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Effects of different concentrations of biocides on biofilm microorganisms in oil pipelines in Irri, Delta State, Nigeria

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Abstract

Biofilm can be defined as microbial communities containing large different numbers of microorganisms, which produce a wide range of biopolymers. This leads to adhesion to surfaces within hours of immersion. The attachment results in pit corrosion of oil pipelines. The study to assess the effect of different concentrations of biocides (0, 1,2,3,4 and 5 per cent) on bacterial diversity associated with corrosive biofilms in steel pipelines in Irri, Delta State was conducted in University of Calabar, Nigeria. Microbial communities associated with biofilms promote corrosion of oil and gas pipelines, which is a major problem confronting these industries. The community structure of bacteria in the biofilm formed in oil pipelines is a basic knowledge to understand the complexity and mechanisms of metal corrosion. To assess bacterial diversity, biofilm samples were obtained from mild steel coupons corroded after 127 days of exposure to normal operation and flow of petroleum. Microbial community analysis in biofilms of oil pipeline was determined by traditional cultivation technique. Most of the bacterial species detected in biofilms of oil pipelines included the following *Halomonas subglaciescola, Pseudomonas aeruginosa, Klebsiella oxytoca, Bacillus subtilis, Serratia marcescens, Bacillus cereus, Acidithiobacillus ferrooxidans and Staphylococcus epidermidis.* The sensitivity test results showed that Ozone (oxidizing biocide) exhibited the best biocidal characteristics and at the concentration of 1 percent, eliminated most of the bacterial species except the Gram positive and spore formers. The next effective biocide was sodium hypochlorite (oxidizing biocide) and then followed by formaldehyde as the least effective biocide. For effective control of biofilm bacteria, Ozone (oxidising biocide) and formaldehyde (non-oxidising biocide and persistent) should be used simultaneously as these biocides are effective against biofilms, bacteria and other microbes and are also environmental friendly biocides. The elimination or inhibition of these organisms in oil pipelines will keep our agricultural lands free of contamination from oil spills cause by corrosion.

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Introduction

The Niger Delta has suffered decades from oil spill which occur both on land and offshore oil spoils on land destroy crops and damages the quality and productivity of soils that communities use for faming. Oil in water damages fisheries and contaminates water that people use for drinking and other domestic purposes. Oil spill is said to results from corrosion of oil pipelines, poor maintenance of infrastructure and oil theft (Odu, 1972). Corrosion is caused by the activities of microorganisms. Metal surfaces are rapidly colornized by microorganisms in contact with natural or industrial aquatic environments, giving rise to a complex and strongly adhering microbial community, termed as biofilm (Melchen, 2002). The biofilm accumulation not only protects microbial cells from the external environment, but it is also detrimental to the underlying substratum, thereby causing physical degradation or biodegradation of the metal surface. This phenomenon is widely recognized as biocorrosion or microbiologically influenced corrosion (MIC) (Stoodley *et al.,* 2002). In the oil pipelines, biocorrosion takes place when complex microbial consortia interact with metallic surfaces through the establishment of multispecies biofilms in which different microorganisms influence the corrosion through a cooperative global metabiolism (Frenchel, 2002). Thus, sulphate reducing bacteria (SRB) (*Desulfovibronaceae and Sulfobacteriaceae*), *Clostridiaceae* members and *Methanogens* have been detected in marine bio-films (Zhang and Fang, 2001; Zhang *et al.,* 2003).

Usually, biofilm development is controlled by the addition of biocides and surfactans (Lechevallier *et al.,* 1988), however, this strategy in only temporally efficient and, eventually, the biofilms induced corrosion continue causing great economic losses to the oil and gas industries and the pollution of the environment (Miguel *et al.,* 2006). Due to persistence, pollution of the environment because of oil spillages caused by microbiologically influenced corrosion of oil pipelines, this research was therefore designed to investigate biofilm microorganisms and the biocides that can effectively control or inhibit their activities in the oil and gas pipelines.

Materials and methods

Sampling of biofilms

To obtain the biofilms, ten mild steel coupons (Fig. 1) (surface area: 35.2cm^2 and density 7.5gcm^{-3} each) obtained from a commercial source (metal samples company, Munford, AL). The coupons used have the same chemical properties as the pipelines, with the following chemical compositions 0.06%C, 1.05% Mn, 0.27% Si, 0.006% P, 0.002% S, 0.02% Cr, 0.02% Ni, 0.008% Mo, 0.05% V, 0.02% Ti, 0.05% Al, 9.424 Fe and 0.02% Cu (Lynch 1989). A total of five pipelines were sampled in Irri flow station at the Kwale/Okpail gas plants. Delta State.

The coupons were inserted into the inner surface of the pipelines, through the access values (Fig. 2) and allowed for the flow of petroleum for a period of 127 days. At the end of the 127 days, the coupons were detached from the inner region of the pipelines and the biofilms formed (Fig. 3) were scraped with sterile razor blades and collected into sterile bottles containing 5 ml phosphate buffered saline at pH 7.0 (Sambrock (1989). The bottles were stored in a cooler of ice block at 4^{o}C until arrival in the laboratory (after proximately 24 hours). Each coupon was named after the pipeline in which it was inserted, these were: Irri 02, Irri 06, Irri 07, Kwale 05 and kwale 06 respectively.

Microbiological analysis

Analytical media

The media used for the microbiological studies were:

a) Nutrient agar (Biolab): Yeast extract: 2g; peptane 5g; NaCl 0.1g; agar 15g; distilled water 1000ml.

b) Postgate B: KH_2PO_4 0.5g, NH_4Cl. 1.0g; Na_2SO_4 1.0g; $CaCl_2.6H_2O$ 0.1g; $MgSO_4.7H_2O$ 2g; sodium lactate (60-70%) 5ml; Yeast extract 1.0g; Ascorbic acid 0.1g; thioglycilic acid 0.1g; $FeSO_4.7H_2O$ 0.5g; NaCl 26g, distilled water 1000 ml; pH7.0.

c) Starkey broth: 3.0g kH_2PO_4, 0.2g $MgSO_4$.

$7H_2O$, 0.2g $CaCl_2.2H_2O$, 0.5g $(NH_4)_2 SO_4$, trace $FeSO_4$ in 1000ml distilled water.

Inoculation and incubation

One millilitre of appropriate ten-fold serial dilutions of biofilms were inoculated onto Nutrient agar and postgate B agar plates in triplicates using spread plate technique (Domain and Davis (1999) and Postgate (1984). Inoculated plates were incubated at 37°C for total heterotrophic bacteria counts, while anaerobic bacteria and sulphate reducing bacterial plates were stored in an anaerobic jar and incubated at 37°C for 24 hours and 7 days for enumeration of total anaerobic bacterial and sulphate reducing bacteria respectively.

Maintenance of pure culture

Discrete colonies were purified by repeated subculture unto appropriate agar media. Pure cultures were preserved on Nutrient agar slant and postgate B agar slants and stored in the refrigerator (4°C) for further tests.

Characterization and Identification of microbial isolates

Pure cultures of microbial isolates were identified based on cultural parameters, microscopic techniques and biochemical tests, which included the following: Gram staining, motility, indole, methyl red test, voges-proskauer test, catalase test, oxidase test, citrate utilization, H_2S production, spore formation and starch hydrolysis (Cruckshank *et al.,* 1976). Identification of the bacterial isolates was accomplished by comparing the characteristics of the cultures with that of known taxa as in (Holt *et al.,* 1994).

Sensitivity tests

Tube dilution method was used to determine the sensitivity of the bacteria to the biocides as described by Madigan *et al.,* (2009). The biocides used were ozone, sodium hypochlorite and formaldehyde at the concentrations of 0, 1,2,3,4 and 5 per cent.

Preparation of biocides

Ninety-nine milliliters, 98ml, 97ml, 96ml, 95ml and 94ml for control nutrient broth and postgate B broth were dispensed into test tubes and sterilized by autoclaving at 121°C for 15 minutes before inoculated with the biocides and each of the bacterium before incubation.

Ozone

One per cent was prepared by adding 1ml ozone to 99ml of the broth, 2 per cent prepared by adding 2 ml ozone into 98ml of the broths, 3 per cent prepared by adding 3 ml ozone into 97 ml of the broth, 4 per cent prepared by adding 4 ml ozone into 96 ml of the broth, 5 per cent prepared by adding 5 ml ozone to 95ml of the broth and no ozone was added to the control tubes. The same procedures were used for the preparation of sodium hypochlorite and formaldehyde respectively. After cooling, the biocides were added to the test tubes and then the test organisms inoculated in each test tube. The control test tubes were also inoculated with isolates but with no biocide added. The tubes were incubated for 24 hours for aerobic bacterial counts and the tubes for anaerobic bacteria and sulphate reducing bacteria were packed in anaerobic jar and incubated at 37°C for 24 hours and 7 days respectively. At the end of the incubation periods, the test tubes were tested for turbidity to determine the minimum inhibition concentration (mic) of each biocide against the organisms colorimetrically at 680 nm.

Result and discussion

Microbial sensitivity to ozone, sodium hypochlorite and formaldehyde

Due to the economic losses as well as environmental health and safety hazards caused by the activity of communities of mixed sulphate reducing bacteria, sulphate oxidizing bacteria, iron reducing bacteria, manganese oxidizing bacteria and fungi in many industrial sector such as the oil and gas industries, it is necessary to check the risks resulting from the activity of these organisms.

The growth frequency and the activity of these organisms caused severe corrosion problems in the oil and gas pipelines. The activity of these organisms can be minimized or completely eradicated by the application of antimicrobial agents or biocides. One of the effective ways to measure the effect of biocides on an organism is by determining minimum inhibitory concentration (mic), which prevent growth in a suitable medium. In this study, the effects of three biocides (ozone, sodium hypochlorite and formaldehyde) were tested on the microorganisms isolated.

Fig. 1. Coupons before insertion into oil pipelines.

Fig. 2. Access valve of oil pipeline for insertion of coupons.

Organisms Isolated from Biofilms of oil Pipelines

Halomonas subglaciescola, Pseudomonas aeruginosa, Klebsiella oxytoca, Bacillus subtilis, Serratia marcescens, Bacillus cereus, and *Staphylococcus epidermidis*.

The results in Figures 4-5 show the effects of ozone on *Halomonas subglaciescola, Pseudomonas aeruginosa, Klebsiella oxytoca, Bacillus subtilis, Serratia marcescens, Bacillus cereus,* and *Acidithiobacillus ferrooxidans* and *Staphyloccus epidermidis*. It may be seen from Figure 4 that ozone completely, eliminated *Halomonas subglaciescola, Pseudomonas aeruginosa* and *Bacillus subtilis* from 3% concentration after 24 hours of incubation period, while *Klebsiella* oxytoca was wiped out from 1% level of concentration. The reason for the persistence of the three organisms may be because they are Gram positive bacteria and also are spore formers. Gram positive bacterial cell wall is made up of 25 sheets of Peptidoglycan and murein stacked one upon another. This peptidoglycan layers is protective in function against exposure to toxic substances (Subba , 2011). The spore forming ability is another inherent capacity of these organisms to survive in a stressed environment such as the oil pipelines. Unlike the Gram positive bacteria, the Gram negative bacteria have only one sheet of peptidoglycan, which makes the cell easily attacked by antimicrobial agents.

Fig. 3. Coupons after 127 days in oil pipelines.

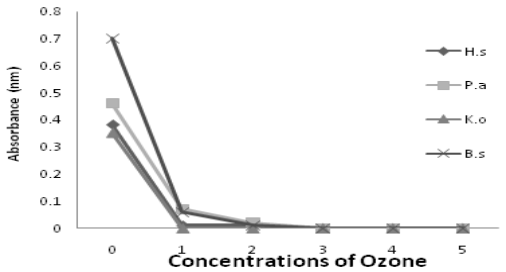

Fig. 4. Effect of Different Concentration of Ozone on *H. subglacesola* (HS), *P. aeruginosa* (P.a.), *K. oxytoca* (K.o) and *Bacillus subtilis* (B.s).

Similarly, in Figure 5 the result shows that ozone depressed *Bacillus cereus, Serretia marcesens, Staphylococcus epidermidis* and *Acidithiobacillus ferrooxidans* in the medium after 24 hours of incubation. Ozone is an oxidizing biocide that is effective against bacteria and biofilms at low concentrations of 0.2-0.5 mg/l. The organisms

persisted up to two per cent concentration. *Serratia marcescens* and *Staphylococcus epidermidis* were eliminated in 2 per cent concentration. Whereas, *Acidithiobacillus ferrooxidans and B. cereus* persisted. The persistence of *B.subtilis* and *Acidithiobacillus ferrooxidans* up to 2 per cent may be due to their genetically endowed ability of the organisms to resist environmental stresses. The persistence of *Acidithiobacillus ferrooxidans* and *B. cereus* may also be ascribed to their being Grams positive and spore formers. The possession of thick cell wall which consists of peptidoglycan, Gram positive bacteria have up to about 25 sheets of peptidoglycan stacked one upon another, this will protect them from being plastmolysed (Madigan, 2009, Subbao 2011).

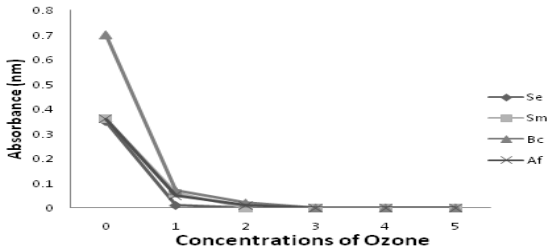

Fig. 5. Effect of Different Concentration of Ozone on *B. subtilis* (B.s), *S. marcescens* (S.m), *B. cereus* (B.c) and *Acidithiobacillus ferrooxidance* (Af).

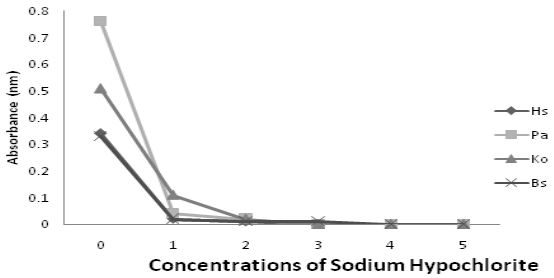

Fig. 6. Effect of Different Concentration of Sodium Hypochlorite on *H. subglacescola* (HS), *P. aeruginosa* (P.a.), *K. oxytoca* (K.o) and *Bacillus subtilis* (B.s).

Figures 6 and 7 showed the effect of sodium hypochlorite against *Halomonas subglacescola, Pseudomonas aeruginosa, Klebsiella oxytoca, Bacillus subtilis, Serratia marcescens, Bacillus cereus, Acidithiobacillus ferrooxidans* and *Staphylococcus epidermidis*. The result in Figure 6 revealed that sodium hypochlorite depressed *H.subglaciescola, P. aeruginosa,* K. *oxytoca and B. subtilis* up to 2 per cent concentration before final elimination at 3 per cent concentration after 24 hours of incubation. The reason for their persistence may be attributed to their ability to degrade the biocide at lower concentration.

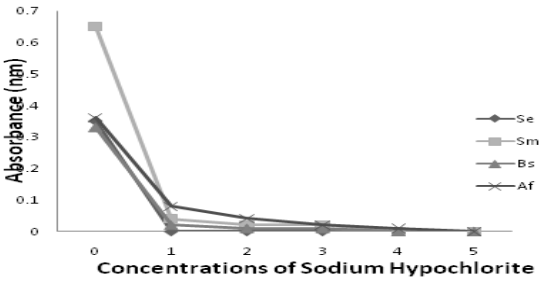

Fig. 7. Effect of Different Concentration of Sodium Hypochlorite on *B. subtilis* (B.s), *S. marcescens* (S.m), *B. cereus* (B.c) and *Acidithiobacillus ferrooxidance* (Af).

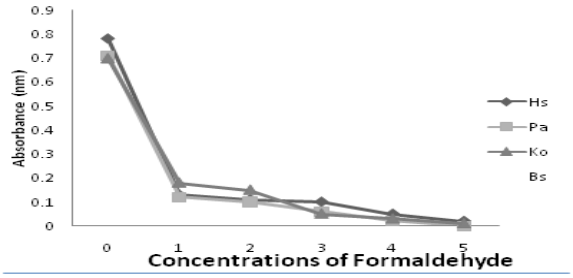

Fig. 8. Effect of Different Concentration of Formaldehyde on *H. subglacescola* (HS), *P. aeruginosa* (P.a.), *K. oxytoca* (K.o) and *Bacillus subtilis* (B.s).

Similarly, in Figure 7 sodium hypochlorite depressed the organisms up to 3 per cent concentration. The Gram positive bacteria persisted before final elimination at 3 per cent and 4 per cent concentrations respectively. Their being Gram positive and spore formers possess them inherent ability to withstand stressed environment such as oil and gas pipelines. The possession of thick cell wall which consists of peptidoglycan gives them the inherent ability to withstand stressed condition. Gram positive bacteria have up to about 25 sheets of peptidoglycan stacked one upon another, whereas Gram negative bacteria have only one sheet of peptidoglycan. This will protect them from being

plasmolysed easily by antimicrobial agents (Madigan, 2009).

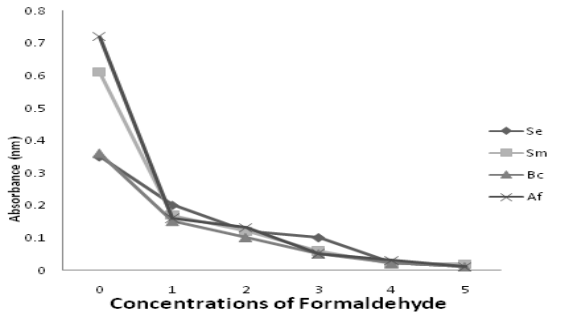

Fig. 9. Effect of Different Concentration of Formaldehyde on *B. subtilis* (B.s), *S. marcescens* (S.m), *B. cereus* (B.c) and *Acidithiobacillus ferrooxidance* (Af).

The result in Figures 8-9 show the effect of formaldehyde against *Halomonas subglaciescola, Pseudomonas aeruginosa, Klebsiella oxytoca, Bacillus subtilis, Serratia marcescens, Bacillus cereus, Acidithiobacillus ferroxidans* and *Staphylococcus epidermidis*. The result in Figure 8 shows that formaldehyde depressed *Halomonas subglaciescola, Pseudomonas aeruginosa* and *Klebsiella oxytoca* up to 5 per cent concentration after 24 hours of incubation. These organisms are both Gram positive and spore formers that are capable of resisting biocides due to the possession of peptidoglycan in their cell wall. Although many biocides may effectively kill nonsporulating bacteria (but not bacterial spores), high concentrations are often needed to achieve this effect.

Similarly, in Figure 9 the result indicated that formaldehyde depressed *Bacillus subtilis, Serratia marcescens, Staphylococcus epidermidis, Bacillus cereus* and *Acidithobacillus ferrooxidans* but did not completely eliminated the organisms in the medium after 24 hours of incubation Videla and Harrera (2005) reported that formaldehyde is a non-oxidising biocide that is effective against bacteria, algae, fungi and biofilms at higher concentrations of 10-70 mg/l. The result further shows that both *Bacillus cereus* are Gram positive and spore formers while *Serratia marcescens* is a Gram negative bacterium. These bacteria persisted in the medium, may be due to their ability to degrade the chemical and use it as source of nutrient. The result further shows that higher concentrations of formaldehyde may be needed to achieve total elimination of these organisms from pipelines.

Conclusion

Laboratory investigations of the effects of different concentrations (0, 1,2,3,4 and 5 per cent) of each of three biocides (ozone, sodium hypochlorite and formaldehyde) against biofilm bacteria in oil pipelines have been presented. It is shown that the performance of each biocide increased as its concentration increases. It is observed that ozone an oxidizing biocide exhibited the best biocidal efficacy against all the biofilm bacteria, followed by sodium hypochlorite, and then formaldehyde (a non~oxidising biocide) being the least effective of the three biocides. Therefore the unique combination of ozone and formaldehyde (which is persistence) will provide excellent means in the control of biofilm bacteria, particularly the Gram positive and spore formers which seem to be more resistance. Ozone is also known to be more environment friendly because of its rapid decomposition to oxygen.

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