured as COD and intermediate alkalinity that have not been identified. These phenomena and the need for a hygienization means that a composting process as a post-treatment is recommended, in order to make the end product suitable for application on soil.

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Evaluation of USEPA Time-Temperature Requirement for *Escherichia coli* Destruction by Standard Culture Method and Flow Cytometry

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ABSTRACT: USEPA time-temperature requirements for fecal coliform destruction were evaluated by both the standard culture method and flow cytometry to investigate if insufficient thermal treatment was the reason for fecal coliforms reactivation observed in a field digester. Results showed that complete inactivation (8-log) of cells can be achieved well below the regulatory requirement. Destruction was found to be permanent where no recovery of viable cell was observed upon further storage up to 2 weeks. Overall, time-temperature treatment is found sufficient to completely inactivate coliform cells but stressors prior to thermal treatment may increase their resistance.

INTRODUCTION

THERMAL treatment of biosolids is a widely used method of reducing biological activity before disposal or beneficial reuse. The United States EPA specifies a set of time-temperature requirements to achieve Class A standard biosolids by thermal treatments above 50°C [1]. These standards are based mainly on work compiled by Feachem *et al.* [2] and the U.S. Department of Health and Human Services requirements [3]. Sewage sludge treated up to or beyond this requirement and that have fecal coliform (FC) levels below 1,000 MPN/g dry solids (DS) or *Salmonella* below 3 MPN/4 g DS are considered class A biosolids and may be reused without site restriction [4].

However, several studies have shown that although FC levels are below class A limits after heat treatment a sudden increase of FC levels occurred immediately after dewatering especially when centrifuge dewatering was used [5–7]. This sudden increase of FC levels immediately after dewatering was believed to be reactivation of viable but non-culturable (VBNC) bacteria which differs from regrowth of bacteria during cake storage [6, 7]. Several researchers have observed the ability of bacteria to enter this VBNC state in which cells cannot be cultured on standard culture media but retain their membrane integrity [8] and/or metabolic activity [9].

This VBNC state can result from bacteria being subjected to a variety of stressors including starvation, chlorine, salinity, and low or high temperatures [8, 10, 11]. While there is still an ongoing debate on whether this state is a stress response or a moribund condition [12], researchers have demonstrated the ability of several species of bacteria including *E. coli, Salmonella, Vibrio, Pseudomonas,* and *Enterococci* to recover after being stressed into a non-culturable state [10, 11, 13, 14]. Reactivation was observed only in plants using thermophilic treatment/digestion but regrowth can occur for both thermophilic and mesophilic processes and is mostly associated with centrifuge dewatering [15]. Though the combination of reactivation and regrowth can potentially reach the level of FC prior to digestion [16], *Salmonella* remained non-detectable in thermophilically digested biosolids [15]. On the other hand and based on molecular DNA methods, several opportunist pathogens were discovered in a Class A biosolids product collected from a temperature-phased anaerobic digester despite having 15–30 days of treatment at 55°C [17]. Therefore, fully understanding the true risk of using biosolids experiencing reactivation remains uncertain.

Since VBNC bacteria are non-culturable culture-independent methods are needed in order to observe their presence. Real-time PCR (rt-PCR) has been used

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to quantify both culturable and non-culturable *E. coli* in liquid and dewatered biosolids. Concentrations of *E. coli* DNA were found to be comparable in both samples despite a 3–5 log difference as enumerated by the standard culture method [6]. The difference between rt-PCR and culture method in liquid biosolids was believed to be due to the presence of VBNC bacteria. This difference was termed "reactivation potential" and was found to have a strong correlation to the level of *E. coli* that increased after dewatering [15]. The drawback of the rt-PCR method is that it can also include DNA from dead cells and thus may over-estimate level of VBNC bacteria.

In the last decade, flow cytometry has gained popularity with microbiologists and environmental researchers due to its ability to assess large amounts of organisms in a fraction of seconds (i.e., less than 1,000 sec^{-1}) and down to single cell levels. Flow cytometry in addition allows for cells to be evaluated on several parameters at one time including particle size, surface condition, and physiological conditions [18–20]. Moreover, inclusions of viability stains in flow cytometric analysis allowed researchers to quickly observe formation of VBNC bacteria under an assortment of physical/chemical challenges [8, 21–24]. The Live/Dead® BacLight™ kit (Invitrogen, Carlsbad, CA) is one of the commonly used commercial kits used to differentiate a cell's viability under the assumption live cells would have an intact cell membrane while the membrane of dead cells are compromised [25]. The test kit contains two fluorescent stains, propidium iodide (PI) and SYTO-9, where SYTO-9 can enter both live and dead cells and exhibit green fluorescence while PI can only penetrate dead cells which then quench SYTO-9 and exhibit red fluorescence. Conjunction of the viability test kit and flow cytometry would then allow researchers to determine each individual cell's viability by level of membrane damage.

Despite growing use of flow cytometry limited work has been conducted to analyze bacterial cell responses within time and temperature ranges over which fecal colfirom reactivation are reported. Allegra *et al.* [26] used flow cytometry to visualize VBNC cell formation of *Legionella* at 70°C with up to 60 min of heat treatment. Foladori *et al.* [27] discovered a significant increase in membrane damage in activated sludge after 30 min of treatment at 55 to 80°C. However, culturability of bacteria was not assessed. Khan *et al.* [8] on the other hand observed a 4-log drop of culturability from 10^9 cfu/ml *E. coli* O157:H7 but only analyzed membrane integrity with a single PI stain. Therefore, a more systematic analysis of fecal coliforms with side-by-side flow cytometric and standard culture methods and especially at ranges reporting reactivation is necessary to elucidate suitability of digestion.

Observation of the fecal coliform reaction after digestion and dewatering leaves doubt that the USEPA time-temperature equation is sufficient in completely destroying indicator bacteria or if it somehow creates a population of VBNC bacteria. Moreover, recent risk analysis concluded that a rigorous biosolids treatment process is the most efficient way to reduce pathogen exposure and infectious risk [28].

Therefore, the objective of this research was to determine if the USEPA's time-temperature equations used in thermal treatment can completely inactivate *E. coli,* a dominant group of bacteria in wastewater fecal coliforms, by analyzing their viability with both standard culture methods and flow cytometry in conjunction with the Live/Dead® BacLight™ kit. Commonly used thermophilic and pre-pasteurization treatment temperatures at 55, 65, and 75°C were tested and impact of background solids on their inactivation was also evaluated.

METHODOLOGY

Bacterial Strain and Culture

A wild strain of *E. coli* was isolated from a biosolids sample collected from a wastewater treatment plant that demonstrated a sudden increase in FC after dewatering courtsey of Dr. Matthew Higgins at Bucknell University, PA, USA. The day before each test trial bacteria were batch cultured in nutrient broth at 37°C for 24 h until a stationary phase was reached. This incubation time was based on a previously produced growth curve in our lab and was double-checked by OD_{670} readings from the culture where no more than 5% change was observed over a 30 min time period.

Instantaneous Heat Treatment

Stationary phase bacteria were harvested from batch culture by centrifugation at 10,000 × g for 5 min, washed twice in sterile phosphate buffered saline (PBS, pH 7.2), and resuspended in PBS at 1/10th of their original volume to concentrate the sample. Cells were spiked and diluted to their initial strength (10^8 cfu/ml) in 2.0 ml centrifuge tubes containing either preheated PBS buffer or autoclaved anaerobically digested liquid bioslids from a local wastewater treatment plant. Tubes

were pre-heated to one of 3 temperatures of 75, 65, and 55°C in a heating block (Labnet intl., Woodbridge, NJ) to ensure heat treatment began immediately. Temperature of the heating block was monitored prior to as well as throughout the experiment and temperatures were always within 0.5°C of the target temperature. Samples were treated at 75°C for 0, 6, 30, 60, and 120 min at 65°C for 0, 0.5, 1, 3, and 7 h and at 55°C for 0, 0.5, 2, 6, 12, 24, and 240 h. Liquid level of the sample was always below the top of the heating block to ensure the entire sample was subjected to the same level of stress and all samples were analyzed in triplicate. All samples were carefully placed into each of the centrifuge tubes to avoid contamination of the lid since the lid does not have direct contact with the heating block and thus can be a potential source of contamination in the subsequent recovery tests. After heat treatment, samples were removed from the heating block and chilled immediately in an ice bath to stop the reaction

Gradual Heat Treatment

E. coli were also challenged at 55°C using a gradual heat increase prior to reaching the target of 55°C. The same stationary phase cells were washed and diluted back to original strength in PBS buffer and each 120 µL sample was placed inside a 200 µL PCR tube heated in a Techne Endurance TC-312 Thermal Cycler (Staffordshire, UK). The heating program was set to go from 25°C to 55°C in 4 h using a 5°C increment every 30 min. After reaching 30 and 60 min samples were chilled immediately on ice and analyzed for surviving *E. coli*. They were analyzed again after being stored at room temperature for 7 days. The thermal cycler was also equipped with a heated lid providing 105°C direct contact with tube lids and thus avoiding potential contamination issues. Triplicate tubes were prepared and analyzed for each of the treatments.

Standard Culture Enumeration of *E. coli*

After treatment, each sample was serially diluted and plated on nutrient agar using a membrane filtration technique [29]. Plates were incubated at 37°C for 24 hours and membranes were subsequently transferred to another petri dish containing 1.8 mL of m-FC broth for 2 hours. *E. coli* will show up as blue colonies. The reason why m-FC was not used directly was due to concern that selective media may also reduce FC growth under stress conditions creating biases in culturability. Triplicate samples for each treatment time were analyzed immediately after treatment and again after 7 days of incubation at room temperature in the dark to determine if culturability can be restored after removal from stress. Samples treated at 55°C were analyzed once more after 14 days of incubation. All plates were periodically recounted up to 12 days to see if any new colonies emerged.

Fluorescent Staining and Flow Cytometric Analysis

E. coli in PBS Buffer

After heat treatment, cells were diluted to 1/100th of their original concentration in PBS buffer and stained using the Live/Dead® BacLight™ kit. Cells were stained with Propidium Iodine (PI), SYTO-9, or using a mixture of the two stains. Staining was performed as suggested by the manufacturer where each 1 mL sample was stained with either 1.5 µL of an individual stain or 3 µL of a 1:1 mixture of the two stains. Samples were then incubated in the dark for 30 min and analyzed using a BD FACSCantoII Flow Cytometer (Becton Dickinson, Sydney, AU) with excitation from a 20 mW argon laser emitting light at 488 nm. Green fluorescence was measured in the FITC channel at 525 nm and red fluorescence was measured in the PE-A channel at 575 nm. Using standard culture methods samples were analyzed immediately after treatment. A subset of samples were stained and analyzed after 7 days of incubation at room temperature in the dark. Again, samples treated at 55°C were analyzed once more after 14 days incubation at room temperature. All flow cytometry analysis was performed at the Penn State Hershey Medical Center Flow Cytometry Core Facility, Hershey PA.

To determine concentration from the recorded number of events, standard counting beads were used as the internal standard to compensate any sample to sample variations (Spherotech Inc., Lake Forest, IL). The concentration of the beads used in this experiment was 1×10^6/ml. The beads were counted by the cytometer in a separate region of plot that does not interfere with target cells. Each sample run stopped when exactly 500 standard beads were counted. All flow cytometric data was analyzed and plotted using FlowJo 7.6.3 PC version (Treestar, Inc., Ashland, OR).

E. coli in Autoclaved, Liquid Biosolids

Samples were diluted after heat treatment to 100× in PBS buffer. Then, 40 mL of diluted sample was soni-

cated using the MetOne WGS-267 Particle Analyzer (Grants Pass, OR) until a specific energy of 20 kJ/L was reached to liberate cells from the flocs. This is an adaptation of the protocol detailed in Foladori *et al.* [27] which indicated that specific energy released the maximum net amount of cells and beyond when cell counts decline due to increased rupture. Samples were then filtered through a 20 μm membrane filter (Partec, Gorlitz, Gerrmany) after sonication to remove any remaining large particles. Finally, they were stained and incubated in the same manner as the buffer-suspended samples.

RESULTS AND DISCUSSIONS

Buffer Suspended Samples

Recent research has proposed the sudden increase of indicator organisms after dewatering may be due to formation of a viable but non-culturable (VBNC) state in which bacteria are not killed by treatment but rather lose their culturability using standard culture media [6, 7]. To further examine if this state can be induced using the current heat treatment as specified by the USEPA, *E. coli* isolated from a treatment plant that exhibited reactivation in the past were isolated and treated using several time-temperature regimes. Then, they were examined for their viability using both standard culture methods as well as the culture-independent LIVE/DEAD® BacLight™ Bacterial Viability Kit in conjunction with flow cytometry. It was observed that when *E. coli* cells were subjected to 75°C treatment none of the cells could be recovered using culture methods for any treatment time used (detection limit 1 cfu/mL) ranging from 6–120 min. This represents an 8-log reduction from the initial 10^8 cfu/mL before treatment. Based on the USEPA's time-temperature equation, 6 min is the minimum time needed at 75°C for Class A inactivation. Therefore, results confirmed adequacy of the equation based on the culture method. Shorter times not tested in this experiment may also be sufficient. Treated samples were also stained by the live/dead fluorescent kit and analyzed using flow cytometry to check individual cell's viability based on membrane integrity. Results showed that cells for all treatment times shifted from the bottom-right gated area representing live cells to the top-left area implicating dead cells (Figure 1). This shift is due to the compromised (dead) cells allowing red fluorescence PI (Y-axis) to enter which simultaneously quenched green fluorescence SYTO 9 (X-axis). Compared to intensities from the live cells this resulted in approximately a 10-fold increase in red fluorescence and a 10-fold decrease in green fluorescence. Total cell counts remained constant within 1-log variation throughout all heated treatments and controls. Intermediate cells reported to contain VBNC bacteria were also not observed in addition to no cell remaining in the live gate [26]. Treatment times up to 120 min yielded similar results to 6 min with all cells located in the dead zone which demonstrates cell damage occurs rapidly at this temperature and complete damage occurred when the EPA's time-temperature requirement was met (Figure 1). Previous reports indicated *E. coli* may enter a non-culturable state upon heat treatment and culturability can be restored upon further storage at room temperature [30]. To observe if this phenomenon had occurred treated cells were stored in a dark room under temperature for 7 days and were analyzed again using both culture methods and flow cytometric analysis. Again, no culturable cells were found after this storage and the flow cytogram yielded similar results where all cells remained at the dead area (Figure 1). This suggests cells were indeed inactivated by this heat treatment without formation of any viable but non-culturable cells. Therefore, it confirmed the USEPA equation is sufficient at 75°C treatment.

The 65°C trial yielded results comparable to the 75°C trial where all cells shifted from the live zone to an area implicating a membrane compromised of dead cells upon heat treatment with the shortest treatment time of 30 min used (Figure 1). Again, no culturable cells could be recovered for any of the heat treated samples. At 65°C, the USEPA's time requirement for class A treatment is approximately 57 min and therefore 100% inactivation was achieved in only 52% of the required time (Table 1). After 1 week of incubation under room temperature all cells remained in the dead zone (Figure 1) and media culture showed that none of them regain culturability. Overall, results suggest the USEPA's time-temperature equation is more than sufficient when the treatment temperature is 65°C. Germany's recommended standard for this temperature is more relaxed than the USEPA regulation requiring only 30 min for this treatment temperature [1]. This requirement is also fulfilled based on this result nonetheless.

Treatment at 55°C is the most commonly used treatment for thermophilic digestion in the U.S. A treatment time of 24 hours is required per the USEPA equation. A few of the previously reported plants exhibiting reactivation of non-culturable cells were from treatment systems of this type [15]. Therefore, a further gradation of treatment period including much shorter treatment

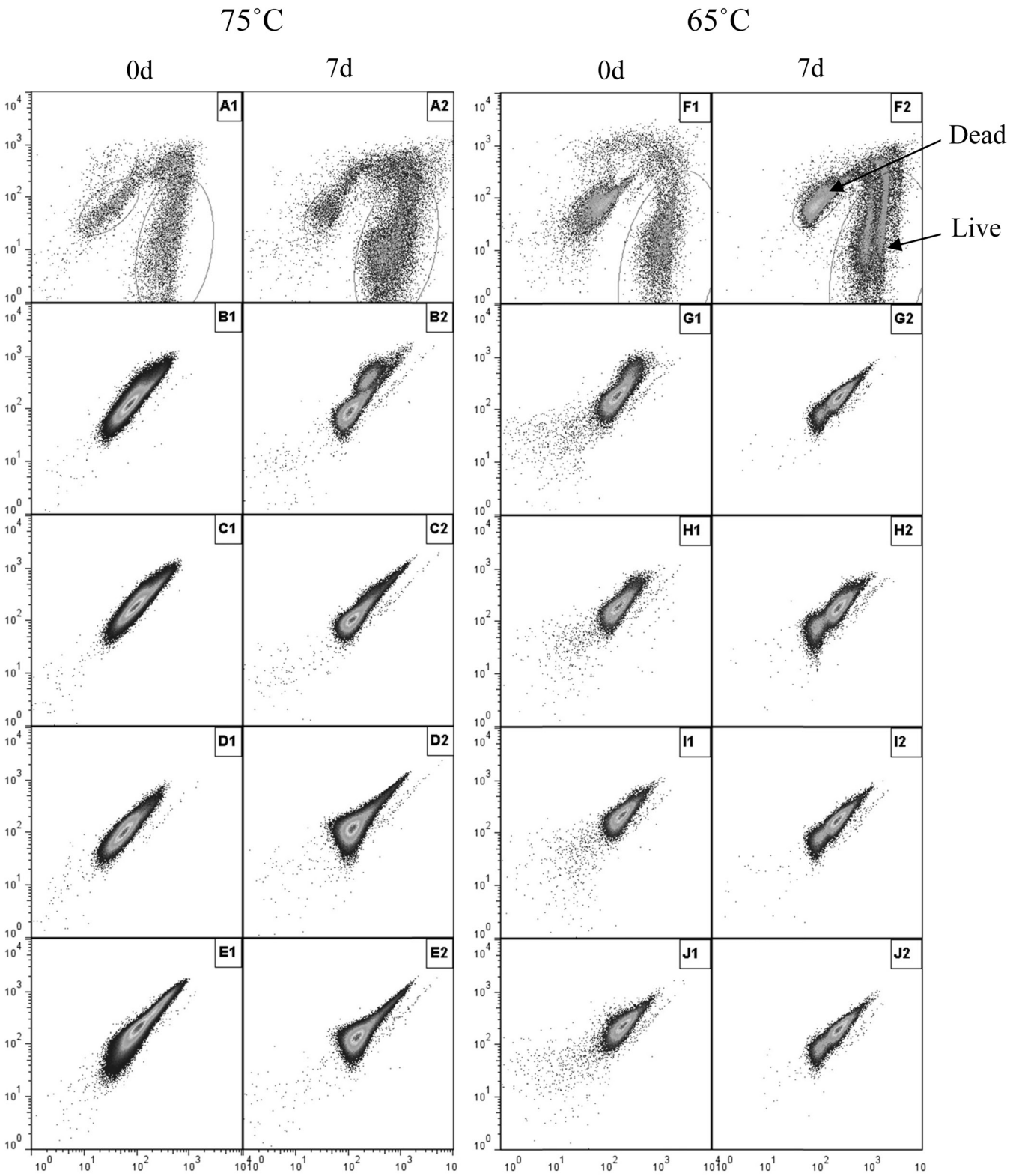

Figure 3. *Flow cytometric dot plots of E. coli treated at 75°C and 65°C. Samples were analyzed immediately after treatment and after 7 days. The 75°C treatments include untreated (A), 6 min (B), 30 min (C), 60 min (D), and 120 min (E). The 65°C treatments include untreated (F), 30 min (G), 1h (H), 3 h (I), and 7 h (J). Live cells are located at the bottom right gate, while dead cells are at the top left gate. All samples were stained with SYTO 9 (X axis) and propidium iodide (Y axis).*

Table 1. Comparison of EPA Time-Temperature Requirements to Observed Data.

Treatment Temp (°C)	EPA Time Temp. Equation Used[a]	Req. Treatment Time Based on Equation	Shortest Treatment Time[b] Used w/ 100% Inactivation	Percentage of Required EPA Treatment Time
75	$D = \frac{131{,}700{,}000}{10^{.14t}}$	6 min	6 min	100%
65	$D = \frac{50{,}070{,}000}{10^{.14t}}$	57 min	30 min	52.6%
55	$D = \frac{50{,}070{,}000}{10^{.14t}}$	24 h	30 min	2.1%

[a]D = days, t = temperature (°C). Treatment time less than 30 min uses a more stringent equation.
[b]Treatment time shorter than these values were not tested in this experiment.

times was used for this temperature to explore possibility of VBNC formation by insufficient heat treatment. Times as low as 30 min (i.e., 2% of required treatment time) were analyzed in this trial in an attempt to capture arrays of cells with varying degrees of damage. Interestingly, all cells immediately shifted to the dead zone despite receiving only 30 min of heat exposure (Figure 2). The shape of the plot, however, is less defined compared to samples with longer treatment times having some cells locating at regions high for both red and green fluorescence. It was previously reported this is due to increased entry of both color stains when only the outer membrane of a cell is damaged at the beginning phase and is termed intermediate cells [22]. Shape quickly became more defined as treatment continued and was very similar for samples treated for 1 hour and up to 10 days. It would be interesting to know that if some of these "intermediate cells" when given time may repair themselves and return to the live region. However, subsequent analysis up to 2 weeks showed damage is permanent. This result is consistent with standard culture methods where no culturable cells could be recovered beyond 30 min of heat treatment. No recovery was observed upon storage. Treatment at 55°C for up to 10 days showed no more significant changes for either culture data or flow cytogram. This further demonstrated that cellular damage occurred very rapidly and damage was permanent well before the specified time-temperature requirement. Overall, results suggested the USEPA equation is sufficient and in fact quite conservative when 55°C is used for heat treatment.

Heat Treatment with Background Solids

Several researchers have observed increased heat resistance for microorganisms in fluids containing high solids [31–33]. In an effort to determine if presence of solids influenced thermal tolerance of *E. coli*, a separate set of samples were prepared by inoculating *E. coli* into an autoclave sterilized anaerobically digested liquid biosolids from a local municipal utility to serve as background solids. They were then subjected to identical tests as described above. All test results were comparable to those treated under a phosphate buffer where none of the cells were culturable at any of the treatment times used. Flow cytometric analysis of biosolids was also attempted and results displayed the population of live cells in untreated samples was visible even with a large amount of background solids were present. This live population disappeared after cells were subjected to heat treatment (Figure 3). Again, no intermediate cells were formed. Unfortunately, the membrane compromised of dead cells was blinded by background fluorescence of autoclaved biosolids particles and thus was unable to provide a clear observation of the fluorescence shift. Despite this limitation and based on voids in the live and intermediate cell region it demonstrates the heat treatment is still sufficient to destroy *E. coli* cells in the presence of biosolids. Results suggest insufficient heat treatment cannot explain the failure of the creation of VBNC cells observed in the field. Other factors must be involved.

Effect of Gradual Heat Challenge

To tightly control treatment time and temperature combinations as specified by the USEPA equation a bacteria culture was spiked into respective carrying fluids that were pre-heated to the target temperature. Therefore, they were subjected to the exact amount of time required at that target temperature. However, this type of immediate heating cannot be accomplished in the actual digestion processes where large quantify of sludge are heated at the same time through a heat

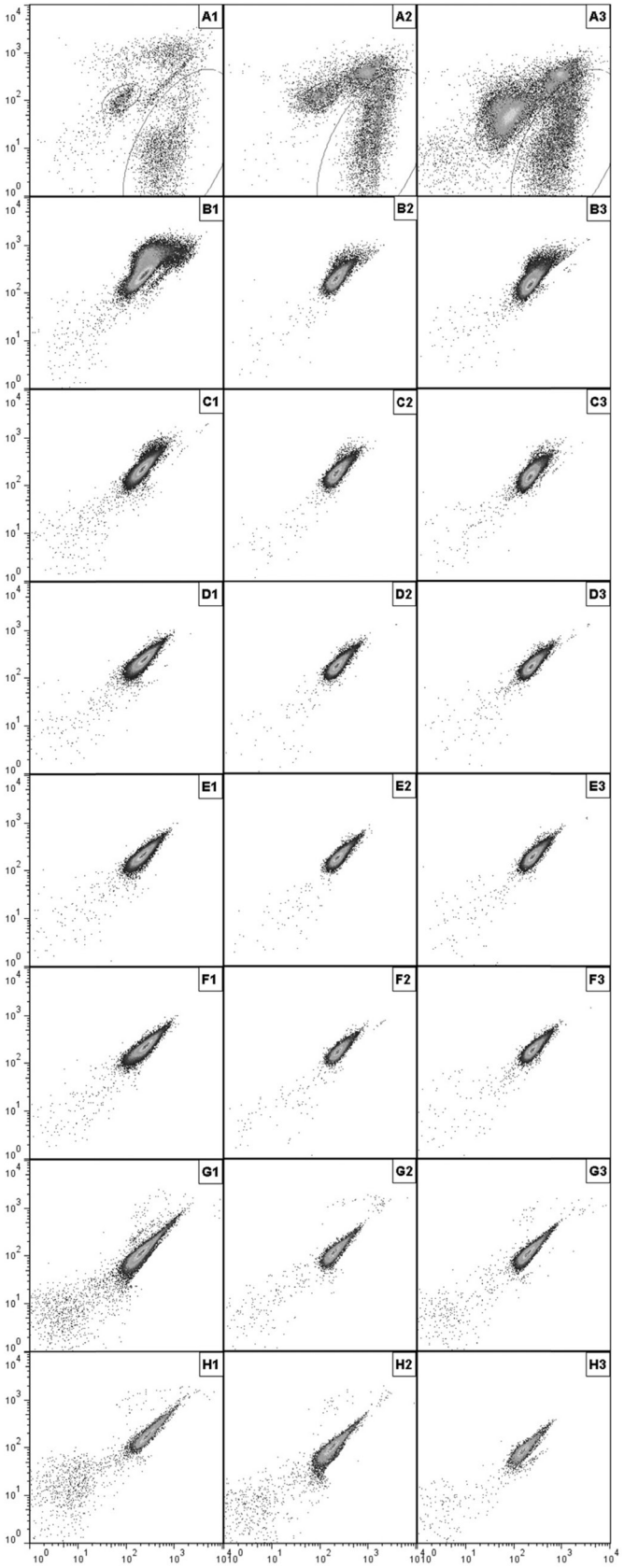

Figure 2. *Flow cytometric dot plots of E. coli treated at 55°C. Samples were analyzed immediately after treatment and after 7 and 14 days. The treatments include untreated (A), 30 min (B), 2 h (C), 6 h (D), 12 h (E), 24 h (F), and 10 d (G). Live cells are located at the bottom right gate, while dead cells are at the top left gate. All samples were stained with SYTO 9 (X axis) and propidium iodide (Y axis).*

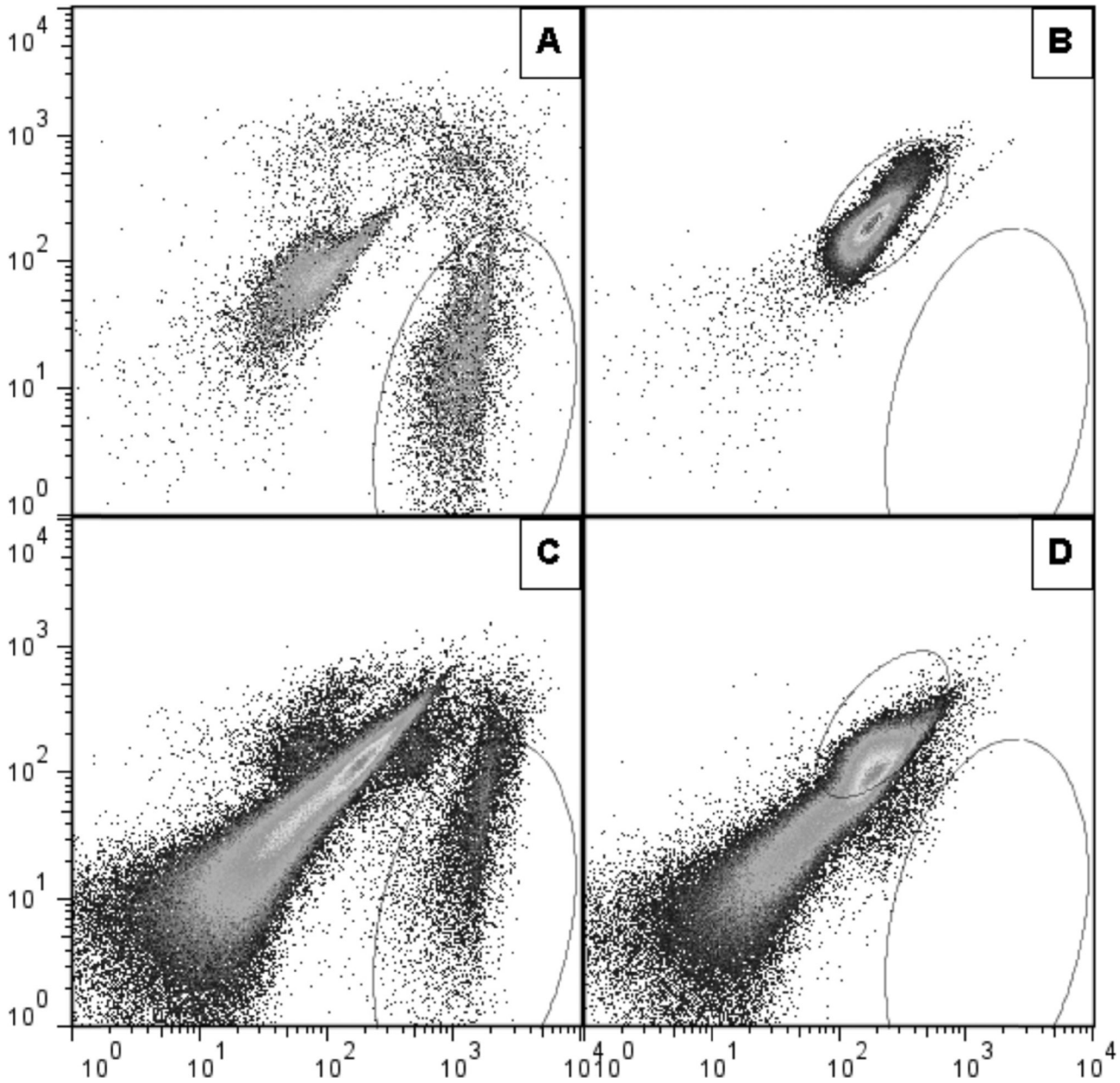

Figure 3. *Examples of flow cytometric dot plots with pure cultures of untreated E. coli (A), heat treated pure culture E. coli (B), untreated E. coli with autoclaved biosolids as the background (C), and heat treated E. coli in autoclaved biosolids. All samples were stained with SYTO 9 (X axis) and propidium iodide (Y axis).*

exchanger. Therefore, field conditions are expected to consist of a gradual heating process rather than an instantaneous one. Studies that examined heat resistance as a function of the diameter of the heating vessel of *E. coli* mixed in mashed potatoes and milk ultra filtrate demonstrated that a larger diameter vessel created a heating-lag and therefore was less effective in inactivating the bacteria [31, 33]. It was also identified that the classical log-linear inactivation curve is only valid when instant heating is achieved and linearity is lost when a heating-lag occurred [31]. While slow heat transfer may contribute to departure from the log-linear behavior it was believed that other factors such as creation of heat-resistant bacteria at a sub-lethal temperature may be even more important [31]. To verify impact of a gradual heat increase, the same *E. coli* sample was heated slowly from 25°C to 55°C using a 5°C increment at every 30 min interval before subjecting it to the target time-temp treatments. Results demonstrated an added resistance to heat inactivation (Figure 4). Instant heat treatment completely inactivated *E. coli* within 30 min while the gradual heat

treatment still contained almost 5-log of *E. coli*. Complete inactivation only occurred after 60 min. Flow cytometric analysis showed some residual live cells as well as formation of intermediate cells with 30 min of gradual heat treatment which mostly merged into the dead zone after 60 min (Figure 5). This result is in line with other researchers' efforts tracking survival of *Salmonella typhimurium* which were heated from 20°C to 52, 55, and 59°C instantaneously and at rates of 0.6°C /min and 10°C/min [34]. Samples heated slowly were always found to be more resistant to heat challenge than those heated at a faster rate. Samples that were introduced to heat instantaneously were always least resistant [34]. Other work that focused on isothermal heat-shock conditions discovered that *E. coli*, both pathogenic and non-pathogenic, became more resistant to heat inactivation if incubated at 42–46°C prior to lethal heat exposure [35–37]. Continued observation of culture plates after 6 and 12 days showed regaining of culturability for samples treated with gradual heat but not instant heat (Figure 4). This showed that not only can gradual heating create bacteria that are more resistant to heat but it also can form VBNC bacteria that are not captured by the standard enumeration with 24h incubation. Therefore, contribution of bacterial heat resistance from heating-lag is a potential concern and cannot be overlooked in process design.

Gradual heating showed signs of increased resistance to heat inactivation but still inactivated the bulk of *E. coli* within 1 h of treatment which is only 4.2% of what is required by the USEPA. Therefore, it is not likely the only reason causing observed VBNC formation in the field. It produces a potential synergistic effect in conjunction with other stressors upstream. Growth conditions will further depart from an optimum as wastewater utilities are moving towards a longer solids retention time and lower food to mircoorganisms ratio for a higher effluent quality that will in turn create stronger stresses to bacteria. This may likely enhance the bacteria's ability to resist heat treatment. If not completely destroyed a surviving *E. coli* population may also potentially become a seed for heat-resistant bacteria for the next batch of digestion. Therefore, to assure complete inactivation of pathogens by thermophilic treatment future work should focus on considering impacts of all prehistory that bacteria may have experienced prior to heat treatment and re-exam efficiency of heat treatment to these stressed populations.

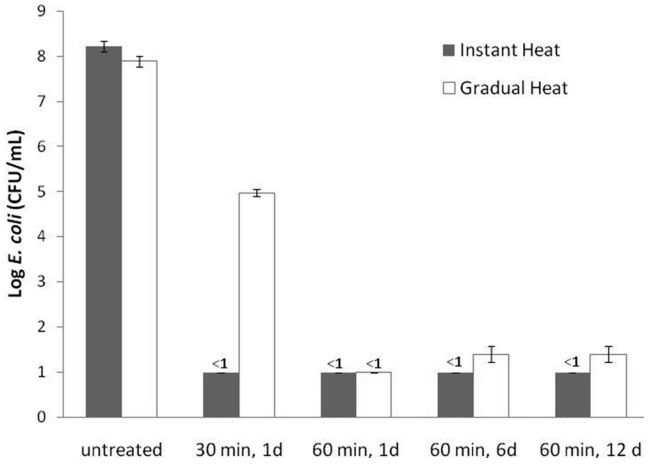

Figure 4. *Culturable E. coli concentration after treating 30 and 60 min at 55°C. The solid columns are samples treated with instant heat, while the hollow columns are samples treated at an average of 0.125°C/min ramp to 55°C and hold for another 30 and 60 min. Samples were counted after 1 day of incubation at 37°C, and recounted after 6 and 12 days of room temperature incubation in the dark.*

CONCLUSIONS

The USEPA's time-temperature requirement for Class A heat treatment at 55, 65, and 75°C was evaluated. Results verified that the requirement is more than sufficient to completely inactivate stationary phase pure culture *E. coli* providing that an instantaneous heat increase to the target temperature is used. This was verified using both standard culture methods and a culture-independent live/dead test kit in conjunction with a flow cytometer. Results are consistent even when *E. coli* were treated in a background of digested biosolids. However, it is difficult to achieve instant heating in practice and results demonstrated that *E. coli* becomes more resistant to heat treatment when a gradual heat increase is used. A slow recovery of culturability was also observed in addition. Overall it appears a gradual heat increase alone is not the sole reason for VBNC bacteria formation observed in a thermophilic digester and continued work focusing on combinations of other stressors upstream will likely resolve this issue.

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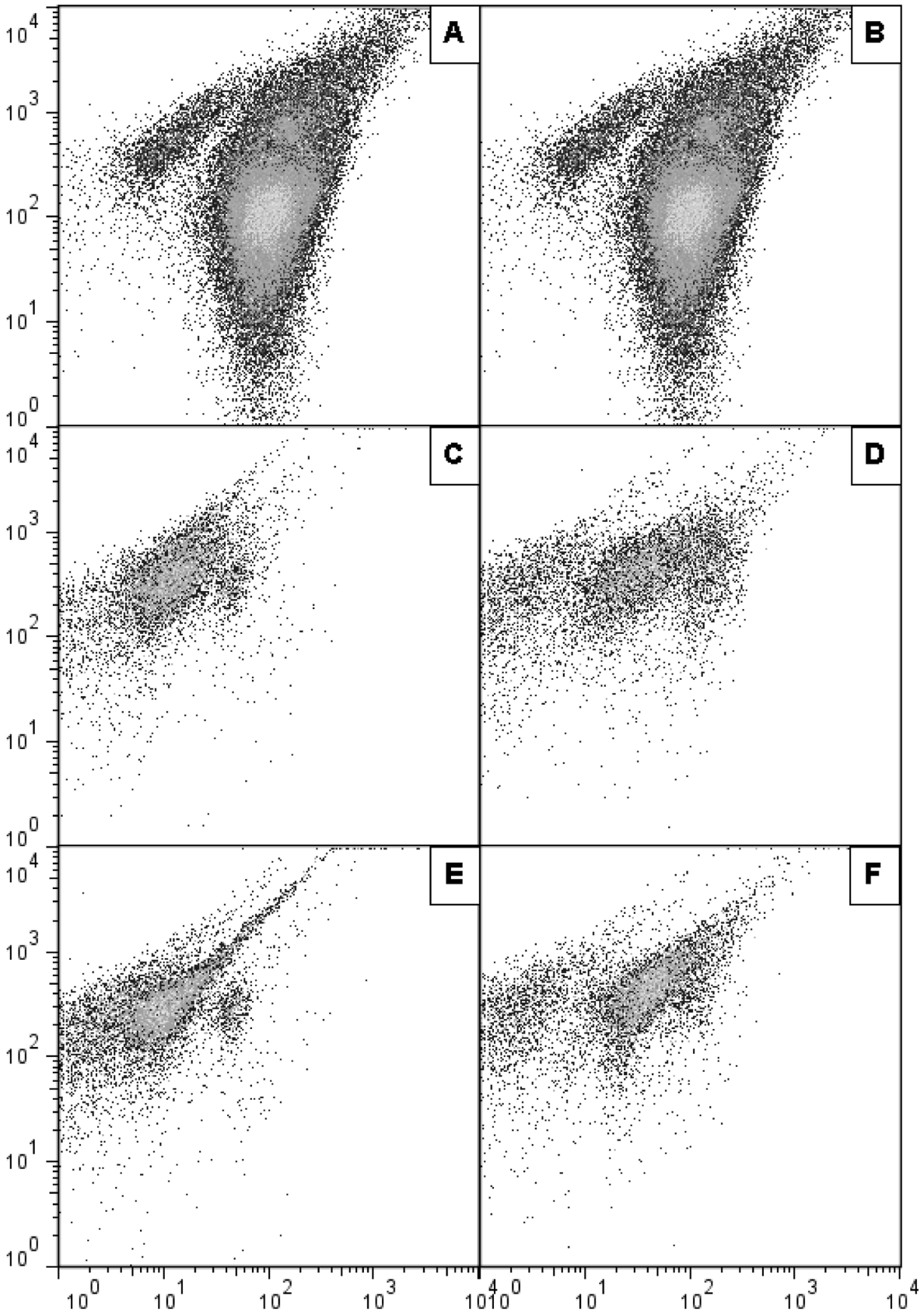

Figure 5. *Flow cytometric dot plots of E. coli treated at 55°C with either instant or gradual heat challenge. The treatments include untreated (A) and (B); 30 min instant heat (C); 30 min gradual heat (D); 60 min instant heat (E); and 60 min gradual heat (F). All samples were stained with SYTO 9 (X axis) and propidium iodide (Y axis).*

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Influence of Humic Acid and Superabsorbent Usage on Lead Phytoextraction from Contaminated Soil by *Brassica napus* L.

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ABSTRACT: Phytoextraction can provide an effective in situ technique for removing heavy metals from polluted soils. The objective of this study was to investigate effects of humic acid and superabsorbent on phytoextraction of lead from contaminated soil by *Brassica napus* L. (i.e., rapeseed). A pot experiment was carried out in a completely randomized block design with three replications in a greenhouse using Pb contaminated soils with 17, 417 and 817 mg $Pb.kg^{-1}$soil. Humic acid (0 and 125 g in 7 kg soil) and superabsorbent (0 and 5 g in 7 kg soil) were applied to lead-contaminated soil. Then, seeds were planted. Results showed that root and shoot dry weight of rapeseed was not affected by increasing lead concentration up to 817 $mg.kg^{-1}$ even with addition of humic acid and superabsorbent. Lead concentration in roots was enhanced significantly ($p < 0.05$) with humic acid usage. Uptake of lead in roots and its translocation to shoots also increased with increasing total lead concentration in soil. Superabsorbent application seemed to be beneficial for in situ remediation of soil via adsorption of the toxic metal on its surface and preventing movement in the soil profile. It decreased uptake of lead in to the plant and reduced phytoextraction efficiency.

INTRODUCTION

SINCE the dawn of the Industrial Revolution humankind has been introducing numerous hazardous compounds into the environment at an exponential rate. These hazardous pollutants consist of a variety of organic compounds and heavy metals which pose serious risks to human health. Heavy metals are primarily a concern because they cannot be destroyed by degradation [1]. Lead may be a ubiquitous environmental pollutant in industrial settings that poses a serious threat to human health. However, it is usually poorly bioavailable [2,3] and only a minor fraction of the total soil Pb^{2+} is in soil solution [4,5].

Phytoextraction has received increasing attention as a promising and cost-effective alternative to conventional engineering-based remediation methods [6]. Phytoextraction is the use of pollutant-accumulating plants to remove metals or organic pollutants from soil by concentrating them in harvestable parts [7]. All plants have potential to extract metals from soil, but some plants have shown the ability to extract, accumulate and tolerate high levels of heavy metals which would be toxic to other organisms. These plants are termed hyperaccumulators [8]. We are interested in the possibilities of *B. napus* L. (i.e., rapeseed) as a candidate phytoextraction crop for several reasons. First, rapeseed is among the oldest cultivated oil-producing plants in Europe and has been cultivated since the 14th century.

Today, rapeseed varieties are mainly used in food applications but to a growing extent they are also used for the production of biofuel. Phytoextraction with *B. napus* L. has the potential to become a profitable enterprise when combined with biofuel production and especially when considering increasing oil prices over the coming years. Its higher biomass production compared to natural metal accumulators in addition contributes to the suitability of *B. napus* L. as a phytoextraction species [9].

Increasing mobility and bioavailability of lead in the soil through certain chelators, organic acids or chemical compounds allows for hyperaccumulation of met-

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als in some plants. Chelators such as humic substances could be used for increasing the solubility of metal cations and thus their bioavailability to plants. They result from decomposition of plant and animal residues [10]. Humic acids are those parts of humic substances which are not soluble in water under acidic conditions but become soluble and extractable at higher pH values. Humic acids contain acidic groups such as carboxyl and phenolic OH functional groups [11] and therefore provide organic macromolecules with an important role in the transport, bioavailability and solubility of heavy metals [12]. A new technique for in situ soil remediationin based on insoluble polyacrylate polymers was proposed over the last decade [13,14,15,16,17]. Polyacrylate polymers are based on acrylic acid and are composed of chains with regularly distributed carboxylic groups, (–CH_2CHCOOH–) n. [18]. The template is a metal ion and with this technique polymers for selective removal of heavy metals ions have been successfully synthesized. Important results were reported for Pb (II), Cd (II) and Co (II) removal special hydrogels (i.e., superabsorbents absorb and store water hundreds times of their own weights) [19]. It is well recognized that hundreds of different gel-polymers exist [20] and they influence soil physical structure, plant growth and yield differently.

MATERIALS AND METHODS

Agricultural soil was collected from Khatoon Abad field in the northeast portion of the Isfahan province located in the center of Iran. The soil was air-dried at room temperature and then sieved through a 2 mm sieve and characterized as displayed in Table 1.

A pot experiment was conducted in a completely randomized block design with three replications including:

1. Pb-contaminated soil for three levels (17, 417 and 817 mg.kg^{-1} Pb: S_0, S_1 and S_2, respectively),
2. Superabsorbent for two levels (0 and 5 g.pot^{-1}:SA_0 and SA_1), and
3. Humic acid for two levels (0 and 125 g.pot^{-1}:HA_0 and HA_1).

The soil was artificially contaminated by spraying (Pb $(CH_3OO)_2.3H_2O$) and then saturated with de-ionized water and air-dried before use. DTPA- extractable concentration of lead (i.e., available Pb) was 129.7 and 342.3 mg.kg^{-1}. Plastic pots were filled with 7 kg of soil and rapeseed (*B. napus*) seeds were cultivated in pots and watered to 75% field capacity. No fertilizer was needed due to adequate amounts of N, P and K in soil. This is displayed in Table 1. All rapeseed plants were grown under controlled environmental conditions with about a 15-hour light period, an average temperature about 32°C and 65% relative humidity. After the first pair of true leaves appeared seedlings were thinned to six plants per pot and kept constant until the end of the experiment. After 8 weeks plants were harvested and soil samples were collected from each pot. Shoots and roots were selected separately and rinsed completely in deionized water and oven dried at 65°C for 48 hours to reach a constant weight. Dry weight was measured and samples were ground and homogenized. Ground shoot and root samples (1 g) were digested in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, by volume) and lead concentration in the solutions was determined [21] using the atomic absorption spectrophotometry method. Soil lead concentration (total and DTPA-extractable) was determined after harvesting [22]. Statistical analysis of the data was performed and the difference between specific pairs of means was identified by the Duncan test.

RESULTS

Data analyses for dry mass yield of rapeseed, lead concentration in soil and plant after harvesting are displayed in Table 2.

Plant Analysis

Shoot dry weight and root dry weight increased in the presence of humic acid and superabsorbent. No decrease in shoot and root biomass was visible but toxicity symptoms such as pale leaves were observed during plant growth. The dry mass yield of rapeseed was not severely affected by increasing concentration of total

Table 1. Physicochemical Properties of Soil Used in Study.

Pb DTPA	Pb Total	P Available	K Available	N Total	O.M	C.E.C	EC	pH	Sand	Clay	Silt
mg.kg^{-1}				%		cmolc.kg^{-1}	dS.m^{-1}	$CaCl_2$	%		
1.67	17	46.05	449.7	0.13	0.25	10.7	2.39	7.9	16.17	20.83	63

Table 2. Means for Measured Parameters in B. napus (rapeseed).

	Dry Weight		Plant Pb		Soil Pb	
	Root	Shoot	Root	Shoot	Total	DTPA
Treatment	(g)		($mg.kg^{-1}$)			
HA_0	1.72a	6.74a	153.66b	11.37a	321.31a	128.44a
HA_1	1.76a	9.04a	167.25a	13.43a	304.13b	134.24a
SA_0	1.61a	7.86a	176.73a	13.42a	309.30a	143.85a
SA_1	1.87a	7.92a	164.18a	11.36a	316.15a	118.83a
S_0	1.78a	7.20a	7.40c	0c	12.17c	1.33c
S_1	1.83a	10.15a	124.21b	14.88b	302.79b	127.58b
S_2	1.60a	6.319a	214.76a	22.17a	603.21a	265.11a

Pb in soil. Nevertheless, seedlings from contaminated soil appeared stunted as compared to those grown on the control soil and their leaves had a purple color with necrotic areas. Concentrations of Pb in roots and shoots of *B. napus* (rapeseed) are shown in Table 2. Pb concentrations ranged from 7.4 to 214.76 $mg.kg^{-1}$ dry weights in roots and 0 to 22.17 $mg.kg^{-1}$ dry weights in shoots. A ccumulation of Pb into the biomass of the tested plants was low for both roots and shoots in the control soil. Results showed a positive correlation between accumulation of Pb in roots or shoots and the Pb concentration in the soil ($p < 0.01$). Humic acid application significantly increased the Pb concentration in roots of *B. napus* under Pb treatments ($p < 0.05$). Pb concentration in shoots also increased but it was not significant. The effect of superabsorbent decreased the Pb concentration in the roots and shoots of the plants but it was not significant.

Soil Analysis

Results from DTPA extraction indicated very low variation in the available lead concentrations between humic acid treatments. Addition of humic acid decreased the total lead concentration of soil significantly ($p < 0.05$) (Table 2). In superabsorbent treatments, DTPA-extractable Pb (i.e., available Pb) and total lead concentrations of the soil showed no significant change after harvesting. This indicated the effect of the superabsorbent was negligible within the period of the experiment. Expected from S_0 to S_2 treatment, the total and available Pb of the soil enhanced significantly with artificial contamination of soil ($P < 0.01$).

Phytoextraction Efficiency

Table 3 displays the bioconcentration factor (BCF), the translocation factor (TF) and the Phytoextraction Rate (PR %) for rapeseed (*Brassica napus* L.). The bioconcentration factor (BCF) is the ratio of metal concentration in plant roots or shoots to metal concentration in soil [23]. The transfer factor (TF) is defined as the ratio of concentration of metals in shoots to that in the roots. These two factors were used to evaluate plant effectiveness regarding metal accumulation and translocation [24]. P hytoextraction ratio (PR %) was calculated as follows:

$$PR\% = (C_{plant} \times M_{plant}/C_{soil} \times M_{rooted\ zone}) \times 100$$

Where, M_{plant} is the mass of the harvestable aboveground biomass produced in one harvest, C_{plant} is the metal concentration in the harvested component of the plant biomass, $M_{rooted\ zone}$ is the mass of the soil volume rooted by the species under study and C_{soil} is the metal concentration in the soil volume [23].

Results obtained from calculations for these factors (Table 3) suggested that chemically enhanced phytoextraction of Pb from such contaminated soils with usage of humic acid had a positive significant effect on the root BCF ($p < 0.05$). It increased from 0.5 in HA_0 to 0.66 in HA_1. However, it was not significant on shoot BCF, TF and PR percentage. Moreover, the superabsorbent application is not a suitable method for enhancing the phytoextraction efficiency due to slight reductions for root and shoot BCF with usage of the superabsorbent and it has no effects on the TF and PR percentage. The highest BCF after the phytoextraction process was obtained for S_0 and reached only 0.72 (root BCF) and decreased with increasing total concentration of Pb in soil. The TF values were very low and the application of humic acid and superabsorbent showed similar results. The TF reached its highest amount in S_1 and decreased again in S_2. The PR percentage values were very low

Table 3. Bioconcentration Factor (BCF), Translocation Factor (TF) and Phytoextraction Rate (PR %) for Rapeseed.

	BCF			
Treatment	Root	Shoot	TF	PR%
HA_0	0.50a	0.03a	0.04a	0.003a
HA_1	0.66b	0.03a	0.05a	0.004a
SA_0	0.57a	0.03a	0.05a	0.003a
SA_1	0.51a	0.02a	0.05a	0.003a
S_0	0.72a	0a	0b	0b
S_1	0.53b	0.04a	0.09a	0.006a
S_2	0.52b	0.03a	0.08a	0.003b

(i.e., maximum 0.006%) due to high total concentration of Pb in the soil. Usage of humic acid and superabsorbent did not affect the PR percentage (Table 3).

DISCUSSION

To make phytoextraction practical one needs to couple a high biomass and a very high metal accumulation in plant tissues. *B. napus* (rapeseed) not only produced considerable biomass but also was able to tolerate moderate (417 $mg.kg^{-1}$) and high (817 $mg.kg^{-1}$) levels of Pb in soil. The dry weight of roots and shoots was not significantly reduced with increasing soil Pb contamination. Plants grown in the 417 mg $Pb.kg^{-1}$(S_1) even had slightly higher root (1.83 g) and shoot (10.15 g) dry biomass than the S_0, (1.78 and 7.2 g respectively). This demonstrated that rapeseed could tolerate high Pb concentrations without a severe reduction in biomass production. However, visual symptoms of toxicity (i.e., necrotic areas) were observed on the leaves and was also reported previously by Kos *et al.* [25].

Addition of humic acid and superabsorbent did not affect the dry biomass of rapeseed significantly. A pplication of humic acid to the soil did not adversely affect dry matter production of *Nicotiana tabacum* plants [7] and *Elodea nuttallii* [26]. However, the results (Table 2) indicate that the superabsorbent could have a slight positive effect on enhancement of dry weight for roots and shoots.

Another requisite to the success of phytoextraction is Pb accumulation in plant tissue and especially in harvestable parts. The uptake of Pb by *B. napus* in S_1 and S_2 was significantly ($p < 0.01$) higher than the control (S_0) for both root and shoot tissues. It was also much more than the recommended human maximum consumption limits for Pb at 3.5 $mg.kg^{-1}$ [27]. Results showed that the increasing content of Pb in the soil leads to the increase of its content in the roots and shoots of *B. napus* (rapeseed). In addition, the accumulation of Pb in plant roots is very high in comparison with shoots. This showed that in contaminated soils the main part of the Pb taken up by the rapeseed roots from the soil accumulated in the roots and small amounts move through the conductive system to the stems.

Lead binding to clay and organic matter and its inclusion in insoluble precipitates make a significant fraction of Pb unavailable for plant. While plants are known to concentrate Pb in the roots, Pb translocation to the shoots is normally very low [28, 29, 30]. Actively growing roots provide a barrier that restricts the movement of Pb to the aboveground parts of plants. This restricted movement of Pb may explain why Pb concentration in shoots was less than in the roots [31]. Results are comparable with Boye [32], Kumar *et al.* [33] and Ebbs *et al.* [34].

Although *Brassica napus* accumulated a significant amount of Pb in its roots, it was least effective in translocation of Pb to the shoots. *Brassica napus* accumulates moderate amounts of heavy metals in shoots and has considerably less potential for phytoextraction in comparison with some wild species in the *Brassicaceae* family which grow on metalliferous soils and accumulate large amounts of heavy metals in their roots and shoots [35, 36]. Lou *et al.* [37] also showed that concentrations of Pb in roots of mays and white been were significantly higher than those in the shoots and most of the Pb absorbed by the plants were concentrated in the roots.

The study suggests that usage of humic acid also could be employed to enhance the potential availability of Pb to plant. As is displayed in Table 2, application of humic acid had significant effect on the root Pb concentration and total Pb of the soil ($p < 0.05$). However, no significant influence of humic acid usage was observed on the concentration of Pb in shoot and DTPA extractable Pb in soil. The effectiveness of phytoextraction for metals is highly dependent on the availability of metals for plant uptake. Plant uptake of metals shows that a marked dependence on the chemical speciation of the metal in solution. Plant response generally correlates best with the activity of the free, uncomplexed metal ion in solution. However, numerous observations indicate that chelating agents such as humic acid are taken up by plants. A direct analytical determination of an intact metal—Humat complex inside plant proved that the metal and the chelating agent form a complex. Therefore, it is suggested that plants are not only able to take up free metal ion but are also able to take up intact chelates (complexes).

pH has a significant effect on the mobility of lead and other metals within the soil. The pH of soil generally ranges between 4.0 and 8.5. Under acidic conditions (pH < 5.5) metal cations are more mobile while anions tend to sorb to mineral surfaces [39]. The capacity of the soil to adsorb lead increases with increasing pH, cation exchange capacity (CEC), organic carbon content, soil/water Eh (redox potential) and phosphate levels [1]. The reason for this enhancement is the decrease in pH resulting in higher lead availability. Another possibility taken into account is that plants may take up lead complexes with humic acid fragments [40].

Moreover as in the case of multidentate chelating agents, the humic-metal complexes are very stable in common soil conditions and do not release their metal ions back into a free form unless there is a drop in soil pH [41]. This signifies that while humic substances are potentially useful to increase plant-availability of heavy metals in soil they concomitantly reduce the environmental mobility of these contaminants [38]. Evangelou *et al.* [7] showed that a humic acid (HA) addition at a rate of 20 $g.kg^{-1}$ significantly enhanced uptake of Cd by tobacco (*N. tabacum*) up to 65% although the theoretical bioavailability of Cd as determined by DTPA extraction did not change. Halim *et al.* [38] stated that soil amendments with exogenous humic matter might accelerate phytoremediation of heavy metals from contaminated soil while preventing their environmental mobility.

Addition of 2% HA generally reduced the extractability of the soluble and exchangeable forms of metals [38]. This effect was directly related to the amount of added HA and increased with time. Conversely, the concentrations of the potentially plant-available metals extracted with DTPA were generally larger with increasing additions of exogenous HA solutions. Variations, though, occurred according to the soil and metal. This was attributed to the formation of metal-humic complexes which ensured a temporary bioavailability of metals and prevented their rapid transformation into insoluble species [42].

Evangelou *et al.* [42] stated that the use of HA and FA increased the uptake of metals into plants but the application of HA and FA on fields is not feasible as the amounts that have to be applied are too high and not applicable. Finally, it should be mentioned that the careful management of soil and the right selection and combination of plants are needed before this remediation technology can be used in field conditions [21].

Although the effect of superabsorbent on all factors in Table 2 was not significant some changes for parameters were observed. Root and shoot dry weight increased with addition of superabsorbent. R esults here were similar to deVarennes and Queda [16] investigations that found insoluble polyacrylate polymer could be used to improve the quality of a copper-contaminated soil. Growth of annual medic (*Medicago polymorpha* L.) was stimulated in the polymer-amended soil such that total biomass produced was three times that of plants from unamended soil. Guiwei *et al.* [18] showed that polymer application rates of 0.2–0.4% were considered sufficient as they led to a large improvement in plant growth and soil quality. In comparison with Guiwei *et al.* [18] the amount of superabsorbent used in our study was lower b y about 0.07%. Therefore, it could be understood that although the superabsorbent enhanced the growth of rapeseed, the amount applied in soil did not affect the measured parameters markedly. According to Bres and Weston [43] differences in effectiveness of gel polymers might be due to gel-polymer type and amount applied. Application of the superabsorbent (Table 2) increased the concentration of Pb in soil after harvesting. This is because of adsorbtion of Pb cations on the surface of the hydrogel making them unavailable for the roots of the plant. Therefore, the amount of available Pb (DTPA-extractable) decreased.

The claim is supported by less accumulation of Pb in roots of the plants cultivated in soil with addition of superabsorbent and reducing the translocation of Pb from roots to shoots. Previous studies indicate that superabsorbent hydrogels assist with environmental cleanup by reducing metal phytotoxicity through binding metals into non-toxic forms [44]. DeVarennes and Queda [16] also found that roots of plants cultivated in the polymer-amended soil had a concentration of copper that was 73% of that in plants from the unamended soil. Biver *et al.* [45] discovered that the process for lowering the content of polyvalent cations in aqueous solutions is adsorption of heavy cations on a superabsorbent polymer. Heavy ions which are bound in the superabsorbent polymer correspond to at least 60% of its absorption capacity for heavy metals ions. These polyvalent cations are copper, zinc, chromium, cadmium and lead.

As Lasat [46] stated, "there is no known natural Pb hyperaccumulator." BCF, TF and PR percentage factors for rapeseed in this study showed a low ability of *B. napus* to be considered as Pb hyperaccumulator but it does has potential for phytoextraction. The root BCF for rapeseed is generally higher from that of the shoot. The root BCF ranged from 0.72 to 0.52 while it was from 0 to 0.03 for the shoot BCF. The high BCF value could be a good indicator of the potential for heavy metal accumulation. However, the BCF values for hyperaccumulators decreased with increasing metal concentrations in soil. The phenomenon may be caused by total body burden [47].

TF values can describe movement and distribution of heavy metals in plants [48]. TF values higher than one indicate higher concentrations of metal in shoots and are one of the factors which indicate suitability of a plant for use in phytoextraction [49]. As displayed in Table 3, the TF value for *B. napus* was less than one. Therefore, *B. napus* had a low potential to move Pb

from roots to shoots. Variations of TF for Pb ranged from 0 to 0.09 which is similar to results of Alloway [50]. Phytoextraction rate (PR %) represents the percentage of an element removed by plant aboveground biomass from total element content in the soil during one cropping season [51]. Accumulation of more than 1% of total heavy metal contents present in the soil into the plant aboveground biomass should be achieved for economically feasible use of phytoextraction [52]. The phytoextraction rates of Pb for tested plant species showed (Table 3) that a very low amount of the soil Pb (0 to 0.006%) has moved to aboveground biomass of rapeseed during 8 weeks.

CONCLUSION

Phytoextraction using hyperaccumulator plants has been proposed as a promising, environmentally friendly and low-cost technology for decreasing heavy-metal contents of contaminated soils. However, uptake of heavy metals by plants is limited by their low solubility in soil solution especially in the case of Pb. Application of humic acid has been proposed to increase Pb concentration in soil solution, Pb uptake by plant and finally plant phytoextraction efficiency. Results also showed that *Brassica napus* L. has a potential for phytoextraction. Plant analyses indicated that although Pb concentration in the roots was considerable for shoots it was comparatively low. Application of insoluble hydrophilic polymers promotes plant growth due to increased soil water holding capacity and decreased availability of toxic metals. In conclusion, application of insoluble hydrophilic polyacrylate polymers can be considered a new method for in situ remediation of contaminated soils by making toxic metals such as lead unreachable for t h e plant and will prevent run off or leaching. Nevertheless, it has undesirable effects on phytoextraction efficiency.

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Spectral Analysis of Cd, Zn, and Pb Adsorption by Extracellular Polymeric Substances from Activated Sludge

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ABSTRACT: Spectral analysis of extracellular polymeric substances (EPS) was conducted on sewage sludge capable of adsorbing Cd^{2+}, Pb^{2+}, and Zn^{2+} using X-ray photoelectron spectroscopy, Fourier-transform infrared spectroscopy, and X-ray fluorescence. Results showed that nitrogen-containing groups played a more important role in Pb and Cd adsorption than in Zn adsorption, and that carboxyl, carbonyl, and amino groups in EPS are the main active groups that bind metals. Amino S- and P- groups function more strongly in Pb adsorption than in Cd or Zn adsorption. EPS bind Pb in a way that produces a less-regular structure whereas EPS bind Cd and Zn in a way that creates a more regular structure. Ion exchange contributes to the ability of EPS to bind metals and plays a more important role in Pb adsorption.

INTRODUCTION

EXTRACELLULAR polymeric substances (EPS) are a major component of activated sewage sludge and account for up to 80% of total mass [1, 2]. They are composed of carbohydrates, proteins, nucleic acids, lipids, and humic acids produced by bacterial excretion, cell lysis, and hydrolysis of organic matter [3–5]. Several studies have demonstrated that EPS can effectively bind metals [6–9]. They therefore show great promise as adsorbants capable of removing heavy metals from wastewater, extracting heavy metals from contaminated soil, and reclaiming heavy metals from industrial wastes [10–16]. EPS are assumed in these processes to have multiple metal-binding sites with different binding strengths [7] and to exhibit a range of binding constants [17] because EPS possess multiple functional groups. They are expected to sequentially bind metals which first occupy sites with high binding energy and then sites with weaker binding energy as metal concentration increases.

Many researchers have investigated mechanisms of metal binding by EPS in activated sludge. For example Brown and Lester's research showed that negatively charged functional groups in EPS such as hydroxyl and amino groups play a major role in complexation with metals [6]. Sutherland suggested ion exchange was a potential mechanism involved in metal binding due to the high amount of negatively charged functional groups such as carboxyl, phosphate, and sulfate groups in EPS [8]. Van der Aa and Dufrene used atomic-force microscopy to probe nanoscale morphology and molecular interactions of polystyrene substrata during adhesion of Gram-negative bacteria to solid surfaces and proved proteinaceous EPS were involved in adhesion of *Azospirillum brasilense* [18]. Guibaud *et al.* suggested the number of binding sites and the complexation constant were strongly related to content of proteins, polysaccharides, and humic substances based on an analysis of the correlation matrix between polymer composition and complexation parameters [19]. They also used static mode-dropping electrode polarography to measure metal complexation potential of EPS and found that carboxylic groups of the EPS played a major role in metal binding at pH 7 [19]. However and despite these findings, information available to help us understand mechanisms responsible for metal adsorption by EPS from activated sewage sludge is still limited.

The aim of the present study was to investigate mechanisms of EPS adsorption of Cd^{2+}, Pb^{2+}, and Zn^{2+}. We used several analytical techniques to reveal interactions between EPS and metals and to identify functional groups that are active in the adsorption process. Changes in the chemical environment were analyzed by means of X-ray photoelectron spectroscopy

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Table 1. Composition of Synthetic Feed Medium.

Constituent	Concentration (mg/L)
Glucose	2175
Peptone	200.0
K_2HPO_4	600.0
KH_2PO_4	300.0
NH_4Cl	225.0
$MgSO_4 \cdot 7H_2O$	112.5
$FeSO_4 \cdot 7H_2O$	3.750
$ZnSO_4 \cdot 7H_2O$	3.750
$MnSO_4 \cdot 7H_2O$	3.750
$CaCl_2$	15.00
$NaHCO_3$	180.0

(XPS) to better understand reactions between EPS and metal ions. Functional groups that were active in the adsorption of Cd^{2+}, Pb^{2+}, and Zn^{2+} were identified by means of Fourier-transform infrared (FTIR) spectroscopy. X-ray diffraction (XRD) was used to highlight the structure of adsorption products resulting from binding of metals with EPS. We also used X-ray fluorescence (XRF) to ascertain effects of heavy metal adsorption on chemical constituents of the EPS.

MATERIALS AND METHODS

Preparation and Characteristics of EPS

A mixed bacterial culture grown in a semi-continuous-flow activated sewage sludge reactor was used for extraction of EPS. Microbial seed was obtained from the primary settling tank effluent of the city of Harbin's wastewater treatment plant. Reactors were fed with a synthetic medium described in Table 1 designed based on results from Durmaz and Sanin's research [20].

The reactor was run intermittently for 12 h using aeration for 10 h with a dissolved oxygen (DO) content of 2.5 to 3 mg/L followed by 2 h of settling. Settled sludge was sampled for EPS extraction.

EPS extraction was carried out by steaming samples followed by centrifugation as described by Brown and Lest [10] and Jang *et al.* [21]. Prior to extraction, the sludge was concentrated using an MR 23i (Jouan, Frence) centrifuge at 4300 *g* for 10 min. Recovered residues were rinsed twice in double-distilled water. The rinsed activated sludge was then steamed at 80°C under 100 kPa pressure for 10 min and centrifuged while still hot at 8000 *g* for 10 min. The supernatant was placed in dialysis tubing with an 8000-Da molecular-weight cut-off (Spectra/Por 7; Spectrum Laboratories, Rancho Dominguez, CA) and dialyzed against double-distilled water with constant mixing for 3 d at 4°C. The water was exchanged eight times to remove low-molecular-weight impurities from the solution in the dialysis tubing. After dialysis, EPS solutions were stored at –20°C until use.

Protein content of the EPS was measured using the Folin method [22] and the carbohydrate content was determined using the anthrone–sulfuric acid method [23]. Initial metal content in the EPS was analyzed by means of inductively coupled plasma–atomic emission spectroscopy (Optima 5300 DV, Perkin Elmer, Tucson, AZ). The Zn, Cd, and Pb contents were determined at wavelengths of 213.86, 226.50, and 220.35 nm, respectively, according to standard methods [24].

Adsorption Tests

We conducted a series of tests to study adsorption of heavy metals by EPS produced by following the procedure previously mentioned. Focus was on adsorption of three heavy metals: cadmium (Cd), zinc (Zn), and lead (Pb).

Adsorption tests were conducted in capped 250-mL glass bottles at ambient temperature. Each bottle contained 50 mL of aqueous solution containing 35 to 40 mg of EPS and an individual heavy metal at a concentration of 50 mg/L. One bottle without any metal addition served as the control. After 24 h of mixing by shaking some of the heavy metals were adsorbed by the EPS. The mixed liquor was then filtered through a dialysis membrane with a molecular weight cut-off of 3000 Da based on results from previous studies [25]. Contents of the residual metals in the filtrate were compared with those in original solutions. The difference represented the quantity of metal adsorbed by the EPS that was retained by the dialysis membrane.

Analytical Methods

Samples of the EPS solution before and after heavy metal adsorption were dried at –20°C using an FTS EZ585Q freeze-dryer (FTS systems Inc., Stone Ridge, NY) and then were pressed into pellets. Pellets were examined using a Quantum 2000 X-ray photoelectron spectrometer. The spectrum of each sample was recorded using monochromatized Al Kα radiation with a binding energy (284.7 eV) of amorphous carbon C1s used as a reference point.

We compared the FTIR spectra of the dried blank EPS and of three samples: EPS exposed to (i) Cd^{2+}, (ii) Zn^{2+}, and (iii) Pb^{2+} at 25°C with a pH of 6.0 for 24

h. Samples used for FTIR analysis were prepared by pressing powdered KBr pellets mixed thoroughly with 5%(w/w) finely ground powder for each EPS sample. Spectra were obtained using a Nicolet 740SX FTIR spectrophotometer. Analysis was performed with an MCT-B detector and spectra were recorded in the region between 4000 and 625 cm^{-1} at a resolution of 4 cm^{-1} with 32 scans.

We examined four samples from the FTIR studies using a Rigaku D/Max-rC X-ray diffractometer (XRD). The pattern was recorded using Cu Kα radiation with λ = 1.5418 Å and a graphite monochromator filtering wave at a tube voltage of 40 kV and a tube current of 30 mA. The scanning covered the region from 0 to 120°C at 6°C min^{-1} with a beam incidence angle of 2θ.

A Kevex Delta 770 energy-dispersive spectrometer was employed for these studies. Fluorescent emission results were taken at the characteristic silicon Kα line at 1.74 keV and were averaged over 24 measurements taken on each tress to obtain average silicon fluorescence intensity. The same four samples used in the XRD analysis were examined.

RESULTS AND DISCUSSION

EPS Characteristics

Composition of the EPS from activated sludge varies widely depending on extraction method [26]. Composition of the feed medium also greatly affects EPS content. As Guibaud *et al.* [19] reported, sugars in EPS are expected to be more active in metal adsorption than proteins. However, there are no standard procedures for EPS extraction from activated sludge. A high ratio of glucose to peptone (= 40:1) was chosen to extract EPS from the activated sludge as Durmaz and Sanin suggested this ratio would extract high sugar products from the EPS [20]. Thermal extraction was chosen because Liu *et al.* suggested the sugar fraction of EPS was more sensitive to this extraction method than to other methods [9].

Protein and sugar contents of the EPS extracted from activated sludge measured with respect to the VSS value are summarized in Table 2. About 50 mg of total EPS was extracted from 1 g of activated sludge of which 20% was proteins and 80% was sugars. High sugar content is believed to have resulted from the high ratio of glucose to peptone in the synthetic feed medium used for cultivation of the sludge.

Background metal contents in the EPS were sufficiently low such that the EPS were suitable for use in the adsorption experiment as they would not significantly affect adsorption results.

Table 2. Characteristics of EPS Used in Adsorption Tests (VSS, Volatile Suspended Solids).

Parameter	Range
Proteins (mg/g VSS)	9.0 ± 0.5
Sugar (mg/g VSS)	40 ± 2
pH	6.0–7.0
Background contents of metals (mg/g EPS)	
Cd	< 0.003
Pb	< 0.030
Zn	0.010 ± 0.002

XPS Characterization of EPS Adsorption of Cd^{2+}, Pb^{2+}, and Zn^{2+}

We determined carbon, oxygen, and nitrogen contents to determine whether binding of Cd^{2+}, Pb^{2+}, and Zn^{2+} affected these components at the EPS surface. Figure 1 presents results from this analysis. The blank EPS conditioned at pH 7 displayed the presence of nitrogen at 400 eV (10.2%), carbon at 284.6 eV (61.1%), and oxygen at 530.3 eV (25.7%). No Cd^{2+}, Pb^{2+}, or Zn^{2+} was detected. The binding energies for nitrogen in the EPS decreased after adsorption of Pb^{2+}, increased after adsorption of Cd^{2+}, and did not change after adsorption of Zn^{2+}.

The C1s spectra of all samples clearly comprised three peaks with binding energies (BE) of 284.92, 286.33, and 287.91 eV, respectively, identified via deconvolution. Based on this sequence these peaks may be assigned to phenyl, alcohol, and ether groups [28, 29] because carbon atoms in the three chemical functional groups (mainly in EPS) possess slightly different electron densities. The area distribution of the three peaks indicated that phenyl carbons were the dominant EPS. Furthermore, Figure 2 shows area ratios of ether carbon in the EPS decreased dramatically after metal adsorption.

The BE of carbons increased after Pb^{2+} binding and became indistinguishable from that of carbons from the alcohol and carboxylate groups. This indicates formation of ether-metal complexes in which the ether oxygen donates electrons to metal ions thereby decreasing electron density at the adjacent two carbon atoms. The ether group would undergo the most remarkable change among the three functional groups as a result of adsorption from the perspective of the change in geometric shape of organic functional groups when

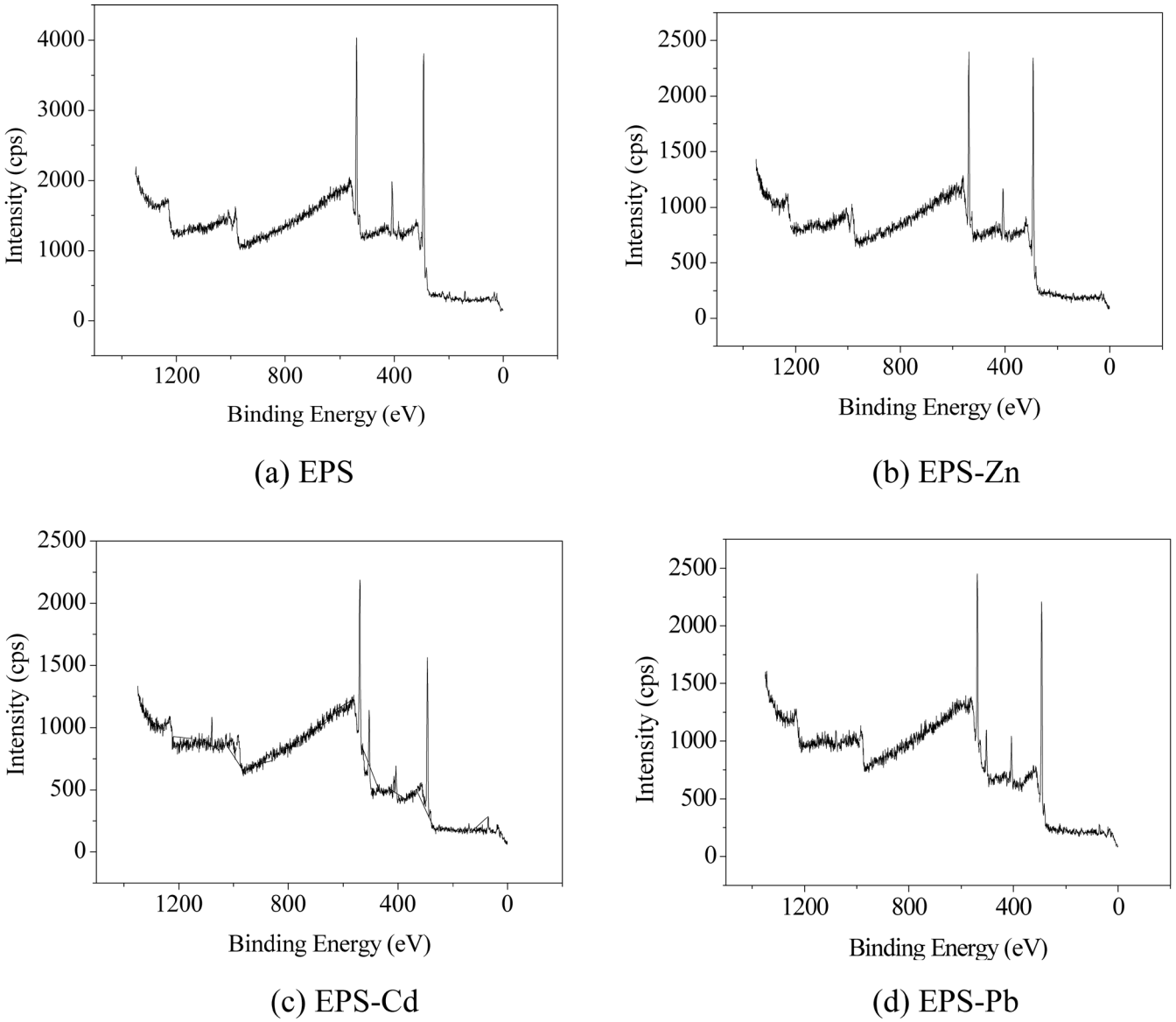

Figure 1. *XPS spectra of EPS before and after heavy metal adsorption.*

they form coordination complexes with metal ions. The C–O–C angle of an ether group will become larger to some extent as a result of its coordination with a transition-metal ion. This adjustment of the geometric shape is another factor responsible for the shift in BE of the ether carbon involved in adsorption. Change of area also displayed that more ether groups in *sp2* than *sp1* participated in binding with the same type of metal ion during the adsorption process.

Contents of methylene and ethers in the EPS decreased after adsorption of Cd^{2+}. The BE of the carbons increased which means that complexation of Cd^{2+} by EPS occurred [29, 30]. After adsorption of Zn^{2+} the BE of carbons decreased. This may be due to electron properties of Zn^{2+} because this ion has full *d* subshells. It was adsorbed through ionic bonding with highly electronegative C. This increased electron density and decreased the binding energy of combined C [28, 31].

N is another element involved in metal adsorption by EPS. Figure 3 displays the XPS spectra of N1s. EPS comprised two peaks with a BE of 399.79 and 401.77 eV identified via deconvolution and indicating presence of $–NH_2$ and $–NH_3^+$. Content of $–NH_2$ was higher than that of $–NH_3^+$.

EPS loaded with Pb^{2+} contains three kinds of functional groups containing N: M–C=NOHC=NOH–M with a binding energy of 399.85 eV, $–NH_3^+$ with a BE of 401.82 eV, and –NH with a BE of 407.08 eV. This suggests proteins in EPS play an important role in adsorbing Pb^{2+}.

Due to overlap of the Cd^{2+} peak and the N peak it is difficult to analyze the function of N from deconvolution. However, based on change in the BE for N it can be deduced that $–NH_2$ is the functional group responsible for adsorbing Cd^{2+}. Considering the d10 structure of the outside electron shell of Cd the electron sphere is not affected by ligands and complex bonding when metal forms complexes with ligands (such as $–NH_2$).

After adsorbing Zn^{2+}, the peak at a BE of 399.83 eV which corresponds to $–NH_2$ increased. This is believed to be due to reaction between $–NH_2$ and Zn^{2+}. $–NH_2$ provided electrons to Zn^{2+} and its electron density decreased resulting in an increased binding energy for N. The peak at a BE of 401.30 eV indicates an amino acid. Its appearance may be caused by hydrolysis of peptide bonds in proteins. The N in EPS appears to be not very important for Zn adsorption based on the peak's shape, the BE, and the half-peak-width.

There was no clear correlation between uptake capacities of the EPS for metal ions and corresponding changes of peak area. Ability of EPS to take up Cd, Pb, and Zn for example varied greatly in binding capacities but corresponding spectra shapes did not change in a predictable way.

FTIR Analysis of EPS after Adsorbing Heavy Metals

Figure 4 displays the FTIR spectra for the region

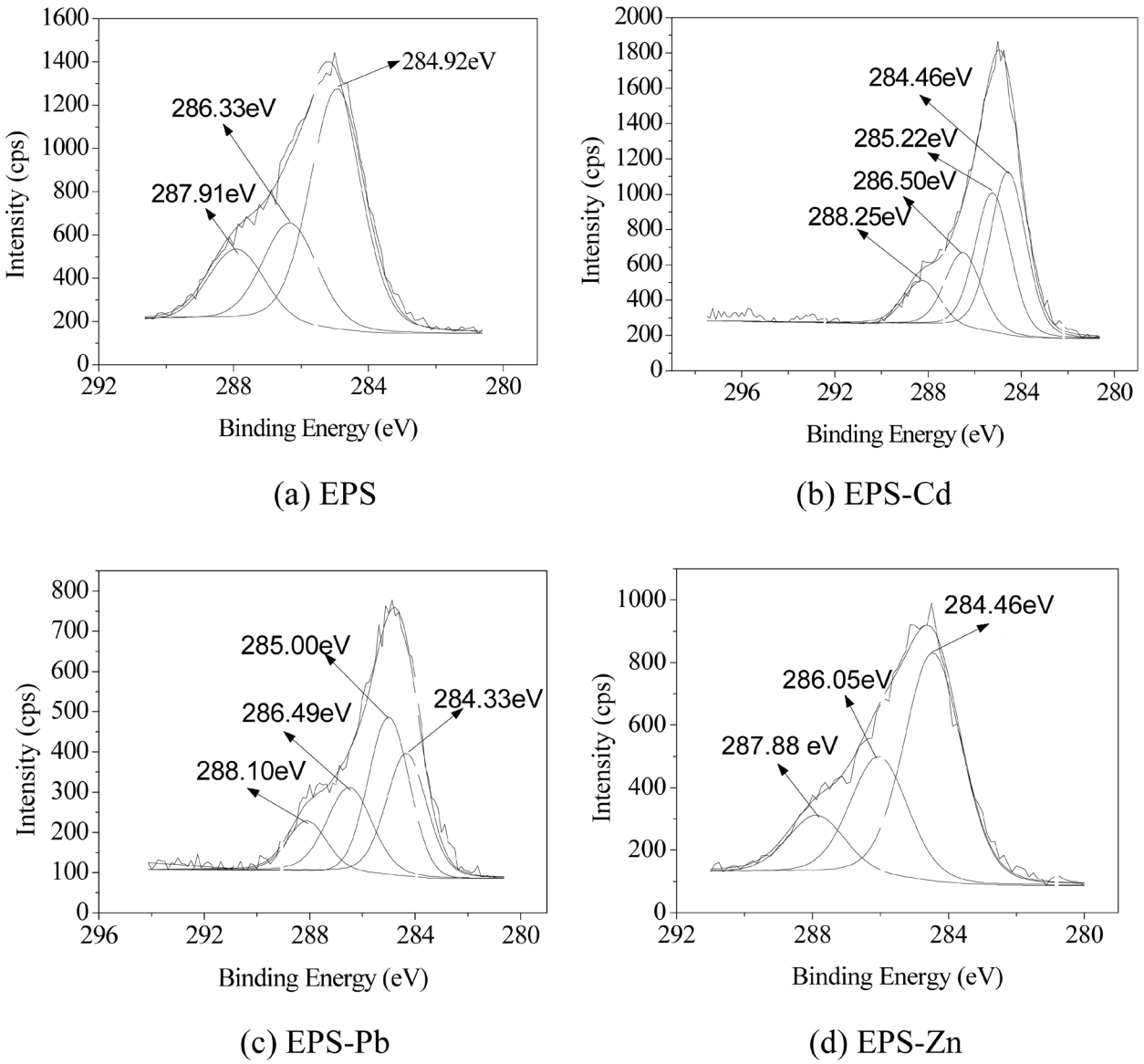

Figure 2. *C1s XPS spectra in EPS before and after adsorption of heavy metals.*

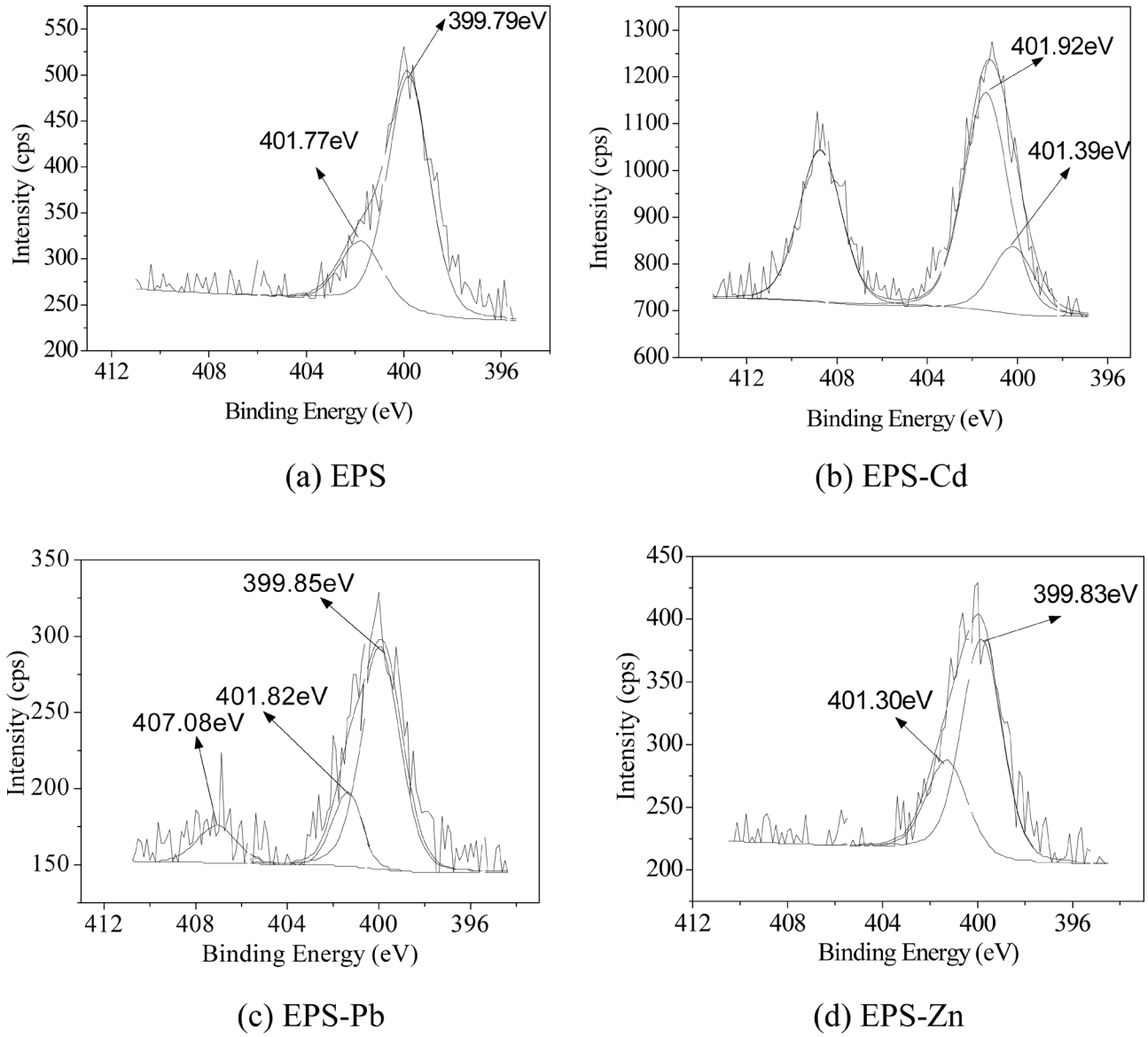

Figure 3. *XPS spectra of N1s in EPS before and after heavy metal adsorption.*

from 400 to 4000 cm^{-1} for the blank EPS and for the EPS exposed to Pb^{2+}, Cd^{2+}, and Zn^{2+}. The FTIR spectra of the four samples were quite similar. Several distinct and characteristic sharp stretching frequencies of chemical groups were visible for the native EPS. The different functional groups observed in the EPS samples agreed with results from Guibaud *et al.* [19]. Table 3 summarizes main stretching frequencies determined from these spectra and biological molecules involved.

Our analysis of IR-spectra revealed presence of numerous functional groups. Several intense characteristic bands can be attributed to functional groups present in proteins and polysaccharides. Some less-intense bands showed carboxylic groups under acidic or basic salt form. When combined with other bands presence of uronic acids (notably for bands characteristic of sugars) and of humic substances was observed.

Some groups of molecules were present in lower proportions in the EPS solutions such as lipids or nucleic acids and were more difficult to detect using FTIR spectra. Some bands observed in the "fingerprint" regions could be attributed to the phosphate group which is one of the functional groups contained by nucleic acids. Presence of $–CH_2$ and of carboxylic groups indicates presence of lipids. Our analysis of the FTIR spectra confirmed the above determined composition of the EPS. The FTIR spectrum of the blank EPS displayed a shoulder peak at 1080 cm^{-1} corresponding to the coupled-vibrations band of the hydroxyl group of polysaccharides.

A comparison of spectra for the native EPS [Figure 4(a)] with that of the Cd-loaded EPS [Figure 4(b)] revealed a characteristic "blue shift" (i.e., negative shift) in the carbonyl, hydroxyl, and ammonia groups, which indicates interaction of Cd with these groups. Disappearance of peaks at 1550 to 1680 cm^{-1} indicates the C–N and N–H bonds in proteins (i.e., peptide bonds) were involved in Cd adsorption. Disappearance of peaks at 1190 to 1000 cm^{-1} for the Cd-loaded EPS indicates stretching vibration of C–OH asymmetric stretching as a consequence of Cd adsorption. These changes suggest the hydroxyl, carboxyl, and carbonyl groups of polysaccharides and the amino and peptide groups of proteins in the EPS were the main active groups for Cd adsorption.

Note that a new adsorption band at 1763 cm^{-1} corresponding to the C=O stretching band of the carboxyl group is visible in Figure 4(b). There are two possibilities that account for occurrence of this new band. One is that the peptide bond was broken by amide hydrolysis. This shortens peptides or amino acids and produces a carboxyl group resulting in occurrence of the corresponding stretching band for carbonyl groups in the FTIR spectrum. The other results from the possibility of a free aldehyde group from reducing sugars. The hydrolysates of polysaccharides are oxidized to produce the carboxyl group [29,30].

The spectrum of EPS loaded with Zn was similar to that of EPS loaded with Cd which means that similar mechanisms are responsible for adsorption of Zn by

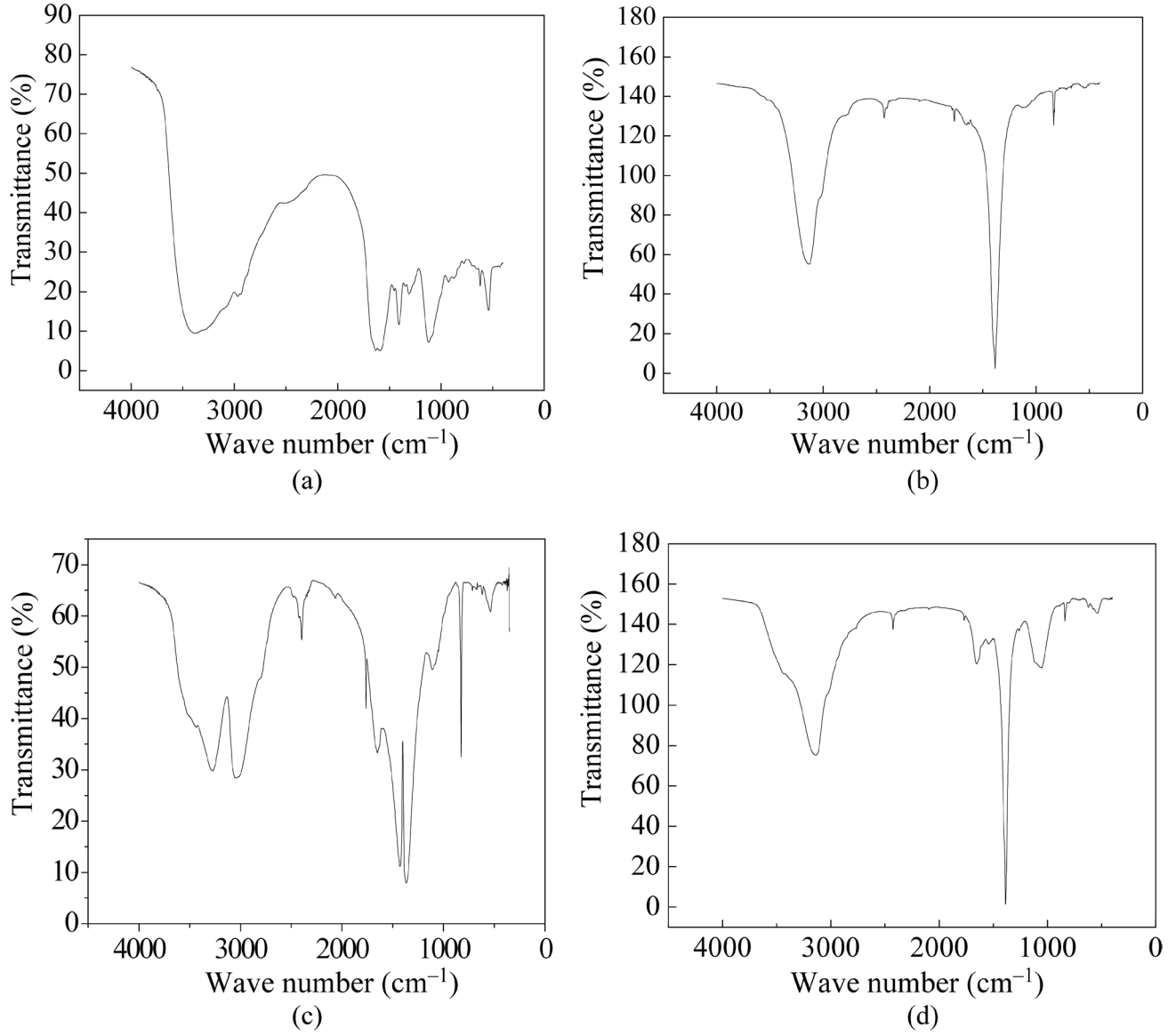

Figure 4. *FTIR spectra of (a) EPS, (b) EPS-Cd, (c) EPS-Pb, and (d) EPS-Zn.*

Table 3. Main EPS Functional Groups Observed in the FTIR Spectra.

Wave Number	Vibration Type	Functional Type
3700 to 3000	Stretching vibration of O–H or N–H	–OH
3000 to 2900	Stretching vibration of C–H	Carboxylates
2926 ± 10	Asymmetric stretching of CH_2	
2853 ± 10	Asymmetric stretching of CH_2	
1720 ± 10	Stretching vibration of C=O	Carboxylic acid
1680 to 1630	Stretching vibration of C=O	Proteins (peptide bond)
	Stretching vibration of C–N	
1630 to 1550	Deformation vibration of N–H	Proteins (peptide bond)
	Asymmetric stretching of $C(=O)_2$	Carboxyl groups
1455 ± 10	Deformation vibration of CH_2	
1450 to 1350	Stretching vibration of C–N	Proteins (peptide bond)
1400 ± 10	Stretching vibration of C=O	Carboxyl groups
	Stretching vibration of OH	Alcohol and phenol groups
1240–1200	Deformation vibration of C=O	Carboxylic acid
1190 to 1000	Stretching vibration of C–OH	Primary alcohol, carboxyl
1030 to 1150	Stretching vibration of C–O–C	Polysaccharides
<1000	"Fingerprint" region	S- and P-containing groups

EPS. After Zn and Cd adsorption new groups occurred at 2424 and 3114 cm^{-1} and represent the stretching bands of C–H, N–H, O–H, and NH_3^+ after adsorption of heavy metals by EPS.

After Cd or Zn adsorption a new peak occurred at 834 cm^{-1}. This indicated Cd or Zn adsorption with oxidated C such as carboxyl and carbonyl groups supporting data obtained in the XPS analysis. At wave numbers lower than 834 cm^{-1} there is little change in the spectra before and after metal adsorption indicating that S- and P- containing groups have little function in the adsorption process of Cd or Zn by EPS. The peak at 1090 cm^{-1} which represents the –OH stretching band in primary alcohols and carboxyl groups disappeared after Cd adsorption but remained unchanged after Zn adsorption. This indicated that the –OH group in primary alcohols and carboxyl groups functions in Cd adsorption but has no function in Zn adsorption.

After Cd or Zn adsorption the carboxyl groups at 1556 and 1405 cm^{-1} disappeared indicating asymmetric and symmetric stretching of $C(=O)_2$ in (COO–). This is typical of complexation of the carboxylate anion functional group by coordination with metal cations [29]. Most of the carboxylate anions must have bound, complexed, or chelated with metal ions because the asymmetrical stretching band at 1338 cm^{-1} moved to a higher frequency [29] that may have been overlaid by other higher frequency bands. This resulted in a deepening of the valley between 1275 and 1511 cm^{-1}.

Mechanisms for Pb adsorption appear to be greatly different from those responsible for Cd and Zn adsorption. After Pb adsorption a new peak occurs at 834 cm^{-1} indicating a reaction between an ester group and Pb^{2+}. The spectral pattern for wave numbers less than 834 cm^{-1} (i.e., the "fingerprint" region) also changes after Pb adsorption indicating that S- and P-containing groups play a role in adsorption of Pb by EPS. This agrees with results from the XPS analysis. A new peak at 1096 cm^{-1} may be caused by the stretching vibration of C–OH in carboxyl groups as a consequence of Pb adsorption. The new peak at 1337 cm^{-1} may result from the stretching vibration of C–N in peptide bonds in proteins. This means proteins in EPS play a more important role in Pb adsorption than in Cd and Zn adsorption. Disappearance of the peak at 1131 cm^{-1} which represents the carbonyl groups in polysaccharides indicates the carbonyl group reacts with Pb and produces a C–O group. Obvious changes at 1436, 1654, and 1754 cm^{-1} indicate the peptide bond of proteins in EPS plays an important role in adsorbing Pb. In addition, the peak at 1654 cm^{-1} which represents the C–N group of a peptide bond disappeared and a new peak occurred at 1355 cm^{-1}. This may be caused by deformation vibration of amine groups or acyl groups as a consequence of Pb adsorption. New peaks at 2375 and 3130 cm^{-1} are believed to result from stretching vibrations of C–H, N–H, O–H, and NH_{3+} caused by metal adsorption.

XRD Characterization of Cd^{2+}, Pb^{2+} and Zn^{2+} Adsorption by EPS

An X-ray diffraction study of EPS before and after

heavy metal adsorption was carried out to ascertain the chemical nature of adsorbed metals (Figure 5). An obtuse peak was observed at 2θ = 22.63° for the native EPS sample indicating the irregular structure of EPS [Figure 5(a)].

Compared with the XRD pattern for the native EPS the Cd-loaded EPS showed sharp peaks at 2θ = 21.54°, 23.46°, 27.38°, 29.50°, 31.74°, 32.84°, 36.36°, 39.38°, 43.12°, 44.54°, and 47.96° [Figure 5(b)]. The peak at 23.46° represents fructose, the peak at 32.84° represents glucose, and the peaks at 27.38° and 36.36° may represent other sugars. Peaks at 29.5°, 39.38°, 43.12°, 44.54°, and 47.96° reflect crystals of a hydrated Cd compound.

After Zn adsorption, peaks show up at 2? = 22.84°, 27.44°, 29.52°, 31.92°, 35.54°, 39.02°, 42.66°, 47.96°, and 55.72 (Fig. 5c). Peaks at 22.84°, 27.44°, and 31.92° may indicate sugars. Peaks at 29.52°, 35.54°, 39.02°, 42.66°, 47.96°, and 55.72° represent crystals of $Zn(H_2PO_2H_2O)$ [32].

There is little change in the XRD pattern for Pb-loaded EPS compared with that of the native EPS except that the intensity values are only about 40% of those in the native EPS. This indicated that Pb adsorption has little effect on the molecular structure of EPS.

Adsorption of Cd and Zn as a whole improved the molecular activity of EPS resulting in a structure with high ordered molecular chains. It appears some functional groups such as carboxyl groups chelate with metal ions resulting in new compounds. These new compounds initially contain water and then lose the water and become either crystals or hydrate crystals

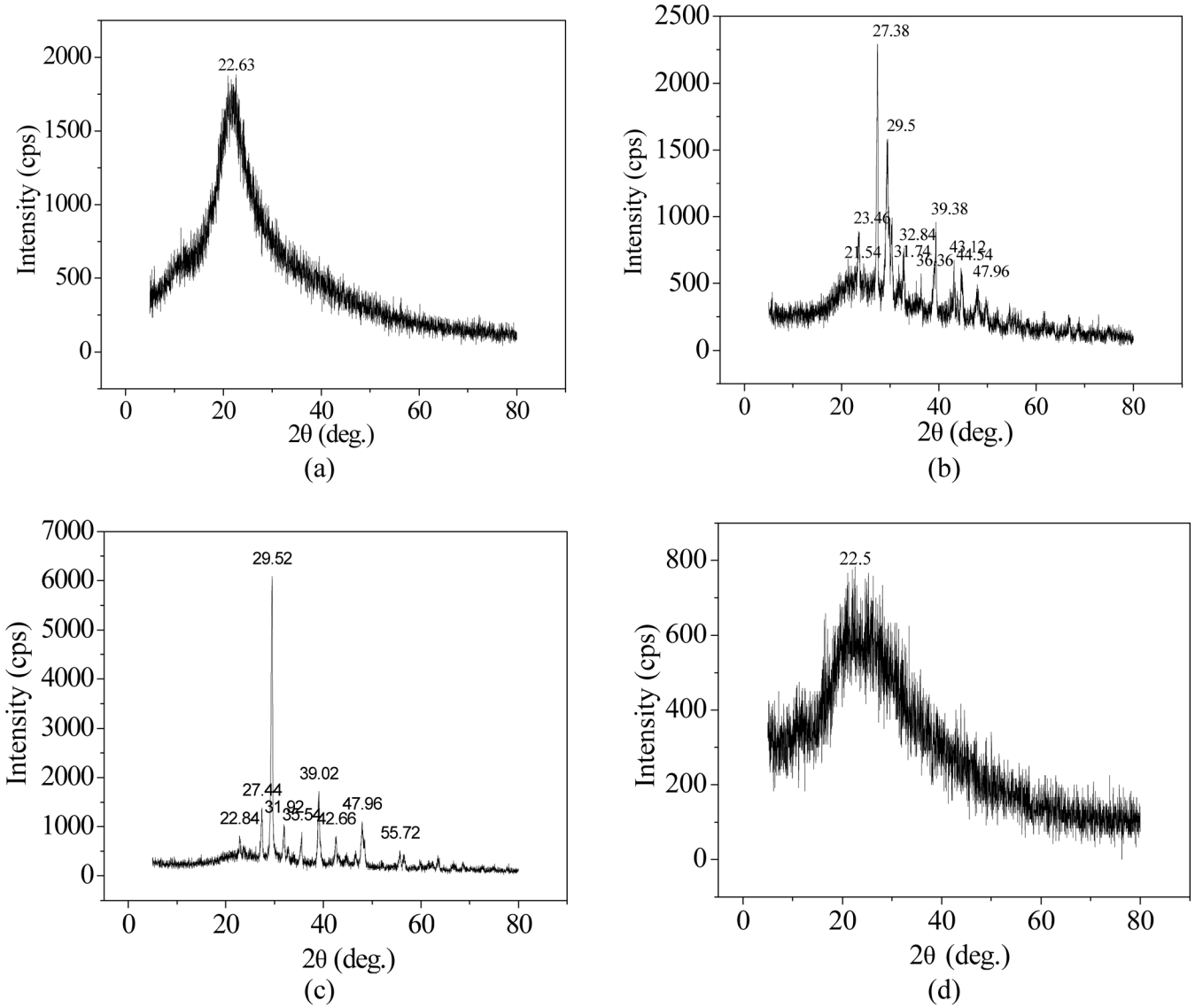

Figure 5. *XRD spectra for (a) native EPS, (b) EPS-Cd, (c) EPS-Zn, and (d) EPS-Pb.*

and turn into crystals [33]. For Zn and Cd adsorption situations are very different. A sharp peak occurs at 2θ = 29.52° for the Zn-loaded sample [Figure 5(c)] and its intensity is much higher at about 4 times that of the Cd-loaded sample [Figure 5(b)]. This indicated Zn adsorption has greater effects on molecular structure than Cd adsorption. This is because Zn adsorption enhances the strength of H bonds. Consequently, the arrangements of molecules in EPS become more regular and the intensity of XRD signals is higher. EPS binds Pb to produce less-regular arrangements in contrast.

XRF Characterization of Cd^{2+}, Pb^{2+} and Zn^{2+} Adsorption by EPS

We carried out an XRF study of EPS before and after heavy metal adsorption to investigate the effect of adsorbed metals on elements in EPS. Table 4 summarizes results.

Contents of several elements change greatly after metal adsorption. Contents of K, Na, Ca, and Mg were 6.2, 15.4, 0.5, and 0.4%, respectively, in the initial EPS sample. After adsorption of Pb, Zn, or Cd contents of these elements generally decreased. This indicated that ion exchange occurs during adsorption of a heavy metal by EPS. The extent of change was in the following order: for Na EPS-Pb > EPS-Zn > EPS-Cd, for Mg EPS-Pb > EPS-Cd > EPS-Zn, for K EPS-Cd > EPS-Pb > EPS-Zn, and for Ca EPS-Pb > EPS-Cd > EPS-Zn. These differences indicate that ion exchange plays a more important role in Pb adsorption than in adsorption of the other two metals.

Note that the P content changes most after Pb adsorption suggesting that P-containing groups play a strong role in Pb adsorption. This agrees with results from the FTIR and XPS analyses.

Compared with results from the XPS analysis the XRF analysis shows that the O contents in all samples were higher whereas the C and N contents were lower. This indicated that O content at the EPS surface is low. After Pb and Cd adsorption N content increases which is opposite of the trend observed in XPS analysis. This suggested that Pb and Cd adsorption cause a transfer of N to sites in the interior of the EPS. This suggests N-containing groups are involved in Pb and Cd adsorption and this is also supported by FTIR and XPS analyses.

The C content decreased and the O content increased after Pb adsorption which is opposite of what is suggested by XPS analysis. This reflects the fact that Pb adsorption results in a transfer of O to sites in the interior of the EPS accompanied by a transfer of C to sites outside of the EPS. Considering that Pb was not detected in the XPS analysis this suggests Pb is bonded i[0]nside the structure of the EPS with N or O.

XRF analysis data displayed changes similar to those indicated by the XPS analysis after Cd adsorption with a slow decrease in C content and an increase in O content. This may suggest Cd is bonded at the surface of the EPS which agrees with results from the XPS analysis.

After Zn adsorption C content decreases slightly and O content increases slightly which is opposite of the trend revealed by XPS analysis. This indicated that adsorption of Zn causes transfer of C and O from the surface of the EPS to the interior of the EPS particles as in the case of Pb adsorption. However, Pb has an effect on this transfer activity more strongly than Zn.

Table 4. Analysis of EPS Elemental Contents Before and After Heavy Metal Adsorption Based on XRF Data.

	Content (%)			
Element	EPS	EPS-Pb	EPS-Zn	EPS-Cd
C	52.8	26.2	50.7	36.9
O	32.1	46.2	33.2	38.0
Na	15.4	0.490	0.492	11.2
Mg	0.410	0.198	0.436	0.243
Al	0.010	0.015	0.093	0.040
Si	0.056	0.200	0.075	0.101
P	3.79	1.88	3.72	2.36
S	0.296	< 0.005	0.813	0.206
Cl	0.733	0.385	0.801	0.473
K	6.25	3.00	6.37	2.81
Ca	0.460	0.213	0.473	0.261
N	2.67	5.96	2.40	7.26
Pb	< 0.003	0.268	< 0.003	< 0.003
Zn	< 0.005	< 0.005	0.411	< 0.005
Cd	< 0.001	< 0.001	< 0.001	0.143

CONCLUSIONS

Carbon and nitrogen are main elements contained in EPS. Nitrogen-containing groups appear to play a more important role in Pb and Cd adsorption than in Zn adsorption. Carboxyl, carbonyl, and amino groups in EPS are main groups that are active in Pb^{2+}, Cd^{2+}, and Zn^{2+} adsorption. The amino, S- and P- groups play a greater role in Pb adsorption than in Cd and Zn adsorption. EPS bind Pb in a way that decreases regularity of the structure and bind Cd or Zn in a way that increases regularity of the structure. Ion exchange is an impor-

tant mechanism in the adsorption of Pb, Cd, and Zn by EPS and plays a more important role in Pb adsorption than in Cd and Zn adsorption.

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Effect of Farmyard Manure and Its Conjoint Use with Microbial Consortium on Chlorpyrifos Residues in Soil, Plant and Percolation Water in Cropped Lysimeters

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ABSTRACT: The efficacies of farmyard manure (FYM) and conjoint use of farmyard manure and microbial consortium (*Bacillus* sp. and *Pseudomonas* sp.) applications were evaluated in lysimeters cropped with maize and rice on chlorpyrifos residues in soil, plants and percolation water. Application of FYM @ 5 t ha^{-1} decreased chlorpyrifos residues in percolation water, crops and surface soil under rice. Conjoint use of FYM @ 5 t ha^{-1} and microbial consortium was more effective than application of FYM alone in reducing the level of chlorpyrifos residues in surface soil, percolation water and crops. Effects were more pronounced under maize as compared to rice crop.

INTRODUCTION

CHLORPYRIFOS (o,o-diethyl-o-3,5,6-trichloro-2-pyridylphosphorothionate) is an organophosphorus insecticide widely used for the control of termites in agricultural crops [1–2] including maize (*Zea mays*) and rice (*Oyriza sativa*). Chlorpyrifos and other organophosphorus pesticides applied to agricultural fields enter natural water bodies through leaching and runoff and pose a potential risk to humans and other organisms [3]. In view of the adverse impact of chlorpyrifos on the environment, it is critically important to reduce the residue levels in soil, percolation water and crops to safeguard human and animal health.

Organic manures influence the sorption and degradation processes of pesticides in soil [4]. Recently, an increasing interest has been focused on assessing the consequences of addition of exogenous sources of organic carbon on the behavior of pesticides in soils [5] because their persistence and potential as environmental contaminant depend on their retention and degradation behavior in soils. Organic manures like FYM and organic sludges restrict the downward movement of pesticides and help their biodegradation in soils [6–10].

Besides organic manures, soil microorganisms can also metabolize organic pesticides either aerobically or anaerobically. In most cases, the microorganisms degrade the molecules and utilize them as a source of energy and nutrients [11–13] or use them through co-metabolism in which an organic compound is not used by the microorganism for growth, but it is metabolized in conjunction with another substrate used for growth [14]. There lies a possibility to use microbial consortium capable of degrading a specific pesticide in a scheduled manner for achieving the accelerated degradation of the pesticide soon after the pest control to ensure lower burden of pesticide residues in soil, percolation water and crops. In the light of these facts, we examined the effect of FYM and conjoint application of FYM and microbial consortium (*Bacillus* sp. and *Pseudomonas* sp.) on chlorpyrifos residues in soil, percolation water and maize and rice crops grown in lysimeters.

MATERIALS AND METHODS

Analytical grade Chlorpyrifos (99.5%) was procured from Dow Agro Sciences, India. For lysimeteric studies, depth-wise (0–15, 15–30, 30–45, 45–60 and 60–90 cm) samples of a mollisol were collected from an agricultural field having no past history of chlorpyrifos application located in Practical Crop production (PCP) block of G. B. Pant University of Agriculture and Technology, Pantnagar, India. The soil samples

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Table 1. General Properties of Depth-wise Drawn Soil Samples Used for Packing the Lysimeters.

Soil Properties							
Soil Depth (cm)	Sand (%)	Silt (%)	Clay (%)	pH (1:2, soil water suspension)	EC (dS m^{-1}, 1:2,soil water suspension)	Organic C (g kg^{-1})	Free Iron oxide (g kg^{-1})
0–15	16.4	44.0	39.6	8.16	0.130	14.5	9.7
15–30	12.4	44.0	43.6	8.35	0.129	11.1	9.7
30–45	1.24	58.0	29.6	8.66	0.122	10.4	10.6
45–60	12.4	42.0	45.6	8.37	0.111	9.6	11.8
60–90	12.4	50.0	37.6	8.60	0.094	9.9	11.4

were air-dried and passed through a sieve having 2 mm openings. Some general soil properties like mechanical analysis, soil pH and E.C. in 1:2 soil water suspension, organic carbon and free iron oxide content analyzed by standard analytical procedures [15] are depicted in Table 1.

Microbial Consortia Preparation

For obtaining the most suitable microbial culture capable of degrading chlorpyrifos, several isolates were screened in Department of Microbiology, G.B.Pant University of Agri. & Technology, Pantnagar, India. Based on these investigation, a final consortium of *Bacillus* sp. (S9) and *Pseudomonas* sp. (P) were selected for the experiment. Loopful cultures of each of the two bacteria (S9 & P) were taken and inoculated in a half litre autoclaved nutrient broth (composition of 3 g Beef extract + 5 g Peptone L^{-1}). The flasks were incubated for 24 h at 30°C on a horizontal shaker to get an active culture broth.

Lysimeter Filling

Lysimeters (110 cm long × 45 cm diameter) were filled with a bottom gravel layer (6–8 cm) followed by 10 cm thick layer of river bed sand. Each lysimeter was provided with a stoppered outlet at the base which was covered with a glass wool pad on the interior side to avoid soil movement. Processed soil samples of different depths (0–15, 15–30, 30–45, 45–60 and 60–90 cm) were sequentially packed in lysimeters. The bulk density of each depth was maintained near to the natural value by a regular gentle tapping with a wooden hammer. Surface of each layer was scratched before packing the next soil layer to avoid undue stratification. After packing the lysimeter, the entire soil mass was initially saturated with water to attain natural soil compaction (bulk density ≈ 1.35-1-5 g cm^{-3}). When the moisture content in the surface soil was near field capacity (24.0% on oven dry weight basis), a basal dose of 75 kg N, 50 kg P_2O_5 and 40 kg K_2O ha^{-1} as urea, diammonium phosphate and muriate of potash was applied to each lysimeter and mixed in the surface layer with help of a screw driver. Six maize seeds (cv. Pragati) were sown in each lysimeter in March, 2009.

After emergence, three plants were maintained per lysimeter. During first 30 d of crop growth only light surface irrigations were given as and when required. After 30 d of crop growth, all lysimeters were treated with chlorpyrifos @ 0.6 kg ha^{-1} as it is the common application rate of chlorpyrifos on maize and rice for controlling termites. In order to ensure a uniform mixing of chlorpyrifos in the surface soil, the required dose of chlorpyrifos was first thoroughly mixed in 500 g fresh soil in a polythene bag layer and uniformly spread all over the soil surface in each lysimeter.

Out of six lysimeters, two were maintained as control while rest four lysimeters received FYM @ 5 t ha^{-1} in the surface layer. In two FYM treated lysimeters, 500 ml of nutrient broth containing microbial consortium of *Bacillus* sp. and *Pseudomonas* sp. was added 7 d after FYM application. A similar quantity of nutrient broth devoid of microbial culture was applied to the control and FYM treated lysimeters. Ten d after microbial consortium application, all lysimeters were flood irrigated and leachates were collected for 3 d after irrigation and the volume of percolated water was measured in each case. Three d after flood irrigation, soil samples were collected from 0–15 and 15–30 cm depth with help of a tube auger. Plant samples were also simultaneously collected from each lysimeter.

Similar experiment was repeated with rice (cv. Pant Dhan 10) crop in August, 2009. After the transplanting of 20 d old six rice seedlings in each lysimeter, 5 cm water was maintained over the soil surface throughout the crop growth period (45 d after transplanting). The methodologies for the application of treatments and

sample collection were similar as described for maize crop.

Extraction of Chlorpyrifos Residues from Percolated Water, Soil and Plant Samples

For the extraction of chlorpyrifos residues from percolated water, 50 mL aliquot of leachates was partitioned with 3 × 25 mL of n-hexane and 0.5 g of sodium sulphate in a separating funnel by shaking the mixture vigorously for 4–5 min, releasing the pressure intermittently and allowing the layers to separate. The upper organic phase was collected in a round bottom flask. The pooled organic phase of three extractions after passing through a column packed with 5 g sodium sulphate was concentrated to dryness in a rotary flash evaporator and the residue was dissolved in 1 mL of n-hexane for the estimation of chlorpyrifos by gas chromatography.

For extraction of chlorpyrifos residue from soil, 10 g aliquot of each soil sample collected from 0–15 and 15–30 cm depths was weighed in duplicate. One sample was oven dried at 105°C for 48 h for the assessment of moisture content. Another 10 g sample was extracted with 50 mL acetone for 1 h on a mechanical horizontal shaker (180 rpm). The contents were filtered by using Buchner funnel and the soil residue was thoroughly washed twice with 20 mL of acetone. The pooled filtrate was concentrated in a rotary flash evaporator at 50°C and the residue was dissolved in n-hexane before being subjected to clean-up by column chromatography. In clean-up process, a small quantity of anhydrous sodium sulphate was added at the bottom of sintered disc column to remove any traces of water. The column was then packed with 5 g silica gel, which was eluted with n-hexane and collected in a conical flask. The eluate was again concentrated in a rotary flash evaporator and re-dissolved in 1 ml of n-hexane for the estimation of chlorpyrifos residues by gas chromatography.

For the extraction of chlorpyrifos residue from plant samples, 5 g homogenized plant sample was taken in 50 mL capacity centrifuge tube, mixed with 3 g anhydrous Na_2SO_4 and 15 ml acetone was added to it. The mixture was blended for 2 min at 3000 rpm in a centrifuge and kept on a mechanical horizontal shaker for 30 min at 25°C. The supernatant was passed through a filter paper. Acidified actived charcoal (0.1 g) was added to the filtrate to remove soluble plant pigments. The filtrate was collected in a round bottom flask and acetone was evaporated to dryness. The residue was redissolved in 1 mL hexane before analysis by gas chromatography.

Estimation of Chlorpyrifos Residue by Gas Chromatography

Chlorpyrifos residue, in water, soil and plant samples, was estimated by the Gas Chromatograph (Chemito Tech. Model Ceres 800 plus) equipped with a packed column 10% SE 30 (8′ length and 1.8′ i.d) and an Electron Capture Detector. The flow rate of N_2 (UHP grade) as carrier gas was maintained at a 30 mL min^{-1}. The temperatures of column, injector and detector were maintained at 180°C, 230°C and 300°C, respectively. One µl aliquot was injected for each analysis. The retention time of chlorpyrifos under the above conditions was 8.7 min. A calibration curve was prepared by plotting the peak area against known concentrations of chlorpyrifos in the range of 0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5 µg ml^{-1}. The limit of detection of the instrument was 0.005 µg ml^{-1}. The concentration of chlorpyrifos in the samples was calculated with the help of the calibration curve.

Statistical Analysis

The residue levels of chlorpyrifos observed in percolated water, soil and plant samples under different treatments pertaining to both maize and rice crops were compared for statistical significance by paired t-test and the significance was tested at $p = 0.10, 0.05$ [16].

RESULTS AND DISCUSSION

The data on chlorpyrifos residues in percolation water, soil and plant samples under different treatment in maize crop are presented in Table 2. It is clearly evident from the data that FYM @ 5 t ha^{-1} reduced significantly the residue levels of chlorpyrifos from 0.241 to 0.138 µg L^{-1} in percolation water and 0.009 to 0.006 mg kg^{-1} in maize fodder as compared to control however, the decrease noted in surface and sub-surface soil under FYM treatment was statistically not significant at $P \leq 0.05$. The effect could be ascribed to greater sorption capacity of FYM for non-ionic compounds like chlorpyrifos [17]. Further, conjoint use of FYM @ 5 t ha^{-1} and microbial consortium of *Bacillus* sp. and *Pseudomonas* sp. was more effective in reducing the residue levels of chlorpyrifos in percolation water, surface (0–15 cm) soil and maize fodder significantly in comparison to the treatment receiving FYM @ 5 t ha^{-1} alone. The residue level of chlorpyrifos in the percolation water under conjoint application of FYM @ 5 t ha^{-1} and microbial consortium was reduced to 0.048

Table 2. Chlorpyrifos Residues in Percolated Water, Surface and Sub-surface Soil and Maize Crop Under Different Treatments Applied to Lysimeters.

	Treatments					
Parameters	**Control**	**Control vs. FYM @ 5 t ha^{-1}**	**FYM @ 5t ha^{-1}**	**FYM @ 5 t ha^{-1} vs.FYM @ 5 t ha^{-1} + Microbial Consortia Culture**	**FYM @ 5 t ha^{-1} + Microbial Consortia Culture**	**Control vs. FYM @5 t ha^{-1} + Microbial Consortia Culture**
Total amount of chlorpyrifos leached in percolated water (in µg L^{-1})	0.241 ± 0.032	*	0.138 ± 0.012	*	0.048 ± 0.0048	*
Chlorpyrifos residue in 0–15 cm soil (mg kg^{1})	0.012 ± 0.001	NS	0.011 ± 0.001	**	0.006 ± 0.001	*
Persistence (%)	(3.49 ± 0.009)		(3.24 ± 0.146)		(2.03 ± 0.12)	
Chlorpyrifos residue in 15–30 cm soil (mg kg^{1})	0.018 ± 0.001	NS	0.015 ± 0.002	*	0.008 ± 0.001	*
Persistence (%)	(5.27 ± 0.25)		(4.45 ± 0.62)		(2.54 ± 0.26)	
Chlorpyrifos residue in rice plant leaves (mg kg^{-1})	0.009 ± 0.0003	*	0.007 ± 0.0001	*	0.006 ± 0.0007	*

Numerals after ± indicate the standard deviation.
* and ** indicate significant difference between pairs at P ≤ 0.05 and 0.01, respectively.
NS—non significant.

µg L^{-1} as against 0.138 µg L^{-1} under application of FYM @ 5 t ha^{-1} alone. Environmental Fate and Effects Division (1999) recommended 0.007 to 0.103 µg L^{-1} level of chlorpyrifos in ground water as revised recommendation for acute or chronic exposure [18]. This level of chlorpyrifos residue in percolation water could be achieved with conjoint application of FYM @ 5 t ha^{-1} and microbial consortium. However, the decrease noted in the residue level of chlorpyrifos in sub-surface soil under treatment receiving of FYM @ 5 t ha^{-1} and microbial consortium in comparison to the treatment receiving FYM @ 5 t ha^{-1} alone was statistically not significant at $P \leq 0.05$.

The data on chlorpyrifos residues in percolation water, soil and plant samples under different treatments in rice crop are presented in Table 3. In general, the treatment effect observed under rice crop were similar to those observed for maize crop except that under rice

Table 3. Chlorpyrifos Residues in Percolated Water, Surface and Sub-surface Soil and Rice Crop Under Different Treatments Applied to Lysimeters.

	Treatments					
Parameters	**Control**	**Control vs. FYM @ 5 t ha^{-1}**	**FYM @ 5t ha^{-1}**	**FYM @ 5 t ha^{-1} vs.FYM @ 5 t ha^{-1} + Microbial Consortia Culture**	**FYM @ 5 t ha^{-1} + Microbial Consortia Culture**	**Control vs. FYM @5 t ha^{-1} + Microbial Consortia Culture**
Total amount of chlorpyrifos leached in percolated water (in µg L^{-1})	0.790 ± 0.035	*	0.571 ± 0.001	*	0.450 ± 0.006	*
Chlorpyrifos residue in 0–15 cm soil (mg kg^{1})	0.0096 ± 0.0002	**	0.0090 ± 0.0002	*	0.0045 ± 0.0008	*
Persistence (%)	(2.817 ± 0.061)		(2.634 ± 0.055)		(1.327 ± 0.231)	
Chlorpyrifos residue in 15–30 cm soil (mg kg^{1})	0.0141 ± 0.0002	NS	0.0116 ± 0.0010	NS	0.0082 ± 0.0007	*
Persistence (%)	(4.115 ± 0.056)		(3.390 ± 0.370)		(2.411 ± 0.211)	
Chlorpyrifos residue in maize fodder (mg kg^{-1})	0.004 ± 0.0001	*	0.002 ± 0.0002	*	N.D.	**

Numerals after ± indicate the standard deviation.
* and ** indicate significant difference between pairs at P ≤ 0.05 and 0.01, respectively.
NS—non significant and N.D.—not detectable.

crop, the use of FYM @ 5 t ha^{-1} was also effective in significantly reducing the residue level of chlorpyrifos in surface soil in comparison to control.

The residue levels of chlorpyrifos in surface and sub-surface soil under maize crop were relatively higher than those observed under rice crop possibly due to considerable degradation of chlorpyrifos in soil cropped with rice [19]. Further, irrespective of the crop taken the residue level of chlorpyrifos was relatively higher in sub-surface soil than in surface soil indicating a tendency of downward movement of this pesticide in soil [20].

A comparison of residue levels of chlorpyrifos in percolation water under rice and maize indicated that in case of rice crop the residue level of chlorpyrifos in the percolation water was more than threefold higher than the level observed in percolation water under maize crop. This could be attributed to higher leaching of chlorpyrifos under submerged soil conditions. The residue level of chlorpyrifos in rice plants was lower than the level observed in maize and the effect could be ascribed to the genotypic differences. In general, the residue level of chlorpyrifos both in maize and rice crops was well within the tolerance limit of chlorpyrifos in foodgrains (0.05 mg kg^{-1}) [21].

CONCLUSION

Results obtained from this study demonstrated that percolation water from chlorpyrifos treated rice fields could be a potential source of ground water pollution. Application of 5 t FYM ha^{-1} reduces the level of chlorpyrifos residues in the percolation water and crops. This treatment also reduces chlorpyrifos residue in the surface soil under rice. The conjoint use of 5 t FYM ha^{-1} and scheduled application of a microbial consortium of *Bacillus* sp. and *Pseudomonas* sp. soon after the pest control is more effective in reducing the chlorpyrifos residues in percolation water, surface and sub-surface soil and crop produce.

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Plant Growth for Sewage Sludge Ecological Stabilization

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ABSTRACT: A pilot scale experiment on the ecological stabilization of sewage sludge was conducted in a bed of 80 m2 for sludge treatment. Sludge loading was performed over the period from June to November 2005 and from May to November 2006, stopped from December 2005 and resumed in May 2006. The sludge was left for natural stabilization since 2007. It was observed that there was more plant biomass growth than in the native ones. In comparison native plant biomass with that of the pilot scale system in 2005 and 2006, the total amount of plant biomass in the system in 2007 increased by about seven times. Totally 2000 g root dry weight per square meter in the stabilized sludge layer was measured, which is two times of root biomass measured in the sand matrix layer in the system. In comparison with native ones, the contents of total nitrogen, total phosphorus, coarse fiber and crude fat of roots were slightly higher. The levels of heavy metals including chromium, lead, arsenic, mercury and cadmium in the stands fed with sewage sludge were slightly higher than those in natural ones. The raw sewage sludge and stabilized sludge contained 4.836 and 3.261 mg/kg of polycyclic aromatic hydrocarbons (PAHs), with a removal of 32.6%; the root and stem of *Phragmites australis* contained 0.185 and 0.272 mg/kg of PAHs, respectively. The results show that the plants played a key role in sludge nutrient recovery and sludge stabilization.

INTRODUCTION

SEWAGE sludge contains valuable plant nutrients such as nitrogen, phosphorus, potassium, and organic matter, thus presents an attractive potential for agricultural land application. However, direct reuse of sewage sludge as fertilizer is not feasible, as it contains pathogens and hazardous substances such as heavy metals, organic pollutants and endocrine disruptors. Another argument against the direct reuse of sewage sludge is its fertilizer properties, for example, the iron content of sewage sludge may reduce the phosphorus availability for agricultural use. All of these arguments led to the research on sludge treatment and disposal.

The sewage sludge ecological stabilization technology is a promising treatment alternative [1–2], and has been given intense attention [3–6]. The core of ecological stabilization studied here is the constructed wetland. The principals of sludge ecological stabilization for drying sludge more rapidly than old unplanted systems are as follows [7]: stem, rhizomes and roots enhance water drainage by providing channels in depth; wind-rock produces holes on the sludge surface at the base of stems; evapotranspiration is enhanced by the presence of leaves; and mineralization. Instead of removing dewatered sludge from the drying systems and hauling it away, sludge can be added continuously for several years before the constructed wetlands are emptied.

It's found that wetland plants can improve sludge stabilization process obviously based on the previous researches [8–14], *Phragmites australis* (common reed) was found to be the best choice for sludge stabilization. It has nodes along the stems for secondary root growth, creates a rich micro-flora in the root zone for helping to break down sludge solids, penetrates the waste sludge and keeps the surface cracked and eventually dry the under layers.

During the last 30 years sewage sludge ecological stabilization technology has been successfully used and improved especially in European countries such as Denmark [15] and Poland [12]. In these systems, sewage sludge was pumped on the surface, where most of its water content is lost by water draining and evapotranspiration of the plants, leaving sludge residue on the surface, where the sludge is dried, the movement of plant stems by wind causes cracking of the surface of the systems and subsequently improves the aeration of the sludge layer [6]. Change of sludge composition

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in time is a result from dewatering processes and the degradation of organic matter, which were also studied by researchers [14].

The aim of this research was to investigate aboveground and underground plant growth in North China and their functions of nutrients transformation, removals of trace metals and organic micropollutants during the process of sewage sludge ecological stabilization, and gain the knowledge on the role of plants in sludge stabilization.

MATERIALS AND METHODS

System Description

A pilot-scale vertical flow constructed wetland with a surface area of 80 m^2, consisting of a 0.6 m deep sand-gravel matrix, supported by a ventilated-drainage system and planted with *Phragmites australis* in 2005, was loaded with thickened sludge generated in a cyclic activated sludge technology (CAST) process in the third wastewater treatment plant (WWTP), Changchun, China. In all vertical flow constructed wetland the draining filter layer is about approximately 60 cm high, and consists in a bottom layer of large gravel (diameter from 3-5 cm) of about 20 cm, a middle layer of small gravel layer (diameter from 1–3 cm) of 20 cm, and upper layers of s fine sand layer (diameter from 0.25–0.5 mm) of 10 cm and coarse sand layer (diameter from 0.5–1 mm) of 10 cm. The drainage system was made from perforated PVC pipes with a diameter of 0.2 m located on the bottom of the wetland. Ventilation pipes with the diameter of 0.1 m were mounted on the drainage pipes, extending 0.5 m above the matrix surface. The wetland was planted with with common reed with a density of 16 plants/m^2.

The thickened sludge was a combination of primary settling and secondary settling sludge, it had a relatively low ratio of volatile solid (VS) to total solid (TS) being 0.347 in 2005 and 2006. During the two-year running (2005–2006), the loaded sludge had TS of 22.34 g/L, VS of 7.76 g/L and moisture content of 97% at average.

Operation and Maintenance

The wetland was constructed before June 1, 2005. *Phragmites australis* harvested from nearby natural wetland, which is located in the adjacent natural pond receiving rain, were replanted in the unit on June 2, 2005 and watered with treated wastewater until plant length reached 1.5 m, spending about forty days. Solid loadings were applied to the system for two weeks from July 12 to 25, 2005 under the loading of 10 tons sludge per day (2.7925 kg TS/d.m^2) in a batch mode with 2 hours of feed and 22 hours of idle, and then fourteen weeks from August to November 2005 under the loading of 10 tons per four days (0.698 kg TS/d.m^2) in the same mode, every cycle had 2 hours of feed time. Operated in a vertical flow mode, the sludge was uniformly distributed on the surface of the constructed wetland. The sludge loadings were stopped in December and resumed in May 2006.

The second year (2006) experiment was conducted from May to November, 25% of area was planted with *Typha angustifolia*. The experiment was divided into three stages:

Stage 1: May to July; loading rate: 10 tons sludge per four days (0.698 kg TS/d.m^2).

Stage 2: August; loading rate: 15 tons sludge per four days (1.047 kg TS/d.m^2).

Stage 3: September to November; loading rate: 7 tons sludge per four days (0.489 kg TS/d.m^2).

The system was leaved for resting from the third year (2007) to the fifth year (2009).

Analyses

The plant total nitrogen, total phosphorus contents were measured with soil and plant nutrients detector of TFC-203 Type, crude fat and coarse fiber contents were measured based on Chinese Standard Examination Methods GB/T 6433-1994 and GB/T 6434-1994, respectively. Cd, Cr, Cu, Pb and As in plants were determined with an atomic absorption spectrometer following the HNO_3-H_2SO_4-H_2O_2 digestion (0.5 g dry plant biomass was digested with 2 ml of concentrated H_2SO_4, 6 ml of concentrated HNO_3 and 6 ml of H_2O_2).

The levels of polycyclic aromatic hydrocarbons (PAHs) in sludge and plant biomass were analyzed by GC/MS. The pre-extraction grinding of sludge and plant biomass was carried out using a warring stainless steel commercial blender. The ground sample was screened on grid (20 meshes). Ultrasonic extraction was carried out in a mixture of hexane/carrene (50/50, v/v). Cleanup was carried out in a silica alumina precolumn, followed by size exclusion chromatography. After final concentration of the sample, deuterated PAH internal standards were added prior to analysis by GC MS.

RESULTS AND DISCUSSION

Plant Growth

In the beginning of system startup, three pipes were used for sludge distribution to prevent local high loading to produce negative impact on plant growth. After a layer of fixed sludge was formed on the surface of the substrata, sludge was distributed with one pipe set at one side of the system.

In summer 2005, weeds grew with *Phragmites australis* together, but the later growth was superior to the former. The researchers found growing period was longer for *Phragmites australis* shoots treated sewage sludge than on natural stands [12]. Similar tendency was found in this research that *Phragmites australis* grew 10–15 days earlier than native ones in 2006, regrowth of plants in Spring 2006 was greater than 90%. For different plants comparison, 20 m^2 in the southwest corner of experimental field was chosen and replanted with *Typha angustifolia* in the spring of 2006. The system ran at full capacity after replanting, it is found that plants survived well, especially for *Phragmites australis*, strong growth was observed in the system under the highest loading of more than 1 kg TS/d. m^2. However, previous researches proved the application of non-stabilized or partially stabilized sludge may result in partial dying of plants, and *Phragmites australis* is susceptible to high rate loadings [3]. This difference may be caused by climate condition and sludge characteristics.

Phragmites australis lengths in the nature and in the system were measured during growing season from May to July, 2006. The plant growth superiority in the system was more obvious with time. Compared to native ones, the plants in the system kept 0.2–0.3 m superiority during early growing season and 0.5–1.0 m superiority during the peak of vegetation growing season. Compared with the system, the nutrients of native ones come from soil, while soil nutrients are limited due to the shortage of external nutrients supply. The system has different situation, the sludge contains plenty of nutrients, fresh sludge and percolate are also nutrient resources. When soil nutrients become the factor of limitation for plant growing, abundant nutrients in the system ensured and stimulated the plant growing.

The temperature of the ground in systems flooded with sludge was 5°C, the critical temperature for reed growth, in February [12]. This may provide for an early start to germination and a late finish to growth, as the process of withdrawing organic matter to the rhizomes is induced by the temperature of the ground, rather than the air. The earlier appearance of young shoots in systems with sewage sludge may result from oxygen deficiency in the ground [16].

Plant Biomass

The experiment selected three sub-plots in the field randomly selected, both in the system and in the natural wetland for biomass comparison, the area of per sub-plot was one square meter. *Phragmites* was harvested in November, 2006 and 2007. The results of plant biomass were listed in Table 1. The mean dry weight of a shoot was always higher in the stands with sewage sludge (17.63–18.52 g) than in the native stand (11.13–12.05 g). The total dry weight of native stands per square meter had no obvious change in 2006 and 2007, while there was a greatly rising in the system from 0.926 kg/m^2 in the second year to 7.342 kg/m^2 in the third year, which demonstrates that plant grew healthy after certain adjustment period, generally taking about two years [3].

Researchers confirm that *Phragmites* grows well in particular systems and can achieve even superior values. Hoffman indicated the harvest of reed from the systems in Ulm, Germany totaled 4 kg D.M./m^2 at the applied relatively low loading of 20 kg D.M./m^2.yr, while lower production of reed was noted at high loadings [17]. Hardej and Ozimek reported that harvest of *Phragmites* in systems in Swarzewo totaled 3.6 kg D.M./m^2, the harvest of *Phragmites* on systems can be twice plant harvest of those in natural conditions [12]. Dry biomass of the harvest aboveground portions of the *Typha angustifolia* ranged from 3.0 to 5.4 kg/m^2 corresponding to net production rates of 43–76 ton/ha.yr [18].

In the stands with sewage sludge the highest dry weight per unit area was observed in this research at the applied highest loading of 0.691 kg D.M./m^2.d, corresponding to the loading of 252.2 kg D.M./m^2.yr, the harvest of the stands on systems got 13.7 times of those in natural conditions. This difference with the previous

Table 1. Biomass of Phragmites on System and on Natural Stand.

	Averaged Single Weight (g D. W.)		Total Amount (g D. W./m^2)	
Year	System	Wild	System	Wild
2006	18.52	11.13	926 ± 40.6	556 ± 25.2
2007	17.63	12.05	7342 ± 256.3	535 ± 20.9

researches may be caused by the sewage sludge and plant species.

Accumulated sludge thickness variation with time in the system was investigated in 2007. Choosing nine even distributed ports as measurement points, the results were averaged monthly from May to October, getting 27.9 cm, 29.8 cm, 31.1 cm, 33.1 cm, 33 cm and 33.2 cm, individually. Accumulated sludge thickness variation could indicate underground plant biomass indirectly because the system was been left for resting since 2007.

It is seen that sludge thickness increased with time from May to August, while tended to be stable from August to October. The data from May to August could be shown in Figure 1. It is interesting to find in Figure 2 that linear regression equation with the slope of 1.69 can indicate accumulated sludge thickness variation, the period corresponded to the main plant growth season. It was also found in the operational period that the rhizomes grew out sideways from the initial plant and developed a very thick root mass (Figure 2), numerous plants branch off from one rhizome and grow vertically up through the sludge. Sludge water contents were measured monthly form May to October, and averaged 62.3%, 59.9%, 42.9%, 39.9%, 35.1%, and 34.9%, individually. Considering the decease in sludge water content with time, it is suggested that the main reason of accumulated sludge thickness increasing was underground plant growth.

One square meter of accumulated sludge in the system was chosen to investigate underground plant biomass in April, 2009. The accumulated sludge was divided into three layers: top layer (from the surface to 10 cm deepness); middle layer (from 10 cm to 20 cm deepness); and bottom layer (from 20 cm to 30 cm deepness), achieved 0.5 kg, 0.7 kg and 0.8 kg (D.W.) of root biomass, individually, corresponding to total 2.0 kg (D.W.) root biomass in the sludge layer, which is two times of those in the sand matrix layer.

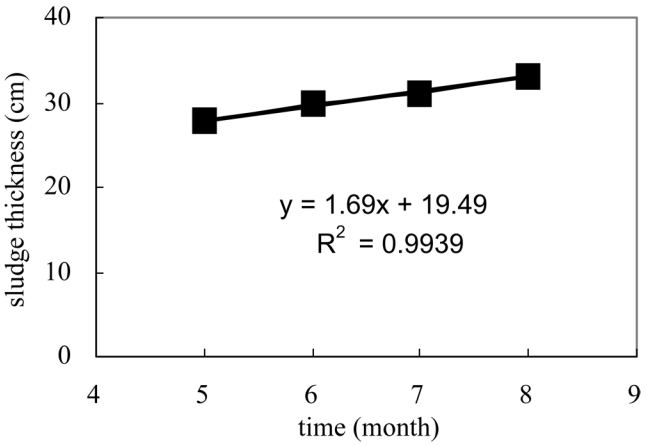

Figure 1. *Accumulated sludge thickness variation in 2007.*

Figure 2. *Stabilized sludge layer in system (2009).*

Based on these, we can conclude that the plant biomass in the system with sewage sludge is higher than that in the natural ones, this may be caused by the later withdrawal of organic matter to the rhizomes in system with sewage sludge [12], and parts of nutrients in the sludge were transformed into plant components, stimulating plant growth. Therefore from the point of source utilization, sludge completes source transformation at the same time of being stabilized.

Plant Components

System flooded with sewage sludge creates a nutritional environment for plants because sludge contains nitrogen, phosphorus, potassium and many trace elements. Researches have detected in underground parts of *Phragmites australis,* during various years of exploitations of systems, average parameters as follows: total nitrogen 3.15% D.M. and total phosphorus 0.39% D.M [19]. Peverly *et al.* reported the contents of total nitrogen 2.03% D.M. and total phosphorus 0.05% D.M. [20]. Thus the nutrient contents of underground parts of plant change greatly in different researching conditions.

In the end of growing season in 2006, samples of *Phragmites australis* and *Typha angustifolia* were taken from the nature and the system, and analyzed in terms of water content, total nitrogen, total phosphorus, crude fat and coarse fiber contents in roots. The detailed results are listed in Table 2. It is worth to note that contents of total nitrogen, total phosphorus, crude fat and coarse fiber in the roots of the system are higher

Table 2. Plant Nutrients Comparison.

	Typha angustifolia	
Item	**Root from Nature**	**Root from System**
Water content (%)	72.50	72.10
Total nitrogen (%DM)	2.23	2.54
Total phosphorus (%DM)	0.21	0.26
Crude fat (%DM)	1.00	1.18
Coarse fiber (%DM)	14.63	17.38
	Phragmites australis	
Item	**Root from Nature**	**Root from System**
Water content (%)	70.56	71.12
Total nitrogen (%DM)	2.05	2.26
Total phosphorus (%DM)	0.20	0.24
Crude fat (%DM)	1.05	1.14
Coarse fiber (%DM)	26.00	33.64

than those of native ones. Nevertheless, water contents of the roots either in the system or in the natural wetland keep the same level.

It is suggested that parts of decreased nutrients in accumulated sludge were converted to plants components. Plants can uptake nutritive components directly from the sewage sludge [13]. Thus from the point of nutrient recovery, the system completed sludge ecological stabilization, at the same time, the transformation of nutrients in the sludge were obtained.

Metals

It is reasonable to assume that metals associated with sewage sludge may concentrate in the sludge. This may be special true for systems because of the reduction in sludge over time. Pollutants concentrated in the reduced biosolids could complicate final disposal because the material may exceed regulatory standards. An attempt in this research was made to determine the fate of selected five metals in the system by analyzing above ground plant tissue in October, 2006.

Table 3. Metal Contents in Sludge and Plant Tissue (mg/kg).

				Phragmites australis	
Parameter	**Sewage Sludge**	**Stabilized Sludge**	**Limit Value**	**In System**	**In Wild**
Cd	0.41	0.41	5	0.041	0.024
Pb	59.66	58.39	300	0.605	0.101
Cr	89.91	86.90	600	6.012	2.917
Hg	9.67	9.51	5	0.012	0.007
AS	17.71	17.65	75	0.274	0.199

Of the five elements studied chromium had the highest content, followed by lead, arsenic, mercury and cadmium in sewage sludge (Table 3). The observation shows that the levels of selected metals are a little bit lower in stabilized sludge than those in the sewage sludge, and a slightly higher in stands with sewage sludge than that of native one, which indicate that the above mentioned metals can be partly transformed by plant tissue.

The roots and rhizomes were neither harvested nor analyzed, only the aboveground portion of the *Phragmites* plants were harvested, thus the contribution of the root biomass to metal attenuation is not considered herein. Even though the metal contents either in sewage sludge or in stabilized sludge meet Chinese standards (Table 4) except Hg, we can also see the positive role of *Phragmites* plants in metal transformation, especially taking the huge increase of plant biomass into consideration which was observed in the system (7.342 kg D.W. /m^2), comparing to the native ones (0.535 kg D.W. /m^2).

One of the main risks in recycling of sewage sludge is the accumulation in agricultural soils of unwanted products, such as organic micropollutants. PAHs are particularly monitored in this context because of their high toxic and high carcinogenic properties even at low concentrations. PAHs are widely distributed in the environment due to the existence of numerous sources

Table 4. Polycyclic Aromatic Hydrocarbons (PAHs) Contents for Phragmites.

		Content (mg/kg)			
No.	**PAHs**	**Raw Sewage Sludge**	**Stabilized Sludge**	**Root**	**Stem**
1	Nap	0.010	0.09	0.002	0.002
2	Acy	0.025	0.0195	0.004	0.004
3	Ace	0.022	0.0132	0.004	0.004
4	Fle	0.053	0.036	0.017	0.015
5	Phe	0.275	0.195	0.029	0.058
6	Ant	0.330	0.234	0.035	0.070
7	Fla	0.847	0.628	0.020	0.050
8	Pyr	0.603	0.481	0.021	0.033
9	BaA	0.954	0.568	0.017	0.006
10	Chr	0.533	0.317	0.010	0.012
11	BbF	0.370	0.243	0.007	0.005
12	BkF	0.277	0.182	0.002	0.001
13	BaP	0.206	0.13	0.005	0.004
14	IcP	0.198	0.103	0.006	0.003
15	DaA	0.042	0.02	0.002	0.003
16	BgP	0.002	0.0014	0.004	0.002
	ΣPHAs	4.836	3.261	0.185	0.272

of production. In the case of wastewater treatment plants, they are easily concentrated into sewage sludge because of their low water solubility and their high affinity for organic compounds. PAHs are known to be highly recalcitrant to biodegradation due to their low bioavailability. Nevertheless, their biodegradation has been widely investigated under aerobic conditions.

The results of PAHs analysis are listed in Table 4. All of sixteen different PAHs were detected, the raw sewage sludge contains 4.836 mg/kg of PAHs, the stabilized sludge contains 3.261 mg/kg of PAHs, the removal of PAHs was averaged 32.6%; the root and stem of *Phragmites australis* contain 0.185 and 0.272 mg/kg of PAHs, respectively; obviously, the stem can absorb more PAHs than roots. The limitation of sludge land application about PAHs in China is less than 3 milligram Benzo(a)Anthracene per kilogram dry sludge, without total amount limitation. However, some developed countries have strict limitation, e.g. Denmark regulated less than 3 mg/kg D.W. of the total PAHs limitation in sludge land application. Obviously, the content of PAHs in the sewage sludge cannot meet the standard, which needs to be removed before land application.

From Table 4 we found that middle heavy PAHs in the sewage sludge have higher contents. For example, Benzo(a)Anthracene has the highest content of 0.954 mg/kg; while least and most heavy PAHs have lower contents, Benzo(ghi)perylene has the lowest content of 0.002 mg/kg. Corresponding to higher contents in the sewage sludge, the plant root and stem concentrate more phenanthrene, anthracene, fluoraathene and pyrene, which have higher contents in the stem than in the root. Benzo(a)Anthracene is an exceptional, which has higher content in the root. Other PAHs have lower contents in the root and stem, without obvious difference.

Liu *et al*. reported that Eichhornia crassipes has better capability to treat wastewater with Nap, its huge rhizomes and roots are ideal places for pollutants removal [21]. Muratova et al studied *Phragmites australis* roots for remediating soil polluted by PAHs through pan experiment, getting 68.7% of PAHs removal within rhizomes; the total microorganism and bacteria for PAHs degradation amounts within the rhizomes are 1.3 and 7 times higher than those without the rhizomes, and microorganism activity within the rhizomes is strengthened obviously [22]. In consideration with the results in this research, we drew a conclusion that the main PAHs removal mechanisms are plant absorption and transformation, as well as the function of roots for strengthening PAHs degradation bacteria activity.

CONCLUSION

In this work we studied plant growth, biomass, nutritive components, heavy metals and hazardous organic compounds PAH contents variation both in the sludge ecological stabilization system and in the natural wetland. Results show that plant played a key role in sewage sludge ecological stabilization and resource recovery. After two years' adjustment operation, the total amount of plant in the system in 2007 increased sharply compared to either in the natural wetland or in the system in the first and second year. Total nitrogen, total phosphorus, crude fat and coarse fiber contents of plant roots harvested in the system flooded with sewage sludge were a little higher than native ones. The results of PAHs suggest that the plant may stimulate sewage sludge stabilization and harmlessness from the viewpoint of organic pollution, while the metals in the sludge need to be removed further before used as a fertilizer in agriculture. As a whole, the studied system demonstrates the feasibility of sludge ecological stabilization technology and its potential application in developing countries.

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Table 5. Comparison of state-of-the-art matrix resins with VPSP/BMI copolymers.

Resin System	Core Temp. (DSC peak)	T_E	Char Yield, %
Epoxy (MY720)	235	250	30
Bismaleimide (H795)	282	>400	48
VPSP/Bismaleimide copolymer			
C379: H795 = 1.9	245	>400	50
C379: H795 = 1.4	285	>400	53

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