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Fungi Isolated from Produced Water and Water-Soluble Fraction of Crude Oil

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Abstract. This study was sought to determine the fungi present in the produced water (PW) and water-soluble fraction (WSF) of crude oil as a preliminary approach to determining that fungi can survive in crude oil polluted water and their possible use in bioremediation. Different concentrations of PW and WSF of crude oil samples from Ughelli East Flow Station in Delta State of Nigeria were exposed to onion (*Allium cepa*) primordial cells at different concentrations for twelve days. Thereafter, samples of the PW and WSF were cultured on Potato Dextrose Agar. Isolates of *Thamnidium* sp, *Gelasinospora* sp, *Zygorhynchus* sp and *Colletotrichum* sp were found. *Zygorhynchus* and *Colletotrichum* were associated with PW while *Thaminidium* and *Gelasinospora* associated with the WSF. There were changes in the pH and turbidity of the PW and WSF before and after exposure to *Allium cepa* cells. At 25% level of treatments there were significant differences in pH and turbidity values of the PW and WSF at $P < 0.05$ and $P > 0.01$ before and after exposure to the plant.

Keywords: bioremediation, crude oil, fungi, water soluble fraction, produced water, pollution

Introduction

Over 90% of Nigeria's income comes from crude oil (Edema and Okoloko, 2008). Oil and gas reservoirs have a natural water layer (called formation water) that, being denser, lies under the hydrocarbons. As a result, oil reservoirs frequently contain some water. However, to achieve maximum oil recovery, additional water is usually injected into the oil surface. Both formation and injected water are eventually produced along with the hydrocarbons. This is called Produced Water (PW).

Neff and Anderson (1981) described PW for ocean discharge as containing up to 48 ppm of petroleum because it has usually been in contact with crude oil in the reservoir rock. Due to rapid mixing with seawater, most physico-chemical features of PW (low dissolved oxygen and pH, elevated salinity and metals) do not pose any hazard to organisms in water. Elevated concentrations of hydrocarbons may be detected in surface sediments that contain aromatic hydrocarbons and metals, up to about 1000 m from the discharge. These aromatic hydrocarbons and metals in PW were reported by Neff and Anderson (1981) to be toxic to organisms.

Crude oil contains a small soluble fraction called water soluble fraction (WSF) (Kavanu, 1964) and it is produced after a long period of oil-water contact (Baker, 1970). As long as incidences of oil spills and hydrocarbon pollution due to oil exploration continue, their effects on the living environment will remain of interest. Organisms exposed to WSF of crude oil take up the dissolved hydrocarbons and react to their effects. Severity of the effects depends on the organisms exposed, the concentration of the compounds and the mode of exposure (Overton *et al*., 2001). Therefore, the study of the effects of WSF of crude oil is important in order to understand and possibly prevent their undesirable effects. The effects of WSF on the growth and development of different organisms have been studied (Edema, 2010; Edema and Okoloko, 2008). Consequently, there is increasing interest in the use of living organisms in cleaning up oil polluted areas (a process known as bioremediation) instead of the use of chemicals because of their harmful effects and high costs of the latter. Fungi are diverse in their ecological adaptations. The potentials of certain fungi in bioremediation have been reported earlier (Kacprzak *et al*., 2005; Chigusa *et al.,* 1996). In this study, fungi consistently associated with PW and WSF exposed to onion (*A. cepa*) root primordial cells in a separate experiment to determine the effect of this exposure on the physico-chemical properties of (PW and WSF) were isolated and identified as an approach to determining whether fungi can survive in crude oil polluted waters (PW and WSF) and any indication of their possible use in bioremediation.

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Materials and Methods

Collection of crude oil and preparation of PW and WSF. Crude oil was collected from Ughelli East Flow Station in the Delta State of Nigeria. The PW is the water that comes with the crude oil. The crude oil used had been standing in the Laboratory for 5 years. To separate the PW from crude oil, the mixture of crude oil and PW was allowed to stand in a separating funnel for 24 h, after which the lower phase was collected and used as stock or 100% PW.

The WSF was prepared according to the method of Anderson *et al.* (1974). A sample of crude oil (500 mL) was slowly mixed in 500 mL deionized water in a 2 L conical flask using a Gallenkamp table top magnetic bar. Stirring was done for 16 h at room temperature (28 ± 2 °C). The oil-water mixture was allowed to stand for 24 h in a separating funnel. The lower phase was collected and used as stock or 100% WSF and diluted serially with deionized water to give 50% and 25% strength WSF which were stored in screw-cap bottles prior to use. Deionized water was used as control and the three levels of WSF concentrations (25, 50,100%) were used for further experiments, all in triplicates.

Exposure of WSF samples to *A. cepa.* The outer scales of the onion (*A. cepa*) bulbs were removed and the primordial cells were exposed using a razor blade. Some quantities of PW and WSF samples prepared as above were exposed to the onion root primordial cells for twelve days while the remaining was left for determination of fungi present before exposure. The exposure to onion cells was done in order to mimic the natural environment where plants are exposed to the effects of PW and WSF. Thereafter, samples of the PW and WSF were cultured on potato dextrose agar (PDA) (Oxoid, England).

Determination of fungi present in PW and WSF before and after exposure to *A. cepa.* PDA was prepared according to manufacturer's instructions. Before exposure to onion cells, 1 mL of each WSF concentration was transferred into a petri dish and 20 mL of PDA (containing 50 µg/mL chloramphenicol, to inhibit bacterial growth) was poured into the plate. The plate was swirled round for even mixing and allowed to solidify. The same procedure used for PW and WSF before exposure as described above was also followed after exposure. Control plates contained 1mL deionized water instead of WSF. Also, PW and WSF not exposed to *A. cepa* were plated on PDA to determine presence of fungi. All culture plates were incubated at room temperature (28 ± 2 °C) for 7 days. Culture samples were examined under low (x40) and high (x100) powers of an optical microscope for fungal growth. Fungal identification was done based on colony morphology and microscopic characteristics according to Barnet and Hunter (1998).

Determination of pH. The pH of WSF was taken before and after exposure to *A. cepa*, using a pH meter model PHS-25.

Turbidity. The turbidity of PW and WSF before and after exposure to *A. cepa* was determined with a portable turbidity meter (Hanna I 93 102) at 500 nm.

Statistical analysis. Statistical analysis was carried out using the t-test for paired sample for means.

Results and Discussion

Four genera of fungi were isolated and identified as *Thamnidium, Gelasinospora, Zygorhynchus,* and *Colletotrichum* (Table 1). While *Zygorhynchus* and *Colletotrichum* were associated with PW of the crude oil, *Thamnidium* and *Gelasinospora* were associated with the WSF. Fungal growth was evident in the PW and WSF both before (data not shown) and after exposure to *A. cepa*. The PW and WSF that were not exposed to onion also had the same fungi growing in them, which indicated that fungi were associated with PW and WSF of the crude oil. The mean pH values before and after exposure to *A. cepa* are shown in Table 2. The pH values after exposure were constantly higher though not significantly different from pH values before exposure to *A. cepa.* The pH values changed to slightly alkaline. Statistical analysis carried out using t-test for paired sample for means showed that the mean values of pH obtained before and after exposure of *A. cepa* to PW and WSF were significantly different ($P<0.05$). It has been reported that pH influences the oxidation- reduction equilibrium and solubility of ionic forms of several elements (Wild, 1996). Increased pH of the PW and WSF indicates that there was more cation removal from the PW and WSF than anions, as the presence of excess anion raises pH (Wild, 1996). All the pH values were within the maximum permissible level (pH 6.5-9.8) for safe water by World Health Organisation (WHO, 1995). The pH 7.7 of 100% PW has been reported to support catalase activity in plants (Taylor *et al.,* 1998). Edema and Okoloko (2008) reported a decrease in pH of WSF after exposure. The difference between their and our

findings could have arisen as a result of the different plants used as well as a possible role of the fungi present. However, Edema (2010) reported an increase in pH after exposure, which agrees with our findings here.

Turbidity decreased after exposure for both PW and WSF (Table 3). The 25% WSF concentration had the lowest change in turbidity (1.2) and 100% PW had the highest (8.3). The turbidity of PW and WSF decreased as a result of decrease in the concentration of PW and WSF as in the opinion of Michaud (1991). Decrease in turbidity implies that there was increased uptake of ionic particles (Edema and Okoloko, 2008). Also, the effect of pH change on solubility of ions might have contributed to the decrease in turbidity. Fungal density was high after exposure (data not shown). This uptake may be due to the additional uptake by the fungi.

Table 1. Fungal genera isolated from PW and WSF before and after exposure to *A. cepa*

Fungi	PW	WSF
Thamnidium sp	-	+
Zygorynchus sp	+	-
Gelasinospora sp	-	+
Colletotrichum sp	+	-

Table 2. Mean pH values of PW and WSF before and after exposure to *A. cepa*

Treatments	Before (Mean and SE)	After (Mean and SE)
100% PW	6.66 ± 0.000	*6.4 ± 0.060
50% PW	6.70 ± 0.003	*7.90 ± 0.030
25% PW	6.89 ± 0.003	7.50 ± 0.050
100% WSF	6.67 ± 0.006	*7.70 ± 0.000
50% WSF	6.80 ± 0.003	*6.81 ± 0.006
25% WSF	6.86 ± 0.003	7.30 ± 0.000

*significant difference ($P<0.05$) between the two experimental conditions (before and after exposure); SE = Standard error of mean.

Table 3. Mean turbidity values of PW and WSF before and after exposure to *A. cepa* (NTU)

Treatments	Before (Mean and SE)	After (Mean and SE)
100% PW	95.7 ± 0.03^{a}	87.4 ± 0.06^{b}
50% PW	47.6 ± 0.06	45.5 ± 0.09
25% PW	21.7 ± 0.06	20.4 ± 0.06
100% WSF	47.3 ± 0.05	42.6 ± 0.06
50% WSF	28.5 ± 0.03	24.5 ± 0.12
25% WSF	18.9 ± 0.00	17.7 ± 0.09

NTU = nephe turbidity unit measured at 500 nm; values with different letters in superscripts along the same row are significantly different ($P<0.01$).

Zygorhynchus is a soil-inhabiting fungus belonging to the Class Zygomycetes and Family Mucoraceae and can tolerate high salinity and certain degree of pollution (Ru-Yong, 2002). It is reported to have high resistance to lysis by other microorganisms due to the presence of uronic acid and fucose and phosphate polymers in its cell wall which confer resistance to the cell wall (Ballesta and Alexander, 1971). The ability of this fungus to thrive in PW of crude oil might have been as a result of this attribute. *Zygorhynchus* was isolated from waste-water and sewage sludges by Kacprzak *et al*. (2005). The occurrence of *Zygorhynchus* in heavily polluted areas such as sewage and crude oil may be due to its more adaptation to polluted environment. It is contrasting to its earlier reported slight tolerance to pollution. It could also be as a result of difference in species reported before and the one found in this work.

Colletotrichum was present in PW. It belongs to the Class Ascomycetes (Alexopoulos and Mims, 1979). Species of this fungus are known pathogens causing fruit and leaf rot of plants (Everett, 2003; Saha *et al*., 2002). A few species cause disease in man and other animals (Marcelino *et al*., 2008). This is the first documented report of the isolation of *Colletotrichum* from PW of crude oil.

Thamnidium was present in WSF. It is a Zygomycete and is commonly isolated from desert and cultivated soils (Arduin and Palma, 2007; Stejskal *et al*., 2005).

Gelasinospora, a soil fungus was detected in WSF (Domsch and Gams, 1972). It is an Ascomycete of the family Sordariaceae (Dettman *et al*., 2001). Although adapted to aerobic life, it has been reported to also tolerate anaerobic conditions (Ulacio *et al*., 1998) a property which probably enabled it to survive in WSF.

Kacprzak *et al*. (2005) opined that ability of fungi to survive in nutrient-limited environments such as polluted water is an indication of their possible use in bioremediation. These results indicate potentials of the fungi isolated for bioremediation of petroleum-polluted water.

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