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Full Length Research Paper

The use of selected purple nonsulfur bacteria to remove heavy metals and salts from sediment and water collected from contaminated areas to decrease their phytotoxicity

Saijai Panwichian[1], Duangporn Kantachote[1,3]*, Banjong Wittayaweerasak[2,3] and Megharaj Mallavarapu[4]

[1]Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai 90112, Thailand.
[2]Faculty of Environmental Management, Prince of Songkla University, Hat Yai 90112, Thailand.
[3]National Center of Excellence for Environmental and Hazardous Waste Management- Satellite Center at Prince of Songkla University, Hat Yai 90112, Thailand.
[4]Center for Environmental Risk Assessment and Remediation, University of South Australia, Mawson Lakes Boulevard, Mawson Lakes, SA 5095, Australia.

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The potential of the purple nonsulfur bacteria (PNSB), NW16 and KMS24, to remove heavy metals (HMs) and salts was investigated in a synthetic solution (62.63 Pb^{2+}, 34.60 Cu^{2+}, 58.5 Zn^{2+} and 0.75 Cd^{2} mg/L) containing 3% NaCl, sediment, and water collected from contaminated post cultured shrimp ponds and seed germination of 2 plants were used to assay their plant toxicities after bioremediation. Both light metal ions (85 mg/L Ca^{2+} and 160 mg/L Mg^{2+} to the synthetic HMs solution) significantly decreased the HMs removal efficiency and the mixed culture gave the highest efficiency to remove HMs (removal percentages; 85 Pb^{2+}, 74 Cu^{2+}, 47 Zn^{2+} and 28 Cd^{2+}). The best set for the treatment of contaminated water from shrimp ponds (Cu^{2+}, Zn^{2+}; 0.043, 0.057 mg/L and salinity, 10.23‰) under the conditions of aerobic-dark and microaerobic-light was a set of native with added mixed culture with a decrease of roughly 75, 31 and 77% for Cu^{2+}, Zn^{2+} and salinity, respectively. For the sediment samples, a set of native with added mixed culture also produced the highest efficiency to remove HMs (initial concentrations in mg/kg dry weight; 23.15 Pb^{2+}, 15.05 Cu^{2+}, 22.16 Zn^{2+} and 0.29 Cd^{2+}) and salinity (0.84‰) under aerobic-dark conditions with the removal percentages of HMs; 84.29, 62.52, 43.33 and 40.95, and 100% salinity. Consequently, this set produced the most effective treatment as the germination index was 34.50 and 35.29% for rice seed (*Oryza sativa*) and water spinach (*Ipomoea aquatic*) respectively in the treated water and 115.70 and 139.33% for rice and water spinach respectively in the treated sediment.

Key words: Bioremediation, contaminated shrimp ponds, heavy metals, photosynthetic bacteria, salinity, seed germination index.

INTRODUCTION

The increased demand for shrimp in world markets has encouraged many developing countries to enter into shrimp farming but this can have damaging effects on the local environment (Chua, 1992). The extension of shrimp farming from coastal areas to freshwater areas has affected those areas, some previously used for growing rice, fruit plantations and fisheries. Traditionally, seawater from coastal waters is directly used to rear the shrimp with no additional processes and often these coastal waters are contaminated by many kinds of pollutants including heavy metals (HMs) (Cheung and Wong, 2006;

*Corresponding author. E-mail: duangporn.k@psu.ac.th. Tel: +66-74288333. Fax: +66-74446661.

Visuthismajarn et al., 2005; Cheevaporn and Menasveta, 2003). In addition, water removed during shrimp pond drainage during harvesting is often directly discharged into canals and flows into other cultivated areas, together with the illegal disposal of shrimp pond sediments (Dierberg and Kiattisimkul, 1996). Consequently, this can cause serious pollution to soil in agricultural areas, especially rice-fields and vegetable crops, resulting in an accumulation of HMs, salts and chemical substances.

The accumulation of HMs in agricultural soil is of increasing concern due to food safety issues and potential health risks because they cannot be biodegraded and may be leached to surface water run-off, groundwater storages, plant absorption, etc. HMs are frequently accumulated by agriculturally important crops and become concentrated in the plant tissues to produce damaging effects on the plants themselves and may also pose a health hazard to animals and humans (Mokhtar et al., 2009; Yap et al., 2004). Stress from HMs and salts can have a negative impact on processes associated with biomass production and grain yield in almost all major field grown crops and this can result in the reduction of growth rate and pigment content and low productivity (John et al., 2009).

Thailand is the biggest rice exporting country; however, the development of shrimp farming in Thailand has opened the door for shrimp farming away from the coast into the paddy land, particularly in this region of southern Thailand. Hence, low rice yields and the contamination of groundwater aquifers have rendered large areas of land unsuitable for cultivation (Flaherty et al., 1999). Accumulation of HMs in rice may cause some illness such as Itai Itai by Cd (Shimbo et al., 2001). Water spinach (*Ipomoea aquatica*) is an herbaceous aquatic or semi-aquatic perennial plant of the tropics and subtropics. It is a fast growing plant and can be cultivated on most kinds of soils. Contamination of HMs in the water where *I. aquatica* is grown may cause the risk of poisoning to consumers (Gothberg et. al., 2002). There are many advantages for using bioremediation instead of physical and/or chemical processes as it is a natural process, produces harmless end products and any bioremediated soil/water can be re-used (Barker and Bryson, 2002). Bioremediation of HMs from contaminated soil and water would provide decontaminated soil/water that could be used for agriculture. However, toxicity of contaminated soil/water after bioremediation must be evaluated prior to use and bioassay has gained widespread attention over the past few decades. Seed germination assay is one of the most common techniques used to assess phytotoxicity as a rapid method due to its simplicity, sensitivity and inexpensive cost (Kapanen and Itavaara, 2001; Wang et al., 2001). In our previous studies, two purple nonsulfur bacteria (PNSB) strains, NW16 and KMS24, have proven their abilities to effectively remove HMs (Cd, Cu, Pb and Zn) containing 3% NaCl present in contaminated shrimp pond water (Panwichian et al., 2010a, b). Therefore, our aims in this present study were to investigate the potential of these PNSB strains to remove HMs and salts from the sediment and water collected from contaminated shrimp ponds after harvesting and to assay the phytotoxicity of the sediment and water after treatment using a seed germination index of economical plants; rice (*Oryza sativa*) and water spinach (*Ipomoea aquatica*).

MATERIALS AND METHODS

Preparation of heavy metal solutions

The inorganic salts; $CdCl_2$, $PbCl_2$, $CuCl_2$ and $ZnCl_2$ were used for preparing stock solutions of each heavy metal (HM) ion whereas $CaCl_2$ and $MgCl_2$ were used for preparing light metal ions. Each metal was dissolved in deionized water (DI water) to obtain the concentration as designated and then the stock solution was sterilized using a 0.22 µm filter membrane. They were stored at 4°C until used. The concentration of HMs was analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES) (PerkinElmer, Germany).

Preparation of PNSB for uptake of heavy metals

Two PNSB strains, *Rhodobium marinum* NW16 and *Rhodobacter sphaeroides* KMS24, used in this study were isolated from water and sediment samples collected from shrimp ponds contaminated with HMs (Panwichian et al., 2010a). A ten percent inoculum of each active isolate was grown in Glutamate-Malate medium (GM medium) as previously described by Panwichian et al. (2010a) under microaerobic-light conditions (3000 lux). Culture broths were harvested in the log phase of growth because previously it had been established that this was the most effective time for them to remove HMs (Panwichian et al., 2010b). After centrifugation (Sorvall RC 5C Plus, Du-pont, Delaware, USA) at 9,300 x g for 15 min, the cell pellets were washed twice with 0.1% peptone water. The cell pellets were later prepared for uptake of HMs with the optimum biomass equivalent to 4.5 and 5.0 mg dried cell weight (DCW)/ml for NW16 and KMS24, respectively. In this study, mixed culture with the biomass equivalent to 2.5 mg DCW/ml of NW16 and 2.5 mg DCW/ml of KMS24 was also prepared for testing the uptake of HMs.

Effect of Ca^{2+} and Mg^{2+} on the removal of heavy metals by PNSB

Experiments in this study were designed based on the highest concentration of HMs and the average concentrations of Na^+, Ca^{2+} and Mg^{2+} in shrimp ponds (Panwichian et al., 2010a). The mixed solution of HMs containing 0.75 mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}; 34.60 mg/L Cu^{2+}; 58.50 mg/L Zn^{2+} in 3% NaCl solution and with or without added 85 mg/L Ca^{2+} and 160 mg/L Mg^{2+} was prepared. These were used for the treatment and control sets for investigating the effects of both Ca^{2+} and Mg^{2+} on the efficiency of HM removal by both pure cultures and a mixed culture of PNSB. The sets of HMs solution without inoculating with bacterial cells were used as abiotic controls. The optimum conditions for removing HMs by each culture was adopted from one of our previous studies (Panwichian et al., 2010b) as follows; 4.5 mg DCW/ml, pH 6.0, 30°C, 30 min for strain NW16 and 5.0 mg DCW/ml, pH 5.5, 35°C, 45 min for strain KMS24. The mixed culture consisted of 2.5 mg DCW/ml of each culture and

it was tested at both the optimal conditions for removal of HMs by strains NW16 and KMS24. Cell suspensions were shaken in an incubator at a speed of 150 rpm for 30 to 45 min under aerobic-dark conditions. Aerobic-dark conditions were also used because they provided a higher efficiency for removal of HMs when compared to microaerobic-light conditions (Panwichian et al., 2010b). Cell suspensions were centrifuged, and the remaining HMs in each supernatant was analyzed using ICP-OES.

Collection of sediment and water from contaminated shrimp ponds

Post cultured shrimp ponds contaminated with the highest levels of HMs (Cu, Zn, Pb and Cd) in the areas of Ranot, Songkhla province; HuaSai, Nakhon Si Thammarat province and Pak Phayun, Pattalung province were chosen for collecting sediment while shrimp pond water was collected from post cultured contaminated shrimp ponds in the Pak Phayun district (Panwichian et al., 2010a). After shrimp harvesting, sediment sub-samples, each of about 100 g were collected from the bottom of a pond at a depth of 5 cm in two diagonal and a half points from each bank (13 sub-samples/pond). Water sub-samples (13 sub-samples/pond) were collected at the time for shrimp harvesting, roughly 100 ml of water at about 50 cm below the surface water level. All sub samples of sediment and water were kept in a big ice box during transport and then at the laboratory all sub-samples were promptly mixed well to obtain one representative sample each for sediment and water. Concentrations of HMs (Cd, Pb, Cu, and Zn) were analyzed using ICP-OES. In addition, samples of sediment and water were also measured for pH, EC (electrical conductivity) and salinity as described by Panwichian et al. (2010a).

Removal of HMs and salts in the water collected from post cultured contaminated shrimp ponds

The uptakes of HMs and salts by biomass of both PNSB strains were conducted under both microaerobic-light and aerobic-dark conditions due to both incubating conditions having an effect on the efficiency of HMs removal by PNSB (Panwichian et al., 2010b). The cells of NW16 and KMS24 present as a suspension of either a pure or mixed culture were added into the collected water samples that had been sterilized (autoclaving at 121°C, 15 min) and not sterilized (native set). A sterile water set without inoculation of PNSB served as an abiotic control while a single culture or a mixed culture was inoculated into a sterile set, namely a pure culture or a mixed PNSB set. In contrast to the non sterile water sets (namely a native set prepared together with NW16 or KMS24 or a mixed PNSB set with the two strains inoculated together). The uptake of HMs was investigated under optimum conditions as previously described as follows: a pH of 6.0, 30°C, 30 min for strain NW16 and a pH of 5.5, 35°C, 45 min for strain KMS24. This study of uptake of HMs by the mixed culture of NW16 and KMS24 cells was investigated at the optimum conditions of pH 5.5 and 35°C for 45 min based on the result of the previous experiment. The optimum condition of a mixed culture was also used for studying uptake of HMs in both control sets using a longer time of exposure when compared with the optimum condition for strain NW16. In these studies, removal of HMs was focused on only Cu^{2+} and Zn^{2+} as these are 2 cations in the water column of the collected shrimp ponds that exceeded the standard guidelines for aquaculture (Panwichian et al., 2010a). To follow the real situation in shrimp ponds and to test toxicity of salinity and salts, samples of treated water in each set from both incubating conditions were mixed to obtain one sample of each set.

Removal of HMs and salts in sediment collected from post cultured contaminated shrimp ponds

The sediment was made into slurry using sterile DI water at a ratio of 1v:1w (10 ml DI water and 10 g fresh sediment). The uptake of HMs and salts was also investigated using the same protocol as used for the water samples. After incubation, the sediment slurry samples were centrifuged at 9,300 x g for 20 min and the loss of each HM was calculated based on the amounts of HMs in the supernatant together with the amounts determined in the pellet (sediment plus bacterial cells) at zero time and at the end of the experiment. The amounts of HMs were expressed based on the sediment dry weight as the sediment was dried in an oven at 105°C overnight until a constant weight was obtained. To monitor salinity and toxicity, treated sediment samples were prepared in a similar way with the treated water samples as previously described.

Toxicity assessment by seed germination for the sediment and water after treatment

Toxicity of the water and sediment samples from post cultured contaminated shrimp ponds after treatment with PNSB; NW16, KMS24, a mixed culture in the presence or absence of native flora from the previous experiments was tested for their effects on seed germination. The plants used in this study were rice (*Oryza sativa*) and water spinach (*Ipomoea aquatica*). Results were evaluated by comparing the results among sets of treatments and control sets (abiotic control and native control). Briefly for the seed germination test, water samples from each set were filtered with a 0.45 µm filter membrane to remove the organisms. 5 ml of filtered water sample without dilution was added to a 9 cm sterile Petri dish, using Whatman # 1 as a bed, and then 10 grains were placed on the bed. All Petri dishes were incubated in the dark at room temperature for 72 h. The percentages of relative seed germination (RSG), relative root elongation (RRE) and the germination index (GI) were calculated and compared with the distilled water as the control set (Hoekstra et al., 2002). Sediment samples were prepared to obtain the sediment solution by adding 25 ml of sterile DI water into 5 g wet weight of sediment and shaking overnight. Thereafter, it was centrifuged at 13,950 x g for 10 min. The supernatant as the sediment solution was filtered with a 0.45 µm filter membrane and the toxicity was tested by the same method as previously described for the water samples.

Statistical analysis

All experiments in this work were conducted in triplicate. Data are presented as mean and standard deviation. One way ANOVA was used to analyze statistical differences at a p-value < 0.05 and mean comparisons were performed by the Duncan's multiple range test.

RESULTS

Effect of Ca^{2+} and Mg^{2+} on removal of heavy metals by PNSB

As there was no loss of HMs in the abiotic control sets the loss of HMs in the sets after inoculation were caused by the presence of bacterial cells (data not shown). Results of the removal of HMs in 3% NaCl in the presence and absence of 85 mg/L Ca^{2+} and 160 mg/L

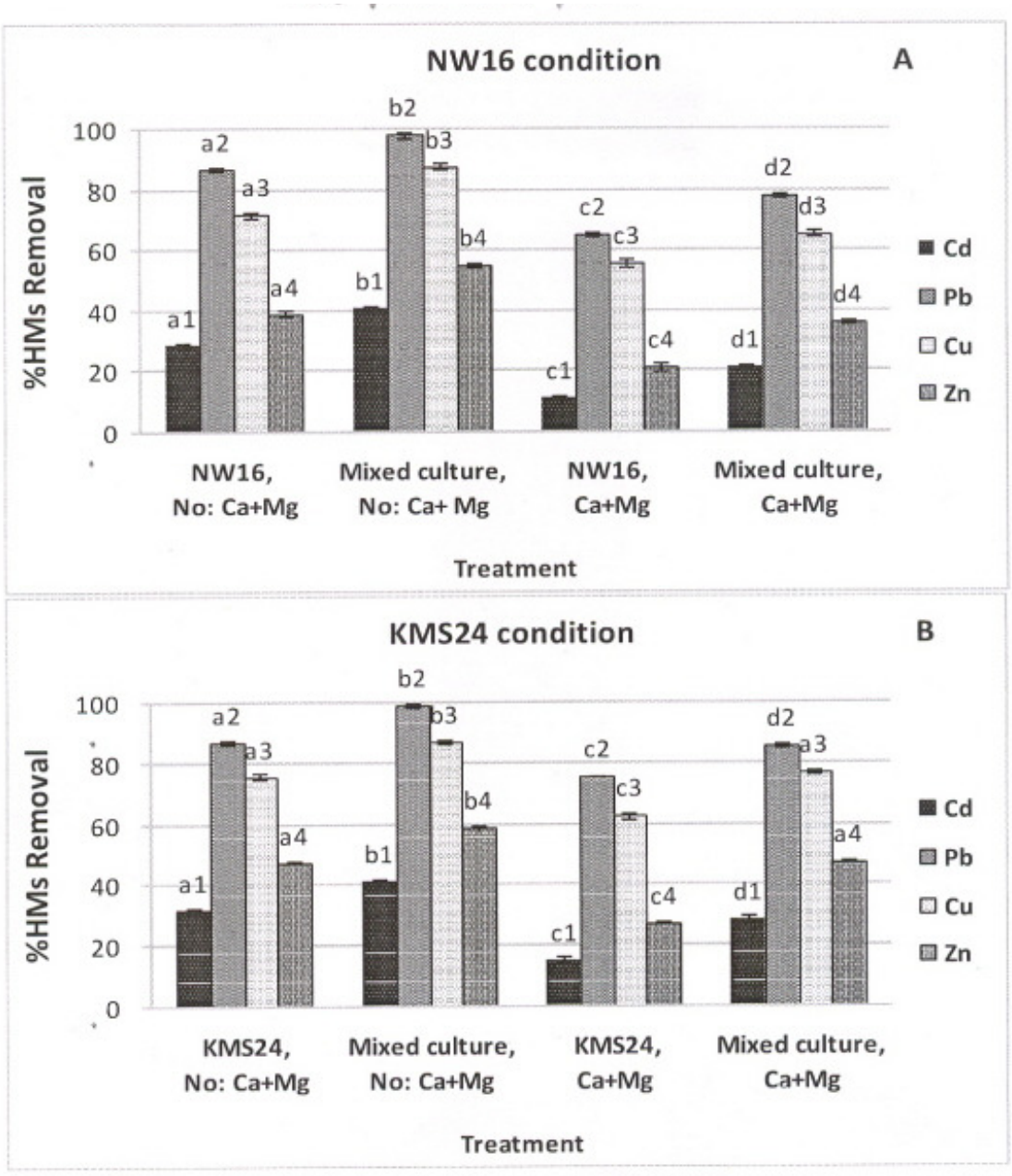

Figure 1. The heavy metals removal efficiency in the synthetic solution (Cd, 0.75 mg/L; Pb, 62.63 mg/L; Cu 34.60 mg/L; Zn, 58.50 mg/L) containing 3% NaCl in the presence or absence of 85 mg/L Ca^{2+} and 160 mg/L Mg^{2+} under aerobic-dark conditions by (A) pure culture of 4.5 mg DCW/ml of NW16 and the mixed of NW16 and KMS24 (2.5 + 2.5 mg DCW/ml) under optimum conditions for removal of HMs of NW16; pH 6.0, 30°C, 30 min and (B) pure culture of 5.0 mg DCW/ml of KMS24 and the mixed culture under optimum conditions for removal of HMs of KMS24; pH 5.5, 35°C, 45 min. Lower case letters with numbers above bars (that is, a1, b1, c1 and d1) using different letters indicate significant differences ($p < 0.05$).

Mg^{2+} by the pure cultures or a mixed culture of PNSB strains (NW16 and KMS24) under aerobic-dark conditions are shown in Figure 1. The mixed culture produced a significantly higher HM removal for all HMs than each of the pure cultures separately, both with and without the added cations (Ca^{2+} and Mg^{2+}). In addition, the extra light metal ions significantly decreased the removal of HMs by all cultures (Figure 1A and 1B). In the presence of Ca^{2+} and Mg^{2+} in the HMs solution containing 3% NaCl with optimum conditions for the strain NW16, the pure culture NW16 removed Pb^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} by about 65, 56, 21 and 11%, respectively but the removal percentages of the mixed culture was roughly 78, 65, 36 and 21%, respectively (Figure 1 A). However,

Table 1. Values of salinity and electrical conductivity (EC) in sediment and water samples from post cultured contaminated shrimp ponds before and after treatment by the selected purple nonsulfur bacteria (PNSB).

Treatment	Water		Sediment	
	Salinity (%0)	EC (mS/cm)	Salinity (%0)	EC (mS/cm)
Before treatment	10.23 ± 0.78^{a}	4.68 ± 0.34^{a}	0.84 ± 0.05^{a}	1.27 ± 0.06^{a}
Abiotic	9.68 ± 0.13^{b}	4.71 ± 0.19^{a}	0.59 ± 0.05^{b}	1.37 ± 0.04^{b}
Native	9.02 ± 0.06^{c}	4.46 ± 0.10^{ab}	0.64 ± 0.05^{b}	1.25 ± 0.04^{a}
Sterile + NW16	7.32 ± 0.21^{d}	4.21 ± 0.28^{bc}	0.27 ± 0.02^{c}	1.04 ± 0.001^{c}
Native + NW16	5.17 ± 0.13^{e}	3.90 ± 0.23^{c}	0.14 ± 0.01^{d}	0.95 ± 0.002^{d}
Sterile + KMS24	6.78 ± 0.20^{f}	3.29 ± 0.25^{d}	0.35 ± 0.01^{e}	1.00 ± 0.013^{cd}
Native + KMS24	5.04 ± 0.13^{e}	2.82 ± 0.04^{e}	0.16 ± 0.03^{d}	0.72 ± 0.002^{e}
Sterile + mixed culture	2.69 ± 0.03^{g}	2.50 ± 0.01^{f}	0.01 ± 0.00^{f}	0.72 ± 0.001^{e}
Native + mixed culture	2.36 ± 0.05^{g}	2.4 ± 0.01^{f}	0.07 ± 0.02^{f}	0.68 ± 0.001^{e}

Values in the same column with different lower case letters indicate significant differences ($p < 0.05$).

Table 2. The removal percentage of Cu^{2+} and Zn^{2+} in the contaminated water from post cultured shrimp ponds by the selected purple nonsulfur bacteria under microaerobic-light and aerobic-dark conditions.

Treatment	% Removal			
	Cu^{2+} (initial concentration, 0.043 mg/L)		Zn^{2+} (initial concentration, 0.057 mg/L)	
	Light	Dark	Light	Dark
Control				
Abiotic	11.87 ± 6.05^{aA}	13.42 ± 1.02^{aA}	7.95 ± 0.89^{aA}	7.78 ± 2.09^{aA}
Native	10.40 ± 4.16^{aA}	11.14 ± 1.72^{bA}	6.44 ± 0.82^{aA}	6.41 ± 1.02^{aA}
NW16				
Sterile + NW16	35.29 ± 1.03^{bA}	40.62 ± 0.97^{cB}	18.60 ± 0.83^{bA}	22.52 ± 1.06^{bB}
Native + NW16	41.18 ± 1.26^{cA}	45.84 ± 1.26^{dB}	22.58 ± 1.11^{cA}	25.73 ± 0.82^{cB}
KMS24				
Sterile + KMS24	39.41 ± 1.41^{cA}	43.23 ± 0.91^{eB}	25.93 ± 1.04^{dA}	25.47 ± 0.74^{cA}
Native + KMS24	45.12 ± 0.96^{dA}	48.40 ± 0.86^{fB}	30.52 ± 1.77^{eA}	29.93 ± 1.38^{dA}
Mixed culture(NW16 + KMS24)				
Sterile + mixed culture	65.91 ± 1.50^{eA}	71.97 ± 1.65^{gB}	29.24 ± 1.77^{eB}	26.35 ± 0.72^{cA}
Native + mixed culture	73.78 ± 1.34^{fA}	75.92 ± 0.52^{hA}	30.40 ± 2.35^{eA}	31.67 ± 1.03^{eA}

Values in the same columns with different lower case letters indicate significant differences ($p<0.05$). different upper case letters in the same row indicate significant differences between light and dark conditions of each set ($p < 0.05$).

the effect of Ca^{2+} and Mg^{2+} had less effect on the efficiency of HMs removal by strain KMS24 itself (Figure 1 B). The removal percentages of Pb^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} by the pure culture KMS24 with its optimum conditions containing both light metal ions were 75, 62, 27 and 15, respectively but the mixed culture performed with a higher efficiency of 85, 74, 47 and 28%, respectively (Figure 1B). Therefore, the optimum condition for HMs removal by the strain KMS24 was applied for use in further experiments in the case of the mixed culture.

Removal of HMs and salts in the water collected from post cultured contaminated shrimp ponds

The water sample used in this study was a composite sample collected from various shrimp ponds contaminated with HMs as previously described and its physicochemical properties were as follows: salinity, 10.23‰; EC, 4.68 mS/cm (Table 1) and pH, 8.07. The composite sample had the following HMs in mg/L; < 0.001 Cd^{2+}, < 0.005 Pb^{2+}, 0.043 Cu^{2+}, and 0.057 Zn^{2+}. As previously stated, only the efficiency for removal of Cu^{2+}

Table 3. The removal percentage of Cd^{2+}, Pb^{2+}, Cu^{2+}, and Zn^{2+} in the contaminated sediment from shrimp ponds after harvesting by the selected purple nonsulfur bacteria under microaerobic-light and aerobic-dark conditions.

Treatment	% Removal							
	Cd^{2+} (0.29 mg/kg)		Pb^{2+} (23.15 mg/kg)		Cu^{2+}(15.05 mg/kg)		Zn^{2+}(22.16 mg/kg)	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Control								
Abiotic	20.2 ± 2.54^{aA}	18.92 ± 3.87^{aA}	21.49 ± 3.41^{aA}	19.64 ± 1.99^{aA}	20.67 ± 2.15^{aA}	20.52 ± 2.36^{aA}	17.48 ± 1.50^{aA}	19.06 ± 2.91^{aA}
Native	19.96 ± 3.19^{aB}	17.76 ± 2.47^{aA}	24.31 ± 5.22^{bA}	24.31 ± 4.22^{bA}	21.09 ± 1.79^{aA}	19.53 ± 2.70^{aA}	20.57 ± 1.40^{bA}	21.67 ± 2.16^{bA}
NW16								
Sterile + NW16	30.28 ± 0.77^{bA}	32.54 ± 0.54^{bB}	60.20 ± 1.99^{cA}	63.24 ± 0.99^{cB}	31.83 ± 0.64^{bA}	34.25 ± 0.65^{bB}	33.76 ± 0.80^{cdA}	35.63 ± 0.54^{dB}
Native + NW16	36.83 ± 1.08^{cA}	40.32 ± 1.07^{cB}	66.95 ± 1.41^{cA}	69.35 ± 0.72^{dB}	41.24 ± 0.68^{cA}	39.63 ± 1.09^{cA}	39.15 ± 0.98^{eA}	40.12 ± 0.90^{eA}
KMS24								
Sterile + KMS24	29.96 ± 1.08^{bA}	32.26 ± 0.49^{bB}	67.66 ± 1.27^{cB}	65.27 ± 1.08^{cdA}	35.28 ± 0.43^{dA}	39.74 ± 0.92^{cB}	30.48 ± 0.86^{cA}	34.82 ± 1.04^{dB}
Native + KMS24	39.34 ± 0.95^{dA}	40.19 ± 1.01^{cA}	71.01 ± 1.23^{dA}	70.97 ± 1.38^{dA}	41.72 ± 0.87^{eA}	45.89 ± 0.94^{dB}	35.40 ± 0.61^{dA}	37.75 ± 0.69^{deB}
Mixed of PNSB cells								
Sterile + Mixed	36.65 ± 1.56^{cA}	39.23 ± 1.22^{cA}	76.20 ± 1.81^{eA}	81.35 ± 2.91^{eB}	57.57 ± 1.02^{fA}	59.16 ± 1.62^{eA}	32.83 ± 2.60^{cdA}	30.02 ± 2.39^{cA}
Native + Mixed	39.80 ± 0.81^{dA}	43.33 ± 0.92^{dB}	78.48 ± 1.12^{eA}	84.29 ± 3.41^{fB}	60.30 ± 2.04^{gA}	62.52 ± 1.07^{fA}	35.48 ± 1.93^{dA}	40.95 ± 1.05^{eB}

The number in each bracket is the initial concentration of heavy metal ions; values in the same columns with different lowercase letters indicate significant differences ($p<0.05$). different upper case letters in the same row indicate significant differences between light and dark conditions of each set ($p < 0.05$).

and Zn^{2+} was studied. The removal of HMs and the control sets is presented in Table 2. There were no significant differences found for the removal of Cu^{2+} and Zn^{2+} under the two incubating conditions (microaerobic- light and aerobic-dark) between the abiotic and native control sets. In contrast, most treatment sets, more HMs was removed in the aerobic-dark than the microaerobic-light conditions although the differences may be not significant for some. The pure culture of KMS24 was performed with a significantly higher effi-ciency to remove both HMs ions than strain NW16 both in the presence and absence of native flora and with both incubating conditions. The presence of the native flora in all cases produced a significant increase in the removal of HMs. The most effective treatment for Cu^{2+} was observed with a mixed culture in the presence of the native flora that removed 73.78 and 75.92% under conditions of microaerobic-light and aerobic-dark, respectively. The mixed culture had a significant increased ability to remove Cu^{2+} from 48.40% for the pure KMS24 culture plus native flora to 75.92%, while the comparable increase for Zn^{2+} removal was from 29.93 to 31.67%, both with dark conditions. In all treatment sets as well as in both controls, salinity significantly decreased, parti-cularly those in a set of mixed culture either with sterile or native cultures (Table 1). However, based on the EC values, significant differences were observed only in the inoculated sets. The highest efficiency to reduce salinity and EC values (roughly 77 and 49%) were found in the mixed culture set as previously stated.

Removal of HMs and salts in the sediment collected from contaminated post cultured shrimp ponds

A composite sediment sample collected from shrimp ponds contaminated with HMs as previously described had the following physicochemical properties: 1.27 mS/cm EC, 0.84‰

Table 4. Germination index of rice and water spinach in sediment and water samples after treatment by the selected purple nonsulfur bacteria.

Treatment	Germination index (% GI)			
	Rice (*Oryza sativa*)		Water spinach (*Ipomoea aquatica*)	
	Water	Sediment	Water	Sediment
Control				
Abiotic	10.50 ± 0.59[a]	59.38 ± 1.32[a]	10.59 ± 0.59[a]	78.30 ± 1.62[a]
Native	15.44 ± 0.29[b]	70.04 ± 0.30[b]	13.41 ± 0.07[b]	92.38 ± 2.09[b]
NW16				
Sterile+NW16	20.47 ± 4.44[c]	80.57 ± 7.44[c]	21.75 ± 2.82[c]	114.83 ± 6.86[c]
Native+NW16	23.86 ± 2.26[c]	85.39 ± 6.29[c]	24.41 ± 1.06[c]	120.71 ± 4.13[cd]
KMS24				
Sterile + KMS24	21.46 ± 0.85[c]	79.41 ± 0.45[c]	22.16 ± 2.54[c]	115.34 ± 3.86[c]
Native + KMS24	24.03 ± 0.66[c]	83.35 ± 2.59[c]	26.91 ± 2.91[d]	126.90 ± 4.61[d]
Mixed of NW16 and KMS24 cells				
Sterile + Mixed	28.33 ± 1.28[d]	106.14 ± 6.56[d]	30.11 ± 3.17[e]	121.89 ± 2.83[cd]
Native + Mixed	34.50 ± 2.23[e]	115.70 ± 3.22[e]	35.29 ± 1.03[f]	139.33 ± 9.51[e]

Values in the same columns with different lower case letters indicate significant differences ($p < 0.05$).

salinity (Table 1) and pH 6.93 (data not shown). The amounts of HMs were in mg/kg dry weight; 0.29 Cd^{2+}, 23.15 Pb^{2+}, 15.05 Cu^{2+}, and 22.16 Zn^{2+}. The removal of HMs by the pure cultures of NW16 or KMS24 or their mixed culture with sterile sediment or non sterile (native) sediment under conditions of microaerobic-light and aerobic-dark is shown in Table 3. Comparing the removal percentages between the abiotic and native control sets under both incubating conditions, there was no significant difference for Cd^{2+} and Cu^{2+}, but a slight significant increase with the native flora was found for Pb^{2+} and Zn^{2+}. In all controls, the percentage removal from the sediment samples was higher than for the water samples especially for Zn^{2+} removal and this was reflected in the values for Zn^{2+} removal with the other pure and mixed cultures. The presence of the native flora with the pure or the mixed culture with both culture conditions caused a significant increase in the removal of all HMs. As was observed from the results with the contaminated water, strain KMS24 was mostly slightly better than strain NW16 at removing HMs (Table 2). Also, the best removal rate was for Pb^{2+} with 84.29% removed by the mixed culture plus native flora in dark-aerobic conditions while the corresponding figure with the same conditions for Cu^{2+}, Cd^{2+} and Zn^{2+} were 62.52, 43.33 and 40.95%, respectively (Table 3). Again the results were similar to those with the water samples, that is, the sediment samples, the efficiency to remove HMs in most treatment sets were higher in the aerobic-dark conditions than in the microaerobic-light conditions. A similar trend was observed in water and sediment samples after treatment as the salinity in all treatment sets and also in both controls were significantly reduced and again the most effective treatment was produced by a set of mixed culture either with sterile or native population (Tables 2 and 3). No significant difference was found for the EC values after treatment in a native control; however, the EC values were significantly reduced in all of the inoculated sets. As expected, the set of the mixed culture with or without native flora in the sediment samples also produced the best reduction of salinity and EC (100 and 45%), as shown in Table 1.

Toxicity assessment by seed germination for the sediment and water samples after treatment

The toxicity of water and sediment samples after treatment by pure cultures of NW16 or KMS24 or their mixed culture was assayed by seed germination and results are presented as a germination index in percent (% GI) with rice (*Oryza sativa*) and water spinach (*Ipomoea aquatic*) (Table 4). All samples from the contaminated water inhibited germination but the inhibition was considerably reduced after any treatment with the most significant reduction occurring after treatment with the mixed culture with the native population. Samples from the sediment before treatment also inhibited germination and again all treatments reduced this inhibition. Water contaminated with HMs after treatment was more toxic than treated sediment samples. Treatment with the mixed culture with and without native flora resulted in an increased % GI. There were no significant differences found between the sets of pure cultures (NW16 or KMS24), but together they did

have a synergistic effect. In all cases, the presence of the native population significantly increased the germination index up to 139.3% after treatment of the sediment samples with the mixed culture plus the native population. Rice was almost always more susceptible to inhibition than water spinach (Table 4).

DISCUSSION

Effect of Ca^{2+} and Mg^{2+} on removal of heavy metals by PNSB

In this study, biosorption by biomass of the selected PNSB strains (NW16 and KMS24) was used to remove HMs from a 3% NaCl synthetic solution (in the presence or absence of the light metal ions; Ca^{2+} and Mg^{2+}), and samples of water and sediment were collected from shrimp ponds contaminated with HMs. The exposure time for binding between HMs and cells was short between 30 and 45 min, so biosorption was likely to be the main mechanism for removing HMs (no energy requirement). This biosorption may require many passive processes such as adsorption, covalent bond formation, complexation, chelation, ion exchange and micro-precipitation (Panwichian et al., 2010b; Ahluwalia and Goyal, 2007). However, some bioaccumulation (requiring energy) of HMs by PNSB cells was possible (Panwichian et al., 2010b). Hence, with the use of both biosorption and bioaccumulation, one explanation for the mixed culture being more efficient than a pure culture might be that different organisms might use different processes resulting in a significant increase in the total removal of HMs when incubated together. The native population may also use alternative processes and might even facilitate both biosorption and bioaccumulation as well.

In general, co-ions interfere and reduce the biosorption capacity of another metal ion such as by competition to use the same processes such as competition for binding sites on the PNSB cells. In this present study, light metal ions, such as Na^{+}, Ca^{2+} and Mg^{+2}, were present in the synthetic solution and the experimental data showed that Ca^{2+} and Mg^{2+} light bivalent cations but not Na^{+} had a big effect on the biosorption of HMs (Figure 1). This indicated that the cell surface binding exhibits a low degree of specificity and similarly charged cations will also increase competition for binding sites on the cell surface. However, the results in this present study indicate that the use of a mixed culture of PNSB cells does have the potential to remove HMs in any of the conditions of the shrimp ponds.

Removal of HMs and salts in the water and sediment samples from post culturing contaminated shrimp ponds

This work was focused on investigating the possibility for removal of HMs and salts in samples collected from shrimp ponds by PNSB prior to testing their use in the field. The physicochemical properties of the composite post cultured water from contaminated shrimp ponds showed a pH of 8.07 (data not shown) and this may have an effect on the solubility of HMs as a high pH normally accelerates their precipitation (Gazso, 2001), particularly for Pb and Cd (< 5 µg/L Pb^{2+} and < 1 µg/L for Cd^{2+}). However, the amount of Cu and Zn ions were present at 43 and 57 µg/L, respectively and they exceeded the standard guidelines for marine aquatic animal cultivation (≤ 8 and ≤ 50µg/L for Cu and Zn) (Pollution Control Department, 2006). In addition, any HM accumulation in the food webs might have an adverse effect on human beings as previously described. Therefore, the HMs contaminated water was treated by the selected PNSB strains under microaerobic-light and aerobic-dark conditions. Fortunately, removal of HMs by PNSB cells did occur under both incubating conditions and in general, the conditions of aerobic-dark produced a better removal efficiency than microaerobic-light conditions. In general, actively growing PNSB cells have been used to treat various wastewaters including that in shrimp ponds (Watanabe et al., 2003). These results show that the application of PNSB cells to clean up water will rapidly remove HMs from both the sediment which has microaerobic-light conditions and in the water column with aerobic-dark conditions at night time. However, biosorption using the biomass of PNSB should be investigated under the conditions of aerobic-light as well, although the biosorption process did occur without requiring energy from the cells.

The results in Table 2 showed that removal of Cu^{2+} and Zn^{2+} in an abiotic control occurred (7.78 to 13.42%) in the water tested under both incubating conditions. This indicates the adsorption of HMs, either Cu^{2+} or Zn^{2+}, to non living organism or inorganic and organic matters in the water. It is well recognized that adsorption can remove metals over a wider range of pH values at lower concentrations, as in this present study than can be removed by alkaline precipitation (Baker and Khalili, 2004). In addition, biopolymers produced by micro-organisms also bind metals strongly (Iyer et al., 2005; Watanabe et al., 2003; Panwichian et al., 2011). However, in this present study there was no significant difference for the removal percentage of HMs by native and abiotic controls. It might be that in this case, biopolymers derived from native flora were not present. Results of this study indicated that the biomass of KMS24 might have a greater affinity for both HMs ions than the biomass of NW16 as it provided higher efficiencies under either a set of pure culture or with native flora. In addition to biosorption of HMs to cells, bioaccumulation of HMs into cells can occur as previously described. This was supported by the removal of Zn^{2+} under dark condition being significantly higher using a mixed culture (26.35%)

than found for NW16 (22.52%) but there was no significant difference with KMS24 (25.47%). This was due to the toxicity of Zn^{2+} on the strain NW16 being higher than the strain KMS24 and this result was in agreement with Panwichian et al. (2011). As each culture may have some different properties for binding or uptake of HMs including HMs tolerance; thereby synergistic removal of HMs was significantly increased with the mixed culture and again with higher efficiencies being obtained in the set with normal native flora.

Results in Table 3 demonstrated that contamination of HMs in the sediment collected from shrimp ponds was at acceptable levels for use as agricultural soil (≤1.5, ≤ 75, ≤ 65 and ≤ 200 mg/kg for Cd^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+}, respectively) (Pollution Control Department, 2004; HKGS, 1998) although, the sediment initially contained higher concentrations of HMs than that found in the water. The abiotic control in the sediment samples removed more HMs than found for the water samples (Tables 2 and 3) due perhaps to the sediment having more organic and inorganic matter including clay particles to bind HMs. Moreover, a higher concentration of HMs in the sediment could increase its removal efficiency as the uptake rate of the metals ions will increase along with its increasing initial concentration when the amount of adsorbent is constant (Wang and Chen, 2006). There was no significant difference found for the removal of Cd^{2+} and Cu^{2+} in both control sets (abiotic and native) but there was a higher significant removal of Pb^{2+} and Zn^{2+} found in the native control. In addition, results obtained from the pure culture sets and the culture of NW16 or KMS24 with native flora confirmed that native flora did increase the efficiency to remove HMs.

As previously described, the strain KMS24 was more resistant to Zn^{2+} in the water tested than strain NW16 and the experiments with the sediment samples also showed that the former strain was more resistant to Cu^{2+} (Tables 2 and 3). This can be explained using the same reasoning that was previously given in the water experiment as bioaccumulation could be involved and thus a pure culture of KMS24 produced a higher efficiency to remove Cu^{2+} in the sediment samples tested. Again, it is not surprising that the mixed culture of both PNSB strains had the highest efficiency to remove HMs from the sediment by a synergistic action as previously explained. Some difference was found for the removal efficiency of HMs from the synthetic solution ($Pb^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+}$) and the sediment samples ($Pb^{2+} > Cu^{2+} > Cd^{2+} \approx Zn^{2+}$) (Figure 1 and Table 3). There are many factors such as organic matter, soil particles like clay in the sediment samples that can affect removal efficiency when compared with the synthetic solution and this is why a higher efficiency was observed in the sediment samples because removal was by both biosorption and also adsorption. Furthermore, those factors also had an impact on the removal of Zn or Cd from the sediment samples.

No significant differences were observed in the sets of control in both incubating conditions; however, a higher efficiency to remove HMs from contaminated water and sediment samples by PNSB cells was observed in the aerobic-dark conditions than in the microaerobic-light conditions (Tables 2 and 3). The possible reason was due to the use of live PNSB cells that acted mainly as a biosorbent for the HMs ions with only a little by bioaccumulation because the contact time was only between 30 and 45 min (Panwichian et al., 2010b). As the binding of HMs to live cells may be toxic and possibly alter their surfaces and their metabolism thus it will affect the HMs removal by cells (Panwichian et al., 2011). In this case both PNSB cells were more sensitive to HMs ions in microaerobic-light conditions than those of aerobic-dark conditions (Tables 2 and 3) and the results were in accordance with Panwichian et al. (2011).

The results of this study indicated that samples of water and sediment from post cultured contaminated shrimp ponds after treatment by the selected mixed PNSB (NW16 and KMS24) in both incubating conditions showed a significant decrease in both salinity and EC values. This was supported by our previous study which reported both strains under conditions of aerobic-dark and microaerobic-light have the potential to remove sodium ion in amounts that were detected in shrimp ponds (Panwichian et al., 2010a). In addition, this study demonstrated that light metal ions (Ca^{2+} and Mg^{2+}) were competitors with HMs for binding with biomass (Figure 1). It is well recognized that the main salt composition of water in shrimp ponds is NaCl, $CaSO_4$ and $MgSO_4$ due to the brackish water used for shrimp cultivation (Panwichian et al., 2010b).

Toxicity assessment by seed germination for the sediment and water after treatment

The seed germination bioassay has been documented as one of the popular techniques for investigating the phytotoxicity of HMs (Ye et al., 2002) and the germination index (% GI) is regarded as the most sensitive parameter that is able to detect low toxicity that affects root growth and seed germination (Zucconi et al., 1981). Results of the germination of rice seed (*Oryza sativa*) and water spinach seed (*Ipomoea aquatic*) in treated water and sediment samples by all treatments was significantly higher than those in both the control sets (native > abiotic) and the % GI of both plants, rice and water spinach in the treated sediment was remarkably higher than that found in the treated water samples (Table 4). Biosorption of HMs and salts by PNSB strains as a pure culture or mixed culture either with native flora did not produce a significant decrease of their toxicity to plants and this corresponded with the removal percentage of HMs and salts.The results in this study demonstrated that in all treatment sets, particularly in a set of mixed culture

with/without native flora, salinity sharply decreased while there was a moderate decrease for the EC values (Table 1). This is because salinity is the saltiness or dissolved salt content (predominantly NaCl and followed by $CaSO_4$ and $MgSO_4$) of a body of water collected from post cultured shrimp ponds whereas EC is the total amount of dissolved ions in the water. As shown in this study, it seemed to be proper to use salinity for interpreting results of phytotoxicity test. It has long been known that increased salinity has an adverse effect on plant growth including halophytes (Khan et al. 2000). Hence, in order to explain why treated sediment had less toxicity to plants, there could be a number of reasons. First, contamination of HMs in the sediment was within the acceptance values for each HMs to allow plants to grow. Secondly, the salinity of the sediment samples (0.07‰) was much less than in the water (2.36‰). Moreover, the presence of higher amounts of nutrients in the sediment may enhance plant growth and help to reduce the toxicity of HMs and salts to both plants.

Conclusion

The presence of light metal ions, Ca^{2+} and Mg^{2+}, had a negative effect on the removal of HMs by biosorption. However, the use of a mixed culture of PNSB (NW16 and KMS24) demonstrated the potential for successful application for reducing HM levels. The results clearly indicated that the biosorption of HMs by the selected PNSB in the water or sediment samples collected from contaminated shrimp ponds after harvesting; particularly with the mixed culture alone or with native flora significantly decreased the toxicity of HMs and salts to plants as demonstrated by an increased GI values. However, toxicity was still found in the treated water but that could be caused by salinity. It is therefore suggested that the wastewater from post culturing shrimp ponds could be treated by bioremediation such as biosorption prior to discharge into the environment.

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Abbreviations

PNSB, Purple nonsulfur bacteria; **HMs,** heavy metals.

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