Bioremediation of heavy metal in crude oil contaminated soil using isolated Indigenous microorganism cultured with E coli DE3 BL21

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Abstract— **Soil contamination from crude oil have often been observed in recent years to increase the heavy metals and some hydrocarbon level in the environment from the soil to the plant and animals from the soil and hence the risk of bioaccumulation of this toxic compounds in the ecosystems which may threaten the human health in the endemic society. Bioremediation potency of individual indigenous bacteria isolated from soil polluted with crude oil was evaluated. Conventional method of identification was used to isolate and identify the indigenous microbes and the following were identified; Bacillus spp, Staphylococcus aureus, Micrococcus sp and Pseudomonas aeruginosa. The microbial accounts of total viable count after bio-augmentation 4.3×108, 2.7×108, 2×107 and 1.6×107 CFU g−1 for Pseudomonas aeruginosa, Bacillus spp, Micrococcus sp and Staphylococcus aureus respectively. Each microbe was bio-amplified in an improvised bioreactor containing nutrient broth and re-inoculated into a 20 gram of sterilized polluted soil with crude oil to ensure mono-bioremediation. The heavy metal analyses were carried out using AS machine in the space of 60 days. There was a significant different at a probability level 0.05 in the degree of bioremediation in all the treatment using t-test, comparing the Bio-Augmented Mechanic Site Sample + PET system and Bio-Augmented Mechanic Site Sample. PET System E. coli DE3 BL21 aided in a synergistic relationship with each selected bacteria to achieve remediation of the polluted soil which may be associated with natural gene sharing and protein amplification by the PET system. Moreover, the gene in each isolated indigenous bacteria encoding bioremediation should be excised and cultured with PET system (E. coli DE3 BL21). The proteins harvested may be used directly to study its bioremediation potentials**

Index Terms— **Bio-augmentation, heavy metal, bioremediation, indigenous microbes, PET System E. coli DE3 BL21.**

I. INTRODUCTION

Heavy metals are universally found in the soil, however, heavy metal contamination in a wide sort of source including industrial waste, refuse dump site and land filling, demolition site wastes, excavation of the soil and weathering of parent materials and oil spillage has been commonly dispersed around the ecosystem due to increase in human activities. The ubiquitous of heavy metals in the ecosystem has been supposed to be due to human and natural undertakings, some which are naturally occurring and are available in an insoluble form which is not readily available for plants or microbial uptakes however, the anthropogenic sources are present in soluble and mobile reactive forms [1]. Most of these heavy metals are recycled in the environment via transportation either release from gas exhausts due to complete or incomplete combustion of crude oil, leading to air pollution and or by oil spillage common via shipping of crude oil and during milling of the ore from the core leading to water pollution. Another common practise is the uncontrollable discharge of spent oil into the soil at the mechanics site and dung hills. This scenario has been reported to further leads to bioaccumulation in water bodies into the aquatic life which are eventually eaten by Man.

Furthermore the alteration of the heavy metals could affect the ecosystem microbiota due to many factor among which include the change in the pH of the soil. Only the acidophilic microbe survives low pH, neutrophilic in the neutral pH and alkalinophile in the basic pH respectively. It is worthwhile to know that most of the heavy metals are toxic to the microbial metabolism. The specific heavy metal toxicity has been studied and findings reveals that copper disrupt cellular function and inhibit enzyme activities [2], [3], Cadmium damage nucleic acid, denature protein, inhibit cell division and transcription, inhibits carbon and nitrogen mineralization [3], [4], lead denatures nucleic acid and protein, inhibits enzymes activities and transcription [3], [5], [6], zinc death, decrease in biomass, inhibits growth [7].

However, microorganism has also been studied to employ varieties of methods to remediate the soil from these heavy metals polluted environment. These include Biosorption, bioaccumulation, biotransformation, and biomineralization. Bioremediation is an innovative technique for the removal and recovery of heavy metal ions from polluted areas, and involves using living organisms to reduce and/or recover heavy metal pollutants into less hazardous forms, using the activities of algae, bacteria, fungi, or plants. It has been employed for the removal of heavy metals from contaminated wastewaters and soils. These organisms help to detoxify hazardous components in the environment. The process can function naturally or can be improved through the addition of electron acceptors, nutrients, or other factors [1]. Soil polluted with heavy metals has been studies worldwide using consortium and specific microbes, however little or no knowledge has been established about the individual potentials in synergy with PET (E. coli DE3 BL21) systems.

II. METHOD

COLLECTION OF MATERIALS

The crude oil contaminated with soil was collected from the soil at Igbokoda, Ilaje local government area Ondo State which has a characteristic of black colours due to oil spillage

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and the soil surface was hardened. Loamy soil was also collected from Rufus Giwa Polytechnic Farm site. The sample was packaged into a sterile polytene bag and was brought to the Science Laboratory Technology Department, at Rufus Giwa Polytechnic Owo for evaluation. The sample was stored at adequate temperature before experimental work.

PREPARATION OF SAMPLES

Preparation of soil sample

200g of the selected soil samples (crude oil contaminated soil, crude oil contaminated sample mixed with cowdungs at 50:50, and loamy/black soil) was weighed using analytical balance into three different containers. 500ml each of distilled water was measured and added into the different container containing the polluted soil and it was mixed vigorously.

ISOLATION OF BACTERIA FROM SOIL SAMPLE

A mixture of 10g of the polluted soil samples were measured into 90ml of sterile water aseptically to make slurry, a drop of 0.1ml of each soil sample slurry were aseptically injected into separate sterile Petri dishes. Sterilized Nutrient agar of about 5ml was poured on the top of the sample to isolate total *Pseudomonas* count, total micrococcus count, Tryptosol agar for total *Bacillus* count and Manitol salt agar for total *Staphylococcus* count. The plates were incubated for 24hours at 37^0C in an inverted position.

IDENTIFICATION OF BACTERIAL ISOLATES

The bacterial isolates were identified and classified using a combination of the methods as recommended by [8], [9], [10], [11], [12]. The identification of bacterial was based on cultural, morphological characteristics, staining properties, sugar fermentation and biochemical characteristics.

MAINTENANCE OF STOCK CULTURE

The pure culture were inoculated into sterile agar slant in McCartney bottles and incubated at 37^0C for 48hours in other to maintain growth of microorganism, the stock cultures were transferred aseptically by sub-culturing from old slants to freshly prepared plates and incubated at 37^0C for 24hours.

BIOSTIMULATION OF ISOLATED MICROBE

Two litres nutrient broth was used to stimulate the growth of each microorganism isolated from the soil polluted with crude oil. The soil samples polluted with crude oil was sterilized to kill all the living soil microorganisms. 20g of the sterilize soil sample was then mixed with each isolated microorganism respectively in 2 litres nutrient broth to form a slurry in a constructed bioreactor [13]. Each soil microorganism was allowed to colonize the soil polluted with crude oil and the heavy metals were monitored. Each isolated microbe was inoculated with PET system *E.coli* DE3BL21 strains in a closed system. The soil sample slurry containing broth was digested with acid H_2SO_4 for heavy metal quantification analysis using A.A.S (Atomic Absorption Spectrophotometer) machine before, during and after the treatment. Treatment of metal by bacterial cells was calculated as the ratio of ions removal.

% R (%)=(A-B)/A × 100

Where is the R=Removal ratio (%); A=Concentration of heavy metal ions in the primary solution (µg mL^{-1}) and B=Concentration of heavy metal ions in the treated solution (µg mL^{-1}) [14].

III. RESULTS

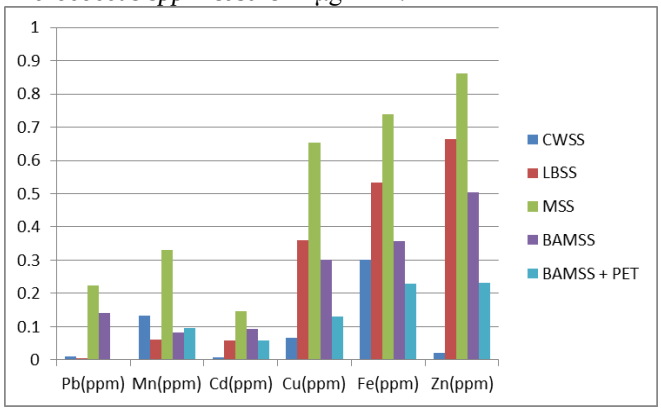
Table 1: Morphology, staining, biochemical and sugar fermentation properties of bacteria isolate from crude oil polluted soil on various selective and general purpose agars

Isolates/ Morphology	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Agar	Nutrient Agar	Tryptose Soy Agar	Nutrient Agar	Mannitol Salt Agar
Shape	Rod	Rod	Cocci	Cocci
Colour	Green	Cream	yellow	White
Margin	Entire	Entire	Entire	Flat
Consistency	Butyrous	Dry	Slimy	Dry
Opacity	Opaque	Opaque	Transparent	Opaque
Gram staining	-ve	+ve	+ve	+ve
Spore staining	-ve	+ve	-ve	-ve
Capsule staining	-ve	-ve	-ve	+ve
Catalase	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	-ve
Glucose	+ve	+ve	+ve	+ve
Lactose	-ve	-ve	-ve	+ve
Maltose	-ve	+ve	+ve	+ve
Fructose	-ve	+ve	+ve	+ve
Sucrose	-ve	+ve	-ve	+ve
Probable organisms	***Pseudomonas spp.***	***Bacillus spp.***	***Micrococcus spp.***	***Staphylococcus spp.***

Table 2: Total viable count of bio-augmented indigenous bacteria isolated from soil polluted with crude oil

Isolates	TVC CFU/g
Pseudomonas spp.	4.3×10^8
Bacillus spp.	2.7×10^8
Micrococcus spp.	2×10^7
Staphylococcus spp.	1.6×10^7

Figure 1: Heavy metal quantity of three selected soil treatment (loamy soil, mechanic site soil and bio-augmented mechanic site) that is inoculated/bio-stimulated with *Micrococcus spp* measure in µg mL^{-1}.



CWSS: Control Without Soil Sample, **BAMSS**: Bio-Augmented Mechanic Site Sample, **MSS:** Mechanic Site Sample, **LBSS: Loamy/Black Soil Sample, BAMSS+PET**: Bio-Augmented Mechanic Site Sample + PET SYSTEM

Figure 2: Heavy metal quantity of three selected soil treatment (loamy soil, mechanic site soil and bio-augmented mechanic site) that is inoculated/bio-stimulated with *Bacillus* spp *measure* in µg mL^{-1}.

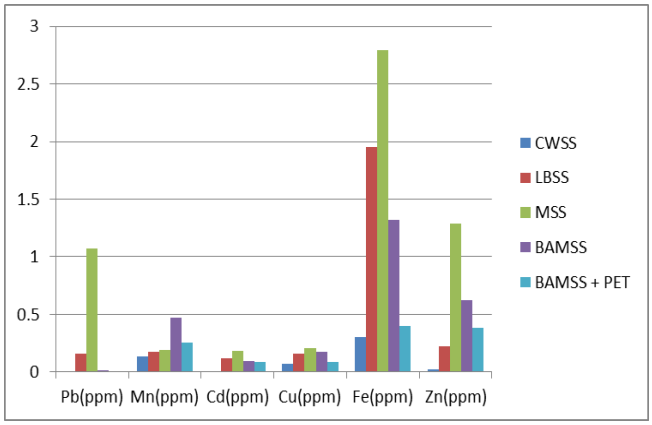


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Figure 3: Heavy metal quantity of three selected soil treatment (loamy soil, mechanic site soil and bio-augmented mechanic site) that is inoculated/bio-stimulated with *Pseudomonas aeruginosa* measure in µg mL^{-1}.

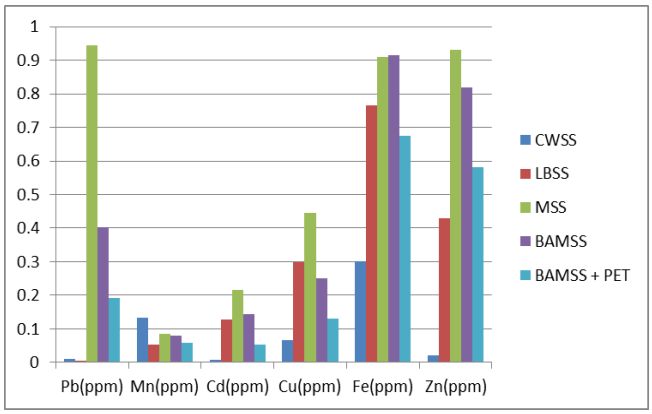

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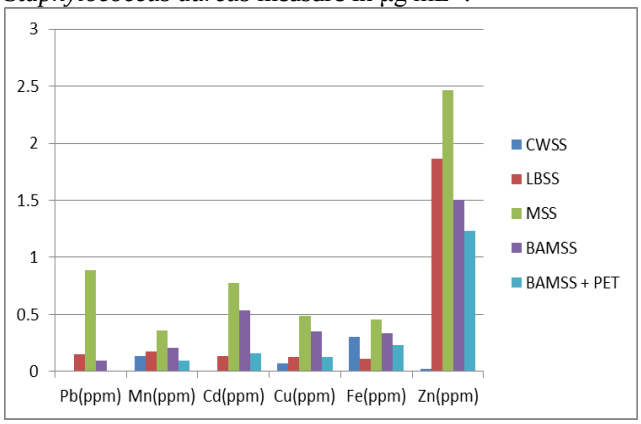
Figure 4: Heavy metal quantity of three selected soil treatment (loamy soil, mechanic site soil and bio-augmented mechanic site) that is inoculated/bio-stimulated with *Staphylococcus aureus* measure in µg mL^{-1}.



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IV. DISCUSSION

The figure 1 above shows the heavy metal quantity of three selected soil treatment (loamy soil, mechanic site soil and bio-augmented mechanic site) The loamy soil as a positive control shows the least contamination with Lead, Manganese, Cadmium, and Copper, however iron and zinc were a bit high in there composition. In the three experimental soil treatment, iron, zinc, copper, manganese were very low while cadmium and lead where under revealing limit. This finding is in line with the findings of [15], which used worms to bio-remediate soil polluted with diesel. Bacteria has proven to have a great biosorbents potentials due to their ubiquity, scope, skill to propagate in an optimize environment [16].

After 60 days of bio-augmentation of indigenous bacteria isolated from each experimental soil sample, most of the heavy metal recorded drastic reduction in the quantity.

Micrococcus spp shows a significant reduction in all the selected heavy metals in figure 1. Micrococcus has been shown to have a high sorption capacity on heavy metals such as lead and copper [17], *Bacillus* was very effective against lead, iron and zinc in figure 2, while *Pseudomonas aeruginosa* was more effective in the bioremediation of lead, copper, zinc and cadmium in figure 3. The ability of *Bacillus* and *Pseudomonas aeruginosa* to bioremediate soil polluted with hydrocarbon could be associated with the presence of gene responsible for alkane monoxygenase enzymes which is involve in the n alkane oxidation [18], [19]. *Pseudomonas* sp has also been studied to comprises of various plasmid capable of bioremediation among which include pDTG1 and pND15 [20], [21]. *Pseudomonas*, *Bacillus*, and *Micrococcus* species has been studied to show excellent sorption capacity and has been associated to their high surface-to-volume ratios and their numerous potential active chemosorption sites, such as the teichoic acid on the cell wall [22].

Staphylococcus aureus, though not necessarily a soil flora, showed a notable bioremediation effect on lead, cadmium and zinc (Figure 4). Ahamed [23] has reported isolation of *Staphylococcus aureus* as one of the petroleum hydrocarbon degrading microbes from soil and water samples of ship-breaking yards at Vatiary and Kumira coast in Chittagong. The ability of *Staphylococcus aureus* to grow on culture containing kerosene, diesel and engine oil as carbon source according to the observation of Ahamed [23] shows that the bacteria possess mechanism to utilize and breaks down the compounds. He further noted that the dominant species among the bacteria that grows on culture embedded with kerosene, diesel engine oil belonged to *Bacillus* followed by *Pseudomonas* and *Staphylococcus* species [23], hence our findings is in line with his own.

Furthermore, the introduction of PET system, E. coli DE3 BL21 cultured in a LB medium shows a significant rate of enhanced bioremediation in all the selected bacteria. This is not a common practice; however this could be as a result of natural sharing of plasmid and genes, between each of the selected bacteria and the PET system (E. coli DE3 BL21). The PET system has been known to be designed to be competent enough to integrate foreign genes in its self and produce up to 80% of the proteins for the incorporated genes. Figure 2 shows great results in the use of bio-augmented *Micrococcus spp* shows great result in lead (Pb), however the value is higher than the acceptable value by [24] of 0.05 µg mL^{-1} and the guidelines for drinking water (WHO, 2004)

concentration of 0.01 μg mL^{-1}. However the combination with PET System shows a further drastic reduction in the lead concentration below and within the acceptable value. Similar trend was also observed with Cadmium (Cd), since the bio-augmented *Micrococcus spp* reduced the value from 0.146 to 0.093 and with the introduction of the PET system; it was further reduced to 0.057 which is within the acceptable value for USEPA [24] of 0.05 μg mL^{-1}. Bio-augmented *Bacillus* sp in figure 3 shows great results in lead (Pb), with value of 0.013 and in synergy with the PET system, it further reduce to 0.003 both of which are within the acceptable specification by USEPA [24] of 0.05 μg mL^{-1}. The value for cadmium was also recorded to be close within the acceptable specification in both soil treatments with bio-augmentation and introduction of PET system. *Pseudomonas* shows a good trend in reduction both in bio-augumented and the introduction of PET system, however the values obtain in lead (Pb) were both higher than the specification. Nevertheless, with the elongation of period from 60 days to 120 days, reduction to specification range may be achievable.

Figure 3 and 4 also follows same trend with *Pseudomonas aeruginosa* showing great impact in the reduction of all the selected heavy metals. The bio-augmented *Staphylococcus aureus* displays high potential of bioremediation, reducing lead from 0.887 μg mL^{-1} to 0.093 μg mL^{-1} as shown **in** figure 4, a value close to the specification of acceptable range in the soil. The introduction of PET system further led to the reduction to lead. However cadmium was a little bit recalcitrant to remediation. All the soil treatment shows that the soil was not really polluted with copper since the values were below and within the acceptable value by USEPA [24] of 1 μg mL^{-1} and 2 μg mL^{-1} guidelines for drinking water (WHO, 2004).

V. CONCLUSION AND RECOMMENDATION

Bio-augmentation is a potentially positive method to enhance indigenous microbes of soil polluted with crude oil to remediate the environments. PET System *E. coli* DE3 BL21 also aided in a synergistic relationship with each selected bacteria to achieve remediation of the polluted soil. However, the gene in each isolated indigenous bacteria encoding bioremediation should be excised and cultured with PET system (*E. coli* DE3 BL21). The proteins harvested may be used directly to study its bioremediation potentials.

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REFERENCE

[1] Ayangbenro, A. S. and Babalola, O. O. (2017) A new strategy for heavy metal polluted environments: a review of microbial biosorbents *Int. J. Environ. Res. Public Health* 14, 94; 1-16 doi:10.3390/ijerph14010094

[2] Dixit, R., Malaviya, D., Pandiyan, K., Singh, .B., Sahu, A., Shukla, R., Singh, B.P., Rai, J.P., Sharma, P.K. and Lade, H. (2015) Bioremediation of heavy metals from soil and aquatic environment: An overview of principles and criteria of fundamental processes. Sustainability, 7, 2189–2212.

[3] Fashola, M., Ngole-Jeme, V., Babalola, O. (2016**)** Heavy metal pollution from gold mines: Environmental effects and bacterial strategies for resistance. *Int. J. Environ. Res. Public Health*, 13, 1047.

[4] Sankarammal, M. and Thatheyus, A (**2014**) Ramya, D. Bioremoval of cadmium using *Pseudomonas fluorescens*. *Open J. Water Pollut. Treat*., 1, 92–100.

[5] Wuana, R.A. and Okieimen, F.E. (2011) Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. ISRN Ecol., 2011, 1–20.

[6] Mupa, M. (2013) Lead content of lichens in metropolitan Harare, Zimbabwe: Air quality and health risk implications. *Greener J. Environ. Manag. Public Saf*., 2, 75–82

[7] Gumpu, M.B., Sethuraman, S., Krishnan, U.M. and Rayappan, J.B.B. (**2015**) A review on detection of heavy metal ions in water—An electrochemical approach. *Sens. Actuators B Chem*, 213, 515–533

[8] Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's Manual of Determination Bacteriology. 9th edn, Williams and Wilkins, Baltimore, USA.

[9] Prescott, Harley, and Klein, (1995).Microbiology.Wm. C. Brown publishers, United States of America.

[10] Singleton, P. (1997): Bacteria in Biology, Biotechnology & Medicine. 4th Edition P. 324-338 John Wiley & Sons Ltd., Chichester ISBN 0-471-97468-4.

[11] Cheesbrough (2006): District Laboratory Practice in Tropical Countries Part 2. 13:48-49,

[12] Oyeleke, S B. and Manga, B. S. (2008): Essentials of Laboratory Practicals in Microbiology Pp 63-65.

[13] Sinha, A., Pant, K.K. and Khare, S.K. (2012,) Studies on mercury bioremediation by alginate immobilized mercury tolerant *Bacillus cereus* cells. *Int. Biodeterior. Biodegrad*. 71, 1–8.

[14] Qin, Y., Shi, B. and Liu, J. (2006) Application of chitosan and alginate in treating waste water containing heavy metal ions. *J Chem Technol* 13: 464-469.

[15] Ekperusi O. A. and Aigbodion I. F. (2015) Bioremediation of heavy metals and petroleum hydrocarbons in diesel contaminated soil with the earthworm: *Eudrilus eugeniae SpringerPlus 4:540 1-13*

[16] Wang, J. and Chen, C. (**2009**) Biosorbents for heavy metals removal and their future. *Biotechnol. Adv*., 27, 195–226.

[17] Puyen, Z.M., Villagrasa, E., Maldonado, J., Diestra, E., Esteve, I. and Solé, A. (**2012**) Biosorption of lead and copper by heavy-metal tolerant *Micrococcus luteus* de2008. *Bioresour. Technol*., 126, 233–237.

[18] Whyte, L., Bourbonniere, L. and Greer, C. (1997) Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (*alk*) and naphthalene (*nah*) catabolic pathways. *Appl. Environ. Microbiol*. 63: 3719-3723.

[19] Feng, L., Wang, W., Cheng, J., Ren, Y., Zhao, G., Gao, C., Tang, Y., Liu, X., Han, W., Peng, X., Liu, R. and Wang, L. (2007) Genome and proteome of long chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. *Proc. Natl. Acad. Sci. U.S.A*. 104: 5602-5607.

[20], Dennis, J. J. and Zylstra, G. J. (2004) Complete sequence and genetic organization of pDTG1, the 83 kilobase naphthalene degradation plasmid from *Pseudomonas putida* strain NCIB 9816-4. *J. Mol. Biol*. 341:753-768.

[21]. Subba, R. M., Kishore, G. and Rambabu, C. (2010) Plasmid mediated Naphthalene Degradation in *Pseudomonas* sp. strain NGK 1. *Curr. Trends Biotechnol. Pharm*. 4: 702-707.

[22]. Mosa, K.A., Saadoun, I., Kumar, K., Helmy, M., Dhankher, O. P. (2016) Potential biotechnological strategies for the cleanup of heavy metals and metalloids. *Front. Plant Sci.*, 7, 1–14.

[23] Ahmed, R. Z., Ahmed, N. and Gadd, G. M. (2010) Isolation of two *Kocuria* species capable of growing on various polycyclic aromatic hydrocarbons. *Afr. J. Biotechnol*. 9: 3611-3617.

[24] US-EPA (Environmental Protection Agency) (1995) Vascular plants as indicators of Prairie wetland integrity.

[25] WHO (2004) Guidelines for Drinking Water Quality. 3rd edn, World Health Organization, Geneva, Switzerland.