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Full Length Research Paper

Responses of selected biota after biostimulation of a vegetable oil spill in the Con Joubert Bird Sanctuary wetland: A pilot study

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An investigation on the effect of a vegetable oil spill was conducted on the biological diversity of the Con Joubert Bird Sanctuary wetland in South Africa before and after biostimulation with different concentrations of fertilizer during 2008. Biostimulation responses were analyzed 30 days after different concentrations of fertilizer were applied to the freshwater wetland at three selected sampling sites. The Con Joubert Bird Sanctuary wetland showed a high degree of contamination after a vegetable oil spill, resulting in a large volume of vegetable oil in the sediment and water column, respectively. Vegetable oil contents differed at each sampling site before biostimulation and each site showed variable responses after biostimulation. In this study, biostimulation results displayed a high yield of microbial activity and vegetable oil degradation at site one and two respectively. However, the degradation of the high vegetable oil concentrations within the sediments at sampling site 3 may have been hampered or retarded by the polymerized state of the vegetable oil. The phytoplankton, protozoan, macroinvertebrates and microorganisms assemblage were affected and showed little improvement at site 3, even after biostimulation with the high fertilizer concentration of 800 g/m^2, in comparison to sites 1 and 2 which showed greater biological activities and degradation of vegetable oil.

Key words: Biostimulation, vegetable oil spill, fresh water wetland.

INTRODUCTION

Con Joubert Bird Sanctuary wetland can be classified as a freshwater wetland with a transitional open water zone (Morant, 1983). The water budget of the wetland is governed by evaporation, precipitation and the inflow of storm water inlets making the wetland a low-energy budget wetland with reduced flushing especially during the dry season. This wetland served as habitat for 250 different bird species, which amongst nearly 400 Greater flamingos. In September 2007, a spill of 250 ton vegetable oil occurred in a nearby vegetable oil production facility when an old vegetable oil storage tank collapsed. The spilled vegetable oil followed the stormwater drains into the adjacent Con Joubert Bird Sanctuary wetland area. Inflatable booms were used to isolate the contaminated area of vegetation from the open water zone to prevent further contamination while free oil between reeds and vegetation on the eastern side of the wetland was collected by means of absorbent material and inflatable booms (Oberholster et al., 2010).

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Vegetable oils lack the acute toxicity components that are present in petroleum and its refinery products such as aromatic and hydrocarbons due to long persistence in the sediment (Li et al., 2005; Mueller et al., 2003). However, non petroleum products, including vegetable oils and animal fats, share common physical properties in water and produce similar environmental effects (US EPA. 1997), such as insolubility, low density, low specific gravity and high viscosity relative to water (Campo et al., 2007). These behavioral characteristics of oils in water lead to the formation of an oily layer on the surface of the water that results in a decrease in dissolved oxygen and increase in biochemical oxygen demand (BOD). This depletion of dissolved oxygen negatively impacts aquatic ecosystems, which is exacerbated by high BOD when microbial biodegradation occurred (Groenewold et al., 1982). The extent of the damage to the aquatic system is dependent on the chemical composition of the oil (Poulton et al., 1997). From the literature, it is evident that spilled vegetable oil has a huge impact on sensitive aquatic organisms and wild fauna such as birds (Mudge, 1995; Bucas and Saliot, 2002), and the immediate effects include coating of bird feathers and animals' fur (US EPA, 1997).

Vegetable oil degradation and reduction of toxicity can be achieved through bioremediation through the addition of organic nutrients to a wetland. Conventional methods such as excavation and mechanical removal of oil from the water often increases ecological damage by destroying vegetation and natural wetland biota habitat (Mueller et al., 2003). Bioremediation, on the other hand increase microbial activity with little physical/mechanical impact on the treated area (Shuhong et al., 2006; Venosa et al., 1996). Balba et al. (1998) and Shuhong et al. (2006) demonstrated that bioremediation is a cost-effective and environmentally friendly methodology depending on the optimal and limiting factors required in the freshwater wetland's ecosystem. However, little is known about the effects of biostimulation on protozoa, macroinvertebrates and phytoplankton as well as the bacteria assemblage.

Most of the microorganisms responsible for vegetable oil degradation in freshwater sediment are anaerobic bacteria (Pereira et al., 2002; Li et al., 2006). However, extremely high concentrations of vegetable oil present in the sediment may reduce biodegradation and this could be relieved by the presence of ferric hydroxide (Li et al., 2005). According to Mudge (1999) and Li et al. (2005) vegetable oils are transformed to methane and carbon dioxide by a complex of microbial consortia involving several groups of microorganisms. The ester bonds of vegetable oil can be hydrolyzed to yield free fatty acids and glycerol. Hydrolysis of vegetable oil is initiated by enzymatic activity of triglycerides to glycerol and long fatty acids, which serves as the growth substrates for several members of the microbial consortia (Aluyor et al., 2009; Dutta et al., 2009).

Bioremediation however, can also have a negative impact on a targeted area since results is dependent upon prevailing environmental physical/chemical status as the addition of organic nutrients to on already autotrophic environment could further damage the wetland (Mueller et al., 2003). The addition of nutrients would cause an increment of primary producers such as algae and other phytoplankton that would produce high concentrations of nuisance toxic compounds to macro invertebrate and vertebrate species (Oberholster et al., 2010).

Wetland restoration is defined as the returning of pre-disturbed wetland conditions and efforts to restore the biodiversity and biological functioning of the ecosystem (Winter, 1988). There is limited information available on the impact of vegetable oils spilled on freshwater wetlands and most of the studies on abiotic and biotic factors that control freshwater ecosystem responses to vegetable oil contamination come from marine environments (Poulton et al., 1997). Because of the difficulties associated with restoration and the lack of pre-spill data on the wetland (Oberholster et al., 2010), we decided to conduct a pilot study before full-scale bioremediation of the wetland. A pilot-study presents a series of steps to be followed that assists in designing a monitoring program to be undertaken in wetlands before full-scale restoration. The objectives of this pilot-scale study were: (1) to determine the applicability of biostimulation to the contaminated freshwater wetland by adding organic fertilizer to the wetland and (2) to determine the effect and responses of biostimulation on aquatic organisms such as phytoplankton, macroinvertebrates, protozoan and microbial assemblage within the water column and sediment in each of the selected sites before and after biostimulation.

MATERIALS AND METHODS

Study area description

This study was conducted on the Con Joubert Sanctuary Bird area (24.69 ha) located near Randfontein South Africa. The 12.96 ha wetland has a maximum depth of 1.2 m (during the raining season) and is dominated by the following emergent macrophytes vegetation (*Typha capensis*. *Schoenoplectus brachyceras*, *Phragmites australis*, *P. mauritianus* and *Persicaria lapathifolia*) and free-floating plants (*Azolla pinnata*. *Spirodela* spp. and *Wolffia arrhiza*).

Site selection for pilot study

Due to the lack of an uncontaminated pre-spill site or pre-pilot data, it was difficult to correlate data generated from this study with a particular reference site. However, three experimental sites (each approximately 2 m^2 in extent) were selected in the most highly contaminated areas where different concentrations of oil were measured during diagnostic risk assessment (Oberholster et al., 2010). These sites are located in close proximity to the storm water inlet into the freshwater wetlands (Figure 1) and their respective

Figure 1. Aerial photograph of Con Joubert Bird Sanctuary wetland study area including the three (1 to 3) sampling sites. Site 1 = 26° 11' 13" S. 27° 41' 16" E; site 2 = 26° 11' 13" S. 27 ° 41' 19" E and site 3 = 26 ° 11' 17" S. 27 ° 41' 18" E.

locations are as follows: Site 1 is located at 26° 11' 13" S. 27° 41' 16" E; site 2 (26° 11' 13" S. 27°41' 19" E) and Site 3 (26° 11' 17" S. 27° 41' 18" E). Samples were collected from the sediment and water column at these sites.

Biostimulation of the wetland

Data obtained for oil concentrations and the presence of microbial organisms within the sediment and water column before and after biostimulation were compared for each site to determine the degree to which vegetable oil degradation had occurred. The concentrations of vegetable oil, redox potential (Eh), biochemical oxygen demand

(BOD), total nitrogen (TN), total phosphorous (TP), dissolved oxygen (DO), and pH were measured before biostimulation and one month after biostimulation with three different concentrations of a slow-release fertilizer in relationship with the low or high concentration of vegetable oil according to Oberholster et al. (2010). The fertilizer used during the study had the following nutrient content ratio: (3:1:5) 87.0 g/kg N: 29.0 g/kg P; 144.0 g/kg K 3:1:5. The fertilizer was added to the respective sites as follows: Site 1 (200 g/m^2); site 2 (400 g/m^2); and site 3 (800 g/m^2). Pre-biostimulation data generated at each site was used as control for biostimulation responses.

Physical and chemical parameters

Integrated water column samples collected at site(s) 1 to 3 were used to assess the extent of oil concentrations as well as the overall water quality through chemical analyses before and after biostimulation. Sediment samples were collected according to Oberholster et al. (2005) at all three sites with a sediment core (5 cm in diameter) to investigate the spatial extent of sediment contamination before and after biostimulation with organic fertilizer over a period of one month. Physical and chemical parameters such as phosphates and nitrates were performed to determine the condition of the wetland using classical spectrophotometric methods (American Public Health Association, American Water Work Association and Water Pollution Control Federation, 1989). Other *in situ* water quality parameters such as temperature, pH, DO and conductivity were measured with a Hach™ sension 156 portable multiparameter (Loveland. CO. USA).

Chlorophyll measurements

Chlorophyll (chl) as surrogate of phytoplankton biomass was measured using sub-samples (50 ml) of the water column sample (collected at the surface and at depths of 0.5 meters and 1.0 meters) of each site before and after biostimulation. Chlorophyll was extracted using 80% acetone and left overnight for incubation. The chlorophyll *a* and *b* content were determined spectrophotometrically at 664 and 647 nm wavelengths respectively according to Porra et al. (1989). PerkinElmer™ Lambda 25 spectrophotometer was used for absorbance determination.

Sampling methods of biota

Macroinvertebrates

Sampling of macroinvertebrates at the three selected sample sites was conducted using a hand net (300 x 300 mm frame 1000 µm mesh). All available biotopes were identified according to Minnesota Pollution and Control Agency (2008) as well as by de Klerk and Wepener (2011) and sampled at the sampling sites before and after biostimulation. Loose substratum was agitated by kicking to dislodge organisms which were collected in the net. Aquatic and marginal vegetation was swept with the net (for 2 m^2); while sand and mud were agitated by kicking and swept for 30 s. A random sampling procedure was used to reduce hydrobiological variability between sites (Voeltz and Ward 1991). Samples were immediately preserved in 70% ethanol and later washed through a 75 µm mesh sieve to remove fine particles. The samples were then sorted and identified according to Merrit and Cummins (1996) to the lowest possible taxonomic category under an Olympus dissection microscope. The collected organisms were identified to the appropriate taxonomic level (mostly to family level, except for Oligochaeta, Hydrachnellae, Amphipoda and Porifera for which a higher taxonomical level was used). Sorting continued until atleast 300 individuals were counted or the entire sample was sorted. Invertebrate diversity was calculated using the Shannon's diversity index (Shannon, 1948).

Phytobenthos and phytoplankton sampling

For sampling of phytobenthos, a corer was used (diameter 5 cm) after which the sediment water was passed through a 75 µm mesh sieve and later the sample was fixed with buffered 5% (v/v) formaldehyde for determination of phytobenthos composition, community structure and identification of the algal species present. A total of a 100 ml of each of the samples were concentrated in chambers and analyzed under an inverted microscope at 1250 x magnification using the strip-count method (American Public Health Association, 1989). Diatoms were identified after clearing in acid persulfate. The biovolumes of the more abundant taxa were estimated by measuring cell dimensions of at least 20 individuals and using the closest geometric formulae (Willen, 1976). Integrated water column samples from the surface up to 1 m depth of the littoral zone were collected at each of the sampling sites as previously described (Oberholster et al., 2010). The duplicate samples were preserved in the field by addition of 5% formaldehyde to a final concentration of 2.5%. All identifications were made according to Van Vuuren et al. (2006) and Taylor et al. (2007). The total number of phytoplankton and phytobenthos taxa and their frequency of occurrence at each sampling site were categorized according to Hörnström (1999): 1 ≤ 250. 2 = 251-1000. 3 = 1001-5000. 4 = 5001-25 000 cells l^{-1}. The Berger-Parker dominance index (Berger and Parker 1970) was used to measure the evenness or dominance of organisms at each site. In all cases, samples were collected in triplicate and subsequently processed.

Protozoa

A 100 ml sub-sample of the integrated water column phytobenthos sample (5 L) was used for determination of protozoa assemblage before and after biostimulation. Protozoa assemblage was determined and quantified using the live counting technique at 400 x or 1250 x magnification with a light microscope Olympus™ BX40 and identification was based on the quantitative protocol (QPS) method of Montagnes and Lynn (1987a, b) and Skibbe (1994). Triplicate samples were taken and subsequently processed.

Denaturing gradient gel electrophoresis (DGGE)

DNA extraction

Bacterial genomic DNA was extracted from the integrated water column and core sediment samples from each of the three sampling sites before and after biostimulation. The samples were kept at 4°C before genomic DNA extraction was performed. A ZR Soil Microbe DNA Kit™ (Zymo Research CORP) was used to extract the genomic DNA from both water (1 ml) and sediment (0.5 g) samples according to manufacturer's description. DNA concentrations were quantified using a NanoDrop® ND-100 spectrophotometer. Samples were analyzed using a 1% agorose gel (v/v) and electrophoresed in 1×TAE buffer (Tris-acetate-EDTA) at 80 volts. The DNA was visualized by staining with GoldView™ Nucleic Acid illuminated under UV light and photographed on a UVP image system.

Polymerase chain reaction (PCR) amplification

PCR products obtained from all sites (1 to 3) were analyzed on

DGGE according to Muyzer (1999) as modified by Surridge (2007). The thermal cycling protocol was included with an initial denaturation at 96°C for 5 min, followed by 35 cycles. Each cycle began with 30 s at 94°C followed at annealing temperature at 58°C for the 16S rDNA DGGE primer pair (PRUN518r-ATTACCGCGGCTGCTGG (Muyzer, 1999); pA8f-GC-AGAGTTTGATCCTGGCTCAG [Fjellbirkeland et al., 2001]; then an elongation step of 1 min at 72°C was performed. The amplification reactions contained a 10 × amplifications buffer with 1.5 mM $MgCL_2$. 0.2 mM dNTPs. 20 pmol of each primer and 1 unit Taq DNA polymerase. and 3 to 5 ng purified DNA in the final volume of 25 µl reaction. The cycles were followed by incubation at 4°C. The PCR products were again analyzed using agarose gel electrophoresis to verify the fragment size before DGGE.

After excision of PCR fragments from the DGGE gels, the PCR products were cleaned through ethanol precipitation and cloned (pGEM-T Easy Vector. Promega) before bidirectional sequencing (ABI BigDye v3.1. System, Applied Biosystems) by Inqaba Biotech (Pretoria, South Africa). Putative sequence identities were obtained with BLAST (Altschul et al., 1990; 1997) analysis against the non-redundant Genbank database (NCBI. http://www.ncbi.nlm.nih.gov/).

All phylogenetic analyses were done with PAUP 4.0b10 (Swofford, 2002) and Bayesian analysis with MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Heulsenbeck, 2003). Multiple sequence alignments were done with Clustal W version 2 (Larkin et al., 2007) and manually evaluated before further analysis. Ambiguous characters and uninformative nucleotides were excluded from data prior to analysis and all characters were re-weighted to the consistency index. Heuristic searches using random sequence additions were preformed with the tree-bisection-reconnection (TBR) branch-swapping algorithm and MaxTrees set to auto increase. Phylogenetic signal that is consistency index (CI) and retention index (RI) was assessed by evaluating the tree length distributions in each dataset after 100 random generated trees. Only groups with a 70% or more support were retained in bootstrap analyses over a 1.000 replicates.

Statistical analysis of data

All data were recorded on standard Excel spreadsheets for subsequent processing and the statistical analysis was conducted using the SYSTAT® 7.0.1 software package (SYSTAT, 1997). Statistical differences were analyzed through a *t* test using the Sigma Plot (Jandel Scientific) program. Values of $p \leq 0.05$ were regarded as significant in the study.

To determine changes in the biotic (macroinvertebrates, algae and protozoa) community compositions on a temporal and spatial scale, the most appropriate univariate and multivariate statistical analyses were used. Univariate analysis such as diversity was used to describe macroinvertebrate species-abundance relations with the help of the software program PRIMER version 6.0 (Clarke and Gorley, 2006). This included the use of the Shannon diversity index (*H'*) (Shannon, 1948). Multivariate analysis was used to express the results of the diversity and abundance of the different biota as an ordination pattern with the different water quality parameters overlaid which resulted in the placements of the respective sampling sites (before and after stimulation) reflecting certain (dis) similarities between each other (Shaw, 2003). In an ordination plot, the arrows point in the direction of the steepest increase, whilst the angles are used to indicate correlation between variables. These types of ordination plots are called Redundancy Analysis (RDA) plots and are derived from Principle Component Analysis (PCA) plots, but the values used in the analysis are the best-fit data that is estimated from multiple linear regressions between each variable in turn and a second matrix of environmental data. Hence, an RDA was used to determine the relationship between biotic community structures and selected environmental variables with the help of the software program CANOCO version 4.5 (Ter Braak and Šmilauer. 2002).

RESULTS

Physical and chemical conditions of the wetland

The chl *a* increase was highest at sampling site 2 after applying 400 g/m^2 of fertilizer as compared to site 1 (200 g/m^2) and site 3 (800 g/m^2). The most polluted site amongst the three sites was sampling site 3 with a vegetable oil content of 20.970 mg/kg in the sediment before and 20.985 mg/kg after biostimulation in the sediment (Table 1). An increase in TP and TN concentrations was observed after stimulation (Table 1). Similarly, an increase in chlorophyll *a* and BOD levels was also noticed at all three sites after stimulation. From Table 1, it can be seen that the oil concentrations decreased in the sediment and water column at site1 and site 2, whilst the oil concentration in both the sediment and water remained similar after stimulation at site 3.

Response of biota after biostimilation

Phytoplankton and phytobenthos

Phytoplankton species diversity recorded at all 3 sites under investigation prior to biostimulation showed low diversity before and after biostimulation in both water column and sediment (Figure 2). Four phytoplankton divisions were the main representatives after vegetable oil contamination. The diatom *craticula ambique* has been dominant at site 2 and site 3 before biostumilation, respectively. The diatom species *Criticula ambigue* were the most dominant (Berger and Parker Index. 0.413; 0.361; 0.211) of the algal Class Bacillariophyceae at all three chosen sites after biostimulation. High numbers of filamentous cyanobacteria *Oscillatoria princep* (1001 to 5000 cells/l) and the green algal *Chlamydomonas africana* appeared to increase in biomass at site 3. At site 2, there were significant increases in the biovolume (from 8.3 mm^3/l. 1 001 cells/l to 15 mm^3/l. 1 001 to 5 000 cells/l) of *Chlamydomonas africana* after biostimulation despite the low water column temperature (11 ± 2°C). The expression of phytoplankton biomass by average chl *a* (average 3.7µg/l) remained relatively low at all sites before biostimulation in comparison with levels (average of 27 µg/l) after biostimulation. After biostimulation, site 2 had the highest content of chl *a* (39 µg/l). Figure 2 shows diverse phytoplankton assemblage among the 3 sampling sites before and after biostimulation.

Protozoa

Before biostimulation, the observed water column protozoa at all sampling sites were low in numbers (50 to

Table 1. Average physical and chemical parameters before and after biostimulation data analyses**.**

Chemical parameter	Before biostimulation			After biostimulation		
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
DO water column (mg/l)	0.51 ± 0.014	0.63 ± 0.031	0.31 ± 0.470	0.72 ± 0.071	0.83 ± 0.005	0.54 ± 0.110
pH sediment	6.26 ± 0.11	6.39 ± 0.23	6.10 ± 0.90	6.26 ± 1.94	7.63 ± 0.12	6.10 ± 0.98
pH water column	7.42 ± 0.2	7.46 ± 0.31	7.11 ± 0.16	7.49 ± 0.41	7.48 ± 0.28	7.21 ± 0.30
Conductivity (µS/cm)	259 ± 21	298 ± 34	278 ± 28	241 ± 18	274 ± 41	261 ± 61
Chlorophyll *a* (µg/l)	3.9 ± 0.10	3.3 ± 0.11	3.1 ± 0.09	28 ± 9	39 ± 8	31 ± 5
Oil sediment 5 cm deep (mg/kg)	1.295 ± 211	2.900 ± 334	20.970 ± 421	450 ± 63	160 ± 11	20.985 ± 2.177
Oil in water column (mg/l)	30 ± 2	90 ± 6	60 ± 16	10 ± 4	19 ± 11	58 ± 2
Total phosphorus (µg/l)	1.800 ± 310	2.200 ± 416	1.200 ± 110	12.600 ± 421	16.500 ± 1.100	20.000 ± 428
Total nitrogen (µg/l)	5.800 ± 210	4.700 ± 661	789.200 ± 510	12.600 ± 900	19.200 ± 2.100	269.000 ± 43.700
BOD	25.1 ± 3	25.7 ± 8	24.8 ± 7	41.2 ± 5	48.0 ± 3	27.6 ± 11
Eh	-189 ± 18	-164 ± 24	-263 ± 39	-179 ± 31	-170 ± 24	-291 ± 28
Temperature (°C)	11.5 ± 2.0	12.3 ± 3.0	13.6 ± 4.0	11.1 ± 2.0	11.7 ± 4.0	12.3 ± 2.0

100 specimens/L) and consisted mainly of large ciliate species such as *Pseudomicrothorax agilis*, *Paramecium caudatum* and *Didinium nastum* (Figure 3). However, after biostimulation the total numbers of smaller protozoa taxa and their corresponding frequency at site 2 increased. At site 1, the percentage protozoa taxa were very low before and after biostimilation.

Macroinvertebrates

The highest numbers of macroinvertebrate families (eight and six) occurred at site 2 before and after biostimulation. An average of four families was accounted for at sites 1 and 3 before biostumulation. In these families, the dominant class in Annelida was Oligochaeta, which constituted around 50% of the total species at all the sites (Table 2). Diptera was another order that were well represented with families such as Chironomidae and Culicidae present at sites 1 and 3 before biostimilation. However, the numbers of these families decreased after biostimilation at both sites.

Influence of water quality parameters on the selected biotic community structures

Based on the RDA triplot (Figure 4), distinct differences can be seen between the respective sites before and after stimulation. This triplot described 93.9% of the variation in the data with 86.7% described on the first axis and 7.2% on the second axis. At site 1, for example, a decrease in more tolerant invertebrates (for example, Hirudinea and Chironomidae) (Figure 4) and an increase in the overall invertebrate diversity at site 1 (Figure 5) was seen after stimulation when compared to the same site prior to stimulation. The overall diversity of invertebrates at site 2 remained relatively similar before and after stimulation (H' = 1.70 and H' = 1.69 respectively), whilst the diversity at site 3 increased (H' = 0.89 and H' = 1.43 respectively) (Figure 5). Phytoplankton diversity increased at site 1 after stimulation (from H' = 2.04 to H' = 2.27); whilst the diversity at sites 2 and 3 remained relatively similar before (H' = 2.15 and H' = 2.43, respectively) and after stimulation (H' = 2.01 and H' = 2.45, respectively) (Figure 5). In contrast to the trend noticed with the macroinvertebrates and phytoplankton assemblages, no difference in protozoan diversities was noticed at site 1 after stimulation. Site 3 also showed no change in protozoan diversity (from H' = 0.67 to H' = *0*.68), whilst the protozoan diversity increased at site 2 after stimulation (from H' = 0 to H' = *0*.69). When relating these biotic changes to water quality changes (Figures 4 and 5), it can be noticed that the changes in TP and TN concentrations after stimulation affected the chlorophyll *a* and BOD levels.

Table 2. Macroinvertebrate families and orders before (180 days after oil spill) and after (211 days after oil spill) biostimulation.

Macroinvertebrate families and order	180 days after oil spill (*n* = 3)			211 days after oil spill (*n* = 3)		
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
Annelida						
Oligochaeta	0	5	0	0	1	4
Hirudinea	1	0	0	1	0	0
Coleoptera						
Hydraenidae	1	0	0	1	0	0
Hydrophilidae	0	0	0	0	0	0
Diptera						
Chironomidae	10	2	5	6	1	1
Culicidae	8	0	41	4	7	0
Psychodidae	0	0	4	0	4	1
Muscidae	0	0	7	2	3	0
Syrphidae	0	0	0		2	2
Blephariceridae	0	0	0	0	0	1
Odonata						
Gomphidae	0	1	0	0	1	0
Trichoptera						
Ecnomidae	0	1	0	0	0	0
Hemiptera						
Corixidae	0	1	0	0	0	0
Ephemeroptera						
Baetidae	0	1	0	0	0	0
Mollusca						
Planorbidae	0	0	0	0	0	0

Orders = bold; families = standard; n = sampling trips.

Changes in oil concentrations within the sediment and water were also noticed after stimulation. All of these changes influenced the different biotic community structures due to two of the three biotic components were observed to have a noticeable change after stimulation at sites 1 and 2. This is in contrast to site 3 where only the macroinvertebrates showed a change after stimulation.

PCR-DGGE analysis for microbial diversity

The integrated water and sediment samples were analyzed on the 1% agarose gel and also on DGGE to determine dissociation patterns of microbial diversity biomass (Figures S1. S2). Figure 6 shows species simple matching based on the DGGE band pattern similarity before and after biostimulation. The microbial composition in the sediment was highest at site 2 and the lowest at site 3 (Figures 6, S2). The microbial composition increased after biostimulation (Figure S2). Analysis of DGGE banding pattern using simple matching also assisted in determining diversity and phylogenetic affiliation of predominant bacterial consortia probably responsible for vegetable oil degradation. Figure 7 shows a schematic representation of the 16S rDNA gene neighbour-joining relationships tree depicting the phylogenetic relationship of the sequences of microbial assemblages in the respective sampling sites before and after biostimulation. From the 11 DGGE profiles analyzed, four were known cultured bacteria (that is., *Pseudomonas* sp. S1007, *Pseudomonas* sp KBOS, *Saccharopolspora halophila* str. YIM 90500 and *Streptomycetaceae* bacterium) six unknown and one known (*Pseudonocardiaceae* bacterium) uncultured bacteria (Figure 7). Unknown DGGE fragment (29) showed no significant nucleotide similarity on GenBank to known bacterial species, however still grouped with DGGE fragments 7, 8, 10 and 11. These microbes were grouped according to similarities in their genes which

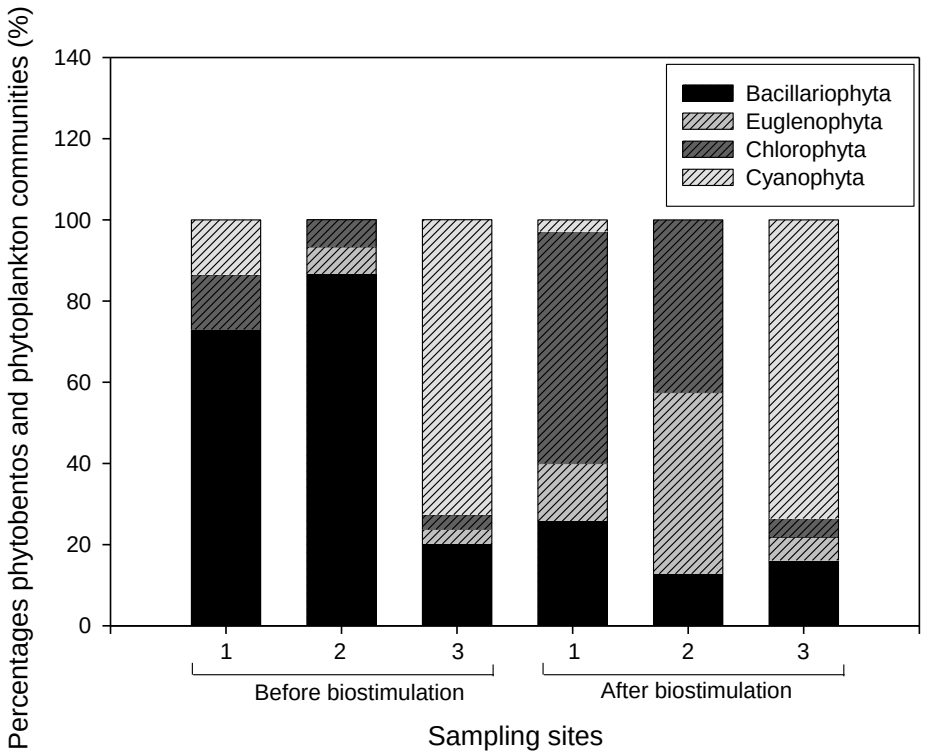

Figure 2. Percentage of phytobenthos and phytoplankton communities before and after biostimulation.

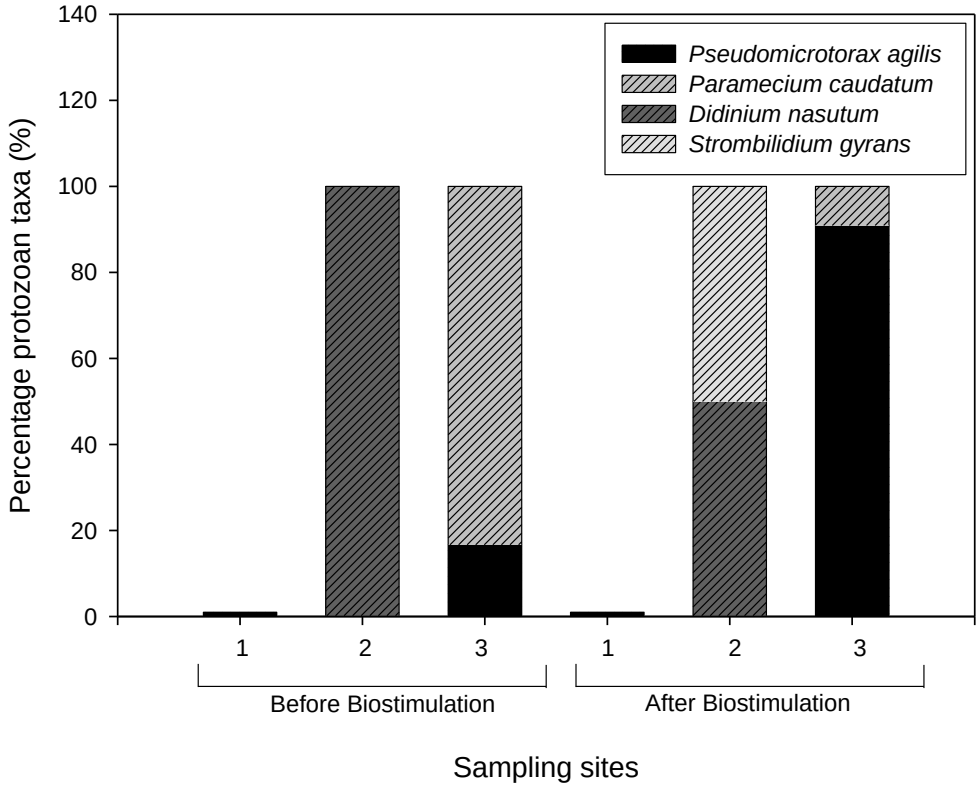

Figure 3. Percentage of protozoa species composition before and after biostimulation.

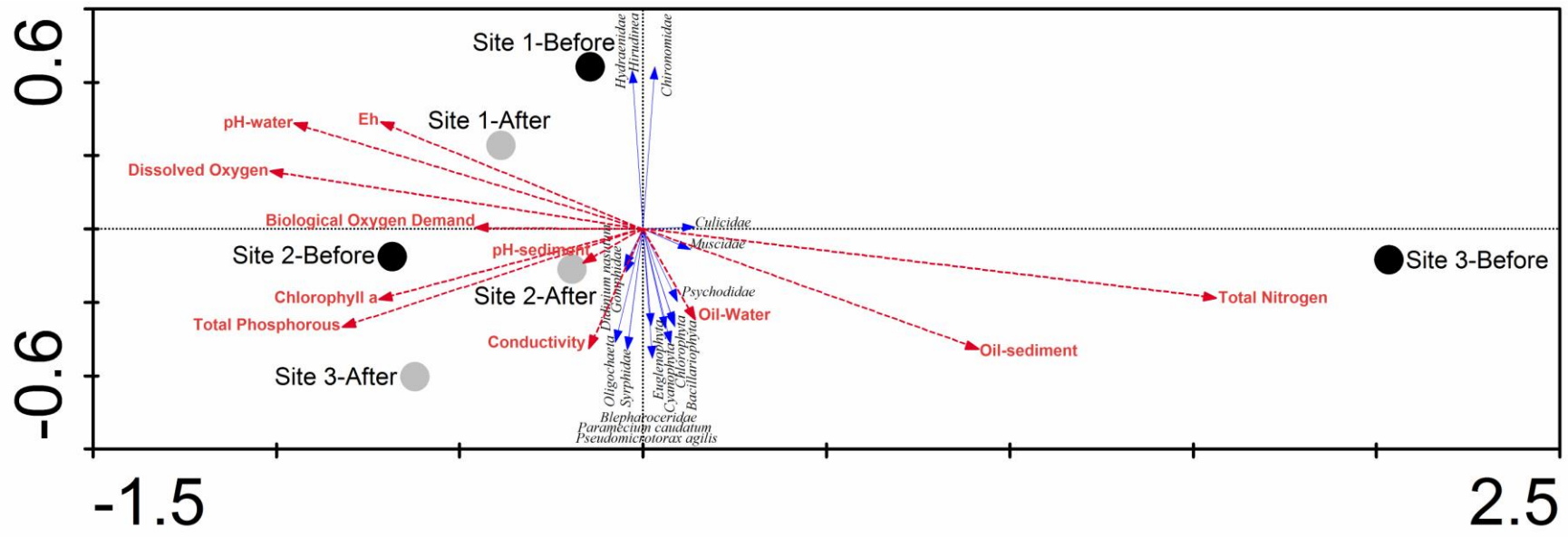

Figure 4. An RDA plot showing the (dis)similarity between the different sampling sites before and after stimulation based on the macroinvertebrate freshwater algae and protozoan abundance data with water quality variables superimposed.

reflect their evolutionary relationships (Muyzer, 1999).

DISCUSSION

Application of a nutrient rich fertilizer was found to significantly increase the biomass of microbes. Despite the observed increase in microbial activity at sites 1 and 2, a similar tendency was not observed at site 3. There may be two explanations for this, firstly, the degradation of the high vegetable oil concentrations within the sediment at sampling site 3 may have been hampered or retarded by the polymerized state of the vegetable oil as this site was severely contaminated (Table 1). In such instance, the vegetable oil was exposed to low energy and an oxygenated water environment that created an anoxic aquatic habitat and decreased microbial activity (US EPA 1997). Secondly, the application of fertilizer at a concentration of 800 g/m^2 exceeded the threshold value of phosphorous and nitrate for the biota and hampered their proliferation. The phytoplankton assemblages, protozoan and microorganisms were affected and showed little improvement at site 3 even after biostimulation with fertilizer concentration of 800 g/m^2 in comparison to sites 1 and 2 which showed great biological activity.

The addition of the organic fertilizer altered the physicochemical conditions within the water column and sediment (Table 1 and Figure 5); for example, there was an increase in TP and TN concentrations after stimulation, whilst pH levels remained relatively similar at all of the sites. US EPA (1997) reported that factors such as pH, temperature, dissolved oxygen, chemical contents, sediment characteristics, nutrient quantities and microbial consortia at the spill site profoundly influenced the degradation of oil. Although sites 1 and 2 showed high content values of vegetable oil in both water column and sediment, they showed high microbial activity after the biostimulation. The higher BOD values at the selected sites after biostimulation were possibly due to vegetable oil biodegradation by the increase in microbial activity (Table 1 and Figure 5). The wetland already showed signs of eutrophication (Table 1) which implies that the addition of fertilizer could cause further depletion

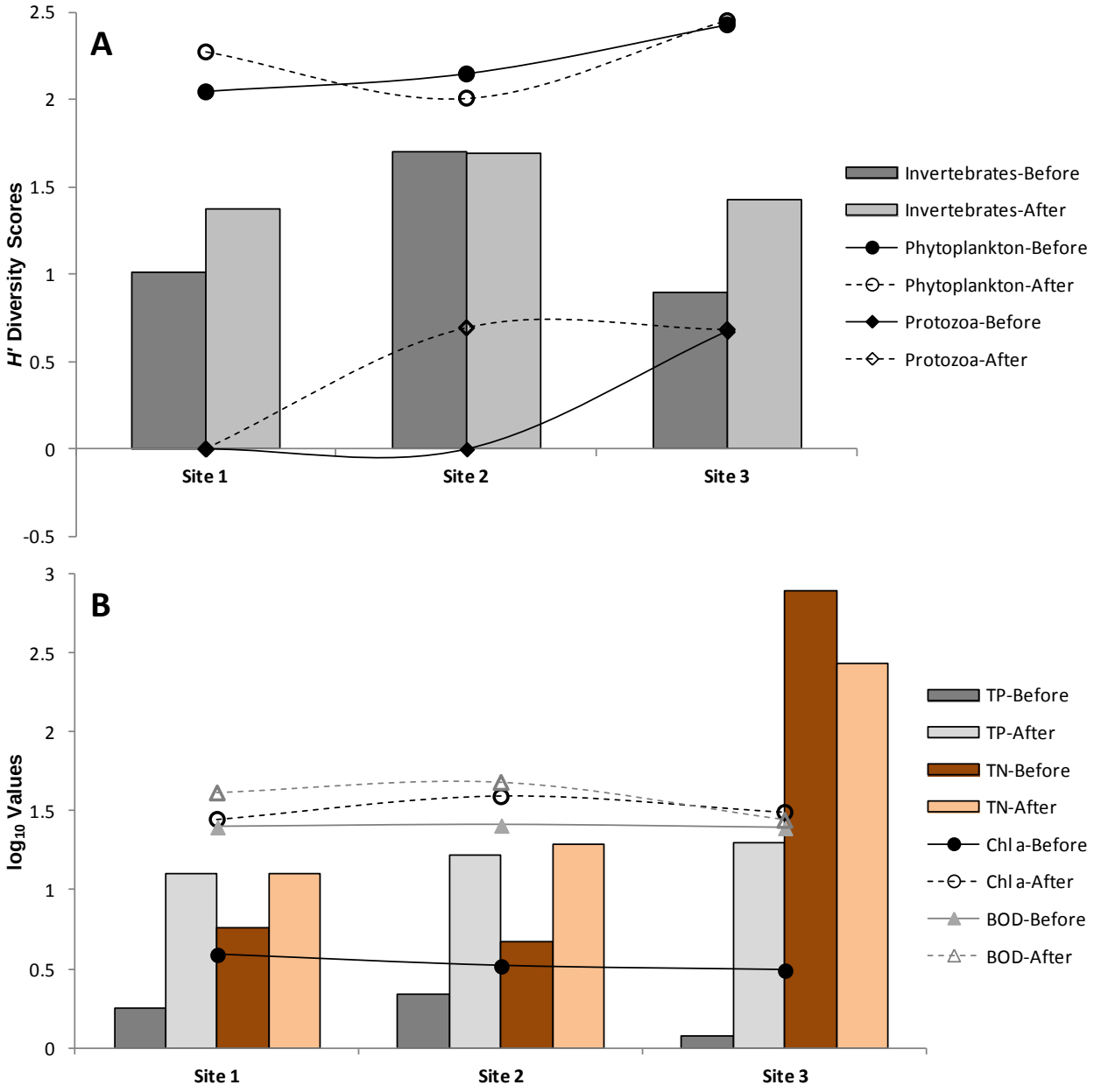

Figure 5. The spatial and temporal (before and after stimulation) changes observed for a variety of different endpoints namely changes in the diversity of the macroinvertebrate. freshwater algae and protozoan communities. **(A)** The changes in total phosphorous (TP) total nitrogen (TN) and chlorophyll a concentrationsas well as the biological oxygen demand (BOD) values after being log10 transformed. **(B).** The changes in the oil concentrations within the sediment and water after being log10 transformed **(C)**.

of the wetland ecosystem. The algae divisions *Bacillario-phyta*. *Chlorophyta* and *Cyanophyta* and *Euglenophyta* were the main algal representative in the wetlands due to their ability to thrive under highly impacted environments with enrichment of primarily phosphorus and nitrate (Wahby and El-Moneim, 1979). An increase in the number of *Chlamydomonas* species at site 2 may be due to the light conditions caused by the water surface oil, limiting light penetration. The high numbers of the *Chlamydomonas* species also might have led to an increase of phagotrophy (that is, species that depend more on ingested bacteria than on photosynthesis) (Oberholster et al., 2010). An increase in phytoplankton biomass as chl *a* after biostimulation at the different study sites might cause a species shift to noxious phytoplankton blooms (for example, blue green algae)

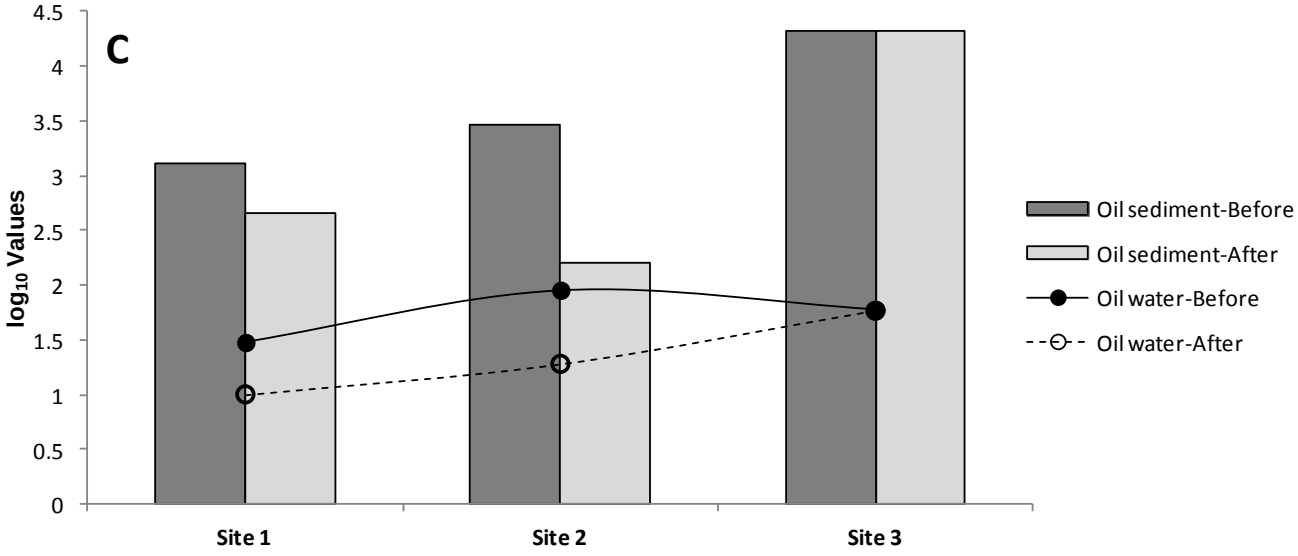

Figure 5. Continued.

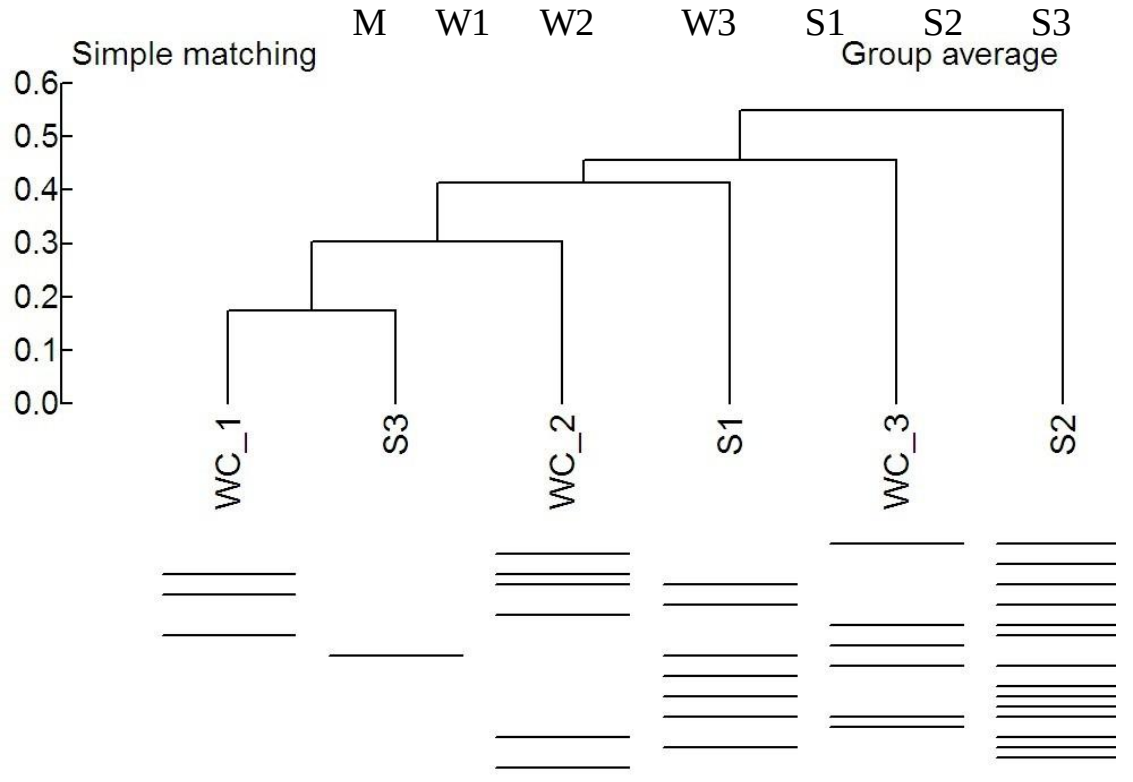

Figure 6. Species simple matching based on the DGGE band pattern similarity before and after biostimulation. WC1, water column of site 1; WC2, water column of site 2; WC3, water column of site 3; S1, sediment of site1; S2, sediment. site 2; S3, sediment. site 3.

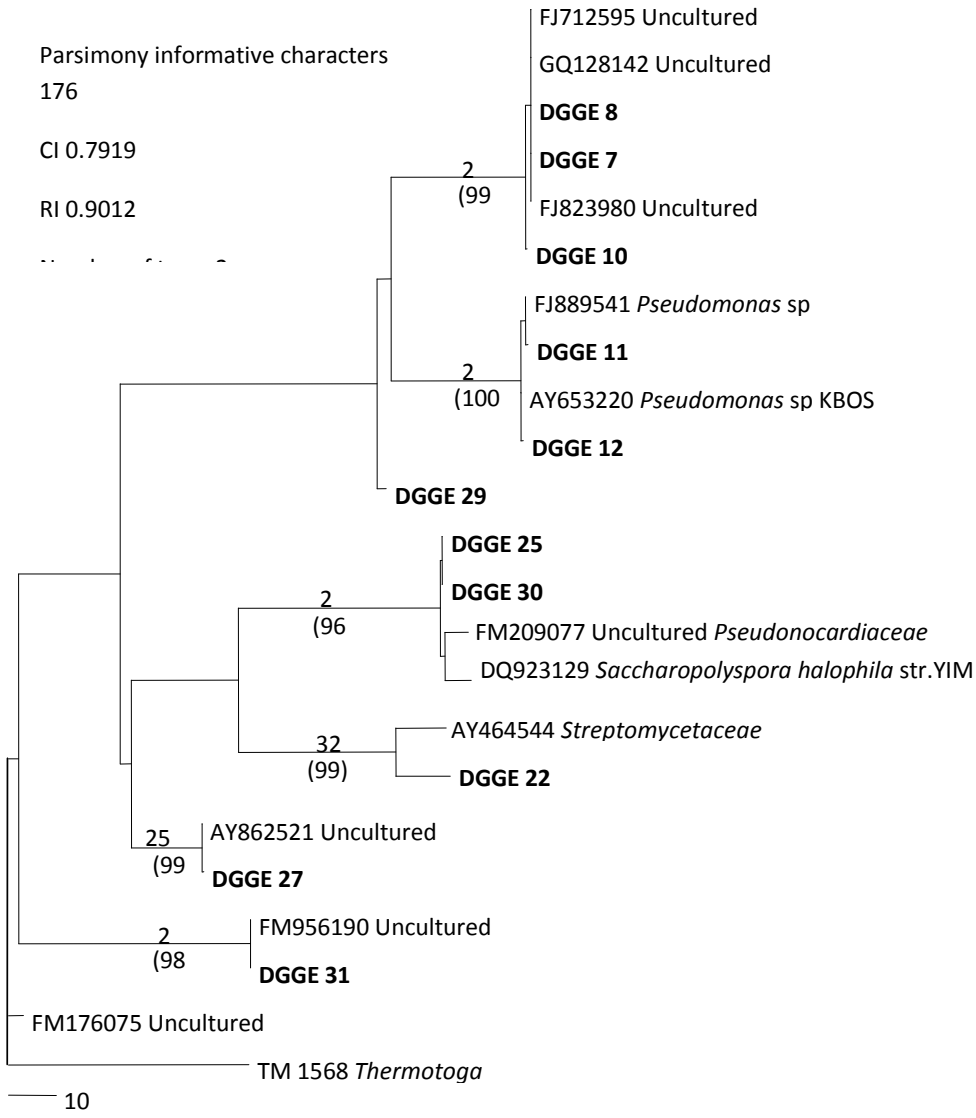

Figure 7. 16S rDNA gene neighbour-joining tree showing the phylogenetic relationship among the microbial amplicon (DGGE and amplicon number) from the three sampling sites obtained in pilot study before and after biostimulation. The species names are preceded by GenBank accession numbers and sequence identity average value of ≥98%. Thermotoga maritime in the main branch was used as supporting branch for the tree. The scale bar represents the changes per nucleotide position.

that could be difficult to reverse.

Protozoa are mostly known as bacterivores (that is, consumers of bacteria) (Kalff, 2002). The larger protozoa such as *Pseudomicrothorax agilis*, *Paramecium caudatum* and *Didinium nasutum* which were present in the water column before biostimulation are likely to be bacterivores and mostly consume algae or other protozoa (Figure 3). According to the study of Fenchel (1988), each species of suspension-feeding ciliate tends to ingest a distinct size-spectrum of particles, related to the form and function of their oral apparatus and this spectrum generally shifts in larger ciliates towards larger particles, that is, algae and other protozoa. However, the low frequency of small ciliates at site 1 and 3 can also be indicators of the absence of bacteria at this site after biostimulation (Figure 6). In contrast, the increase in protozoans at site 2 may be an indication of the increase in bacteria after stimulation at this site.

At site 1, an increase in pollutant tolerant macro invertabrates such as Chironomidae and Hirudinea (Figure 4) were noticed prior to stimulation (Camur-Elipek et al., 2010). This is because of the fact that early changes

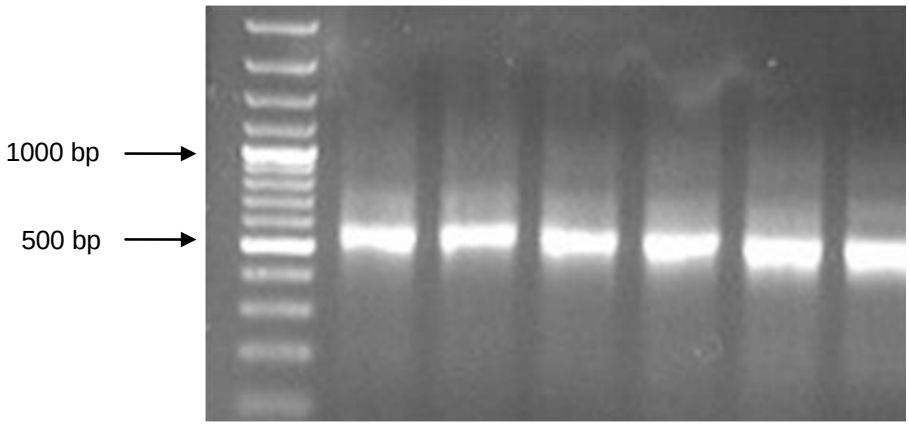

Figure S1 . PCR product of 16S rDNA primers obtained from three sites using genomic DNA from both sediment and water column microorganisms. The gel was loaded as follows: lane M (molecular marker); Lane, W1 (water, site 1); Lane, W2 (water, site 2); lane, W3 (water, site 3); lane S1 (sediment, site1); lane S2 (sediment, site 2); lane S3 (sediment, site 3). Each band on the gel represents lots of species diversity in a particular sampling site.

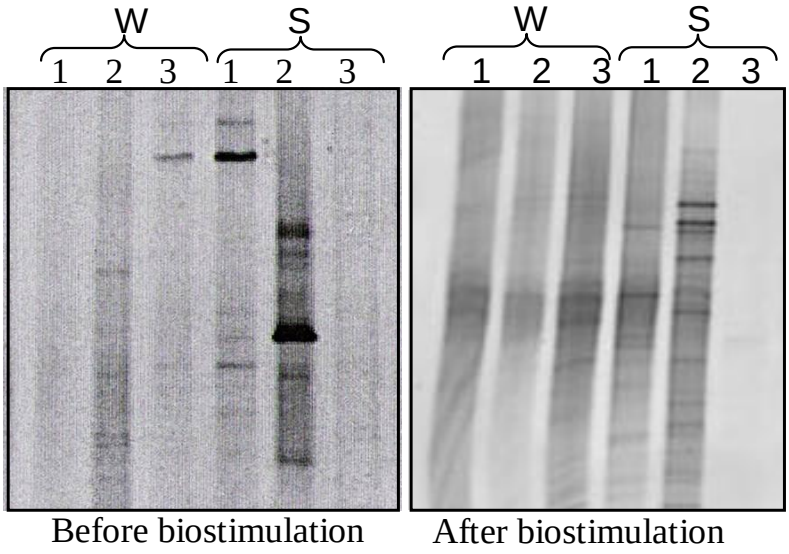

Figure S2 . DGGE band patterns of microbial composition in each sampling site polluted by vegetable oil before and after biostimulation. The individual bands in a single well represent different species. W represent water column sample and S represent sediment sample

in macroinvertebrate community structures due to pollution are usually characterised by shifts from sensitive to less sensitive species (Norris et al., 1982; Clements, 1994). In our study, pollutant tolerant macro invertebrates which generally indicate poor water quality mostly belong to the order Diptera namely, the family Chironomidae (Winner et al., 1980), as well as Culicidae, Muscidae, Psychodidae and Syrphidae. These organisms are then able to dominate the community in the absence of competitors. Hence, as a reduction in oil pollution was noticed at site 1 after biostimulation; an increase in the diversity of macroinvertebrates was noticed which resulted in a decrease in the dominance of the tolerant macroinvertebrates at this site. The increase in macroinvertebrate diversity at site 3 even though oil concentrations did not decrease in the sediment or water could have been the consequence of the polymerization of the vegetable oil on the bottom sediment. In this state, it may be less of a problem to the larger macro-invertebrates as not all aquatic macroinvertebrates are

fully aquatic throughout their entire lifecycles and thus this site may be colonized externally by the dispersion of aquatic macroinvertebrates. The overall macroinvertebrate diversity remained the same at site 2 which may be as a result of the decrease in phytoplankton noticed at the same site (Figure 5) which may have resulted in a food limitation to these organisms and hence hampered the recruitment of these organisms. The abundance of macroinvertebrates is important due to the fact that low abundances of macroinvertebrates may have a long-term adverse effect on fowl in the wetland such as diving duck species that use macroinvertebrates as a major food source during protein demanding periods example during egg-laying.

Overall, the changes noticed in the biotic communities in relation to the water quality variables is in agreement with previous studies by Lee et al. (2002) who showed that the addition of organic fertilizers yield successful improvement in bioremediation oil contaminated environment.

The low abundance of microbial communities at sampling site 3 could be attributed to the presence of high vegetable oil content in the sediment as well as the observed polymerization of vegetable oil residues. The improvement of sediment quality as measurement of oil content at sampling sites 1 and 2 after biostimulation (Table 1) corresponds with the observed bands on the DGGE gel (Figures 6, S2). Sites with higher microbial activities showed higher number of bands on the DGGE gel while site 3 with low microbial activity had fewer bands. The results from DGGE data has been reliably used for molecular community fingerprinting techniques of microbial assemblage diversity (Yu and Morrison, 2004). These results indicate that considerable diverse uncultured microbial assemblages exist in the wetland which is associated with vegetable oil biodegradation. In the present study, the dominant species was related to the genus *Pseudomonas* which agrees with the reported succession of microbial communities after biostimulation (Ogino et al., 2001). Although the use of DGGE for quantification of microbial assemblage is widely accepted, it should be noted that it does have a drawback in that the 16S rDNA specific primes also allows for cross reaction with members of other phylogenetic and physiological groupings in an environmental sample especially when the sample contains a complex microbial gene pool (Rotthauwe et al., 1997). Furthermore, co-migration of short fragments with larger fragments in the DGGE can also yield undesired sequence data and the underestimation of microbial assemblage (Muyzer, 1999).

Conclusion

Biostimulation using organic fertilizer resulted in an increase in microbial consortia activity which promoted vegetable oil degradation at sampling sites 1 and site 2; while site 3 showed less recovery due to the high amount of vegetable oil present even after the addition of higher concentrations of fertilizer (800 g/m^2). Overall, the biostimulation of the wetland affected the different biotic communities studied to varying degrees either directly through the increase in TP and TN concentrations released from the fertilizer or indirectly through altering water quality (example chlorophyll *a* and BOD) which affect the food web. This is as a result of the addition of high concentrations of fertilizer which may promote eutrophication in the wetland aquatic ecosystem and result in significant changes in phytoplankton biomass, this can have adverse effects by causing undesirable bloom formation during low flow periods in the winter months. Therefore, a pilot study is recommended as a first step before full scale biostimulation with fertilizer which may be conducted in the wetland environment.

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