Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.3, No.14, 2013

Synergistic Effect of Monochloramine and Glutaraldehyde Biocides against Biofilm Microorganisms in Produced Water

Ikechi Igwe[1*], Yemainain Tadaerigha Puyate[2]

1. Department of Chemical/Petrochemical and Petroleum Engineering, Rivers State University of Science and Technology, P.M.B. 5080 Npkolu-Oroworukwo, Port Harcourt, Nigeria
2. Department of Chemical/Petrochemical and Petroleum Engineering, Rivers State University of Science and Technology, P.M.B. 5080, Npkolu-Oroworukwo, Port Harcourt, Nigeria

* Email of the corresponding author: igwe.ikechi@ust.edu.ng

Abstract

The synergistic effect of oxidizing biocide (monochloramine) and non-oxidizing biocide (glutaraldehyde) against biofilm microorganisms in produced water system have been investigated. Batch process was used for the investigation of biofilm attachment on polyvinylchloride slides with the aid of chocolate agar (enriched medium), in produced water. The effects of the biocides on biofilm mass were assessed by comparing the biofilm mass of the control samples (without the addition of biocides) to the biofilm mass of the test samples (after the addition of biocides). The organic constituents of the biofilm on each slide were evaluated as spectrophotometric analysis of protein and polysaccharide contents using modified Lowry method and phenol-sulphuric acid method, respectively. The synergy of monochloramine with glutaraldehyde caused a greater reduction in the final biofilm mass, protein and polysaccharide contents, respectively, than monochloramine or glutaraldehyde acting independently. Considering reduction in total protein contents and a 10 day old biofilm, the synergistic biocide caused a 69.6 % reduction, monochloramine caused a 62.9 % reduction, and glutaraldehyde caused a 59.1 % reduction. In terms of reduction in total polysaccharide contents and a 10 day old biofilm, the synergistic biocide caused a 66.6 % reduction, monochloramine caused a 60.6 % reduction, and glutaraldehyde caused a 54.7 % reduction. The result indicates strong antimicrobial value for the combination of oxidizing and non-oxidizing biocides against biofilm-microorganisms.

Keywords: Produced water, biofilm, Standard Curve, biocides, Spectrophotometer

1. Introduction

When a clean surface is in contact with an aqueous environment (water), free-floating microorganisms begin to form, which subsequently generates biofilm microorganisms. Biofilm microorganisms (sessile) excrete extracellular polymeric substances or exopolysaccharide substance (EPS), which hold the biofilm together and cement it to the surface. These polymers trap nutrients and protect microorganisms from biocides and other toxic substances (Dreeszen 2003). A collection of microorganisms surrounded by the slime they secrete and attached to either an inert or living surface is referred to as biofilm (Colghlan 1996). Examples of biofilms are: the plaque on human teeth, the slippery slime on river stone, and the gel-like film on the inside of a vessel which holds water (Colghlan 1996).

Biofilm can be produce by microorganisms on various abiotic hydrophobic and hydrophilic surfaces, such as glasses, metals, and plastic. Several processes, systems, and products can be adversely affected by uncontrolled growth of biofilms. Problems associated with biofilms include accelerated corrosion of metals, accelerated decomposition of woods and other biodegradable materials, restricted flow through pipes, plugging or fouling of valves and flow meters, and reduced heat exchange or cooling efficiency on heat exchange surfaces (Singleton 2009).

Different biocides have been used to control problems caused by biofilms in industrial systems. Various organic and inorganic substances are used as biocides. The type of biocides used in a given system depends on many factors such as the nature of medium to which the biocide is added, the nature of microorganism(s), and specific requirements of the industry, as well as safety and regulatory considerations (Videla and Herrera 2005). Depending on their chemical composition and mode-of-action, biocides are classified as oxidizing and non-oxidizing (Videla and Herrera 2005). Chlorine, ozone and bromine are examples of oxidizing biocides of industrial use. Some non-oxidizing biocides are formaldehyde, isothiazolones, gluteraldehyde and quaternary ammonia compounds. When microorganisms (bacteria) are in films (biofilms), they are very resistant to conventional biocides. They produce more exopolymers after biocide treatment to protect themselves (Dreeszen 2003). Combinations of oxidizing and non-oxidizing biocides or of two non-oxidizing biocides have been

proposed to optimize biofilm control of industrial water systems (Videla and Herrera 2005). This synergistic combination for optimal control of biofilm is the main trust of this work.

Despite the fact that oxidizing biocide or non-oxidizing biocide is periodically applied to industrial systems like produced water system in the oil and gas industry, to control microbial load and growth, biofouling and other problems associated with the presence of microorganisms in such systems still occur. This biocidal inefficiency is mainly due to the inability of the biocide to penetrate microbial biofilms and kill the biofilm microorganisms. Another cause of this biocidal inefficiency is the fact that a mature biofilm is made up of different species of microorganisms, such that a single biocide may have a selective antimicrobial effect on the microorganism and isolate other microbial species in the system.

The inability of a single biocide, whether oxidizing or non-oxidizing to effectively control biofilm growth in industrial system is the problem this work seeks to solve. It intends to do so by combining an oxidizing biocide (monochloramine) with a surface active agent – non-oxidizing biocide (glutaraldehyde) for an optimal effect.

Microorganisms and/or biofilm in a system can be removed and/or destroyed by physical or chemical treatments (Mittleman 1986). Physical methods of removing biofilm include flushing, which is perhaps the most simple, although of limited efficacy. Abrasive or non-abrasive sponge balls are frequently employed in industry. However, abrasive sponge balls can damage the protective passive films, and non-abrasive sponge balls are not very effective with thick biofilms (Videla and Herrera 2005). Also, physical methods require process shutdown for treatment to be carried out. This often results in loss of production time. Recycling the sponge ball is almost impossible in process systems with pressure and flow control devices (valves, orifices, and chokes) and with different process pipe size diameters. The most common chemical method for controlling biofouling in industrial process water systems is by the use of biocides. Biocides are anti-microbial chemicals able to kill the microorganisms or inhibit their growth and reproductive cycle (Videla 1996). The main feature of the use of biocides is that they kill free-floating bacteria and other microorganisms at very low concentrations, although much higher levels are needed to control established biofilms as any EPS tends to deactivate or act as a barrier to the biocide (Videla 1996).

Videla and Herrera (2005) proposed the combination of oxidizing and non-oxidizing biocides or of two non-oxidizing biocides to optimize biofilm control of industrial systems. To assess the value of antibiotic combination of polymyxin B and miconazole, Pietschmann *et al.* (2008) examined the in vitro synergistic potential of the two drugs on Gram-negative and Gram-positive bacteria and yeast. Antifungal activity and antibacterial activity were tested by Minimum Inhibitory Concentration (MIC) of broth macrodilution and urea broth microdilution by fluorescence microscopy and flow cytometry. It was found that E. coli strain XL-1 was susceptible to 7.5 µg/ml polymyxin B; but was susceptible to a concentration of polymyxin B as low as 0.07 µg/ml when combined with 0.07 µg/ml miconazole. There was no effect on bacteria growth if miconazole was applied alone. *Pseudomonas aeruginosa* was susceptible to 0.73 µg/ml polymyxin B and showed no effect to miconazole. The two drugs acting together were inhibitory at a concentration of 0.18 µg/ml (Pietschmann *et al.* 2008). The assessments also show that the Gram-positive *staphylococcus intermidius* was susceptible to 0.73 µg/ml of miconazole and to 2.93 µg/ml polymyxin B. When identical concentration of the two drugs were combined, a twofold lower dose of miconazole and an eightfold lower dose of polymyxin B was effective for growth inhibition (MIC = 0.37 µg/ml). It was discovered that in all the tests, there were no interactions between the drugs. Therefore, it was concluded that if polymyxin B and miconazole are combined, their effect is greater than the sum of the effects observed with polymyxin B and miconazole independently, revealing bactericidal and fungicidal synergy. The results indicated a strong therapeutic value for the combination of these antimicrobial agents against the microorganisms tested for clinical situation where they are involved. However, these antimicrobial agents are clinical drugs applied in vitro (administered to animals or human beings) and in the absence of biofilms. Their synergy on biofilm microorganisms was not stated.

The synergistic effects of haloamine biocide solutions that contained different concentrations of monochloramine and dichloramine were also investigated. In the investigation, an appropriate quantity of cell suspension from bacteria consortium was aseptically transferred to sterile saline. (Singleton 2009). The cells were then challenged with the haloamines and synergistic combination of the haloamines (monochloramine and dichloramine), respectively. The cells were exposed to the following concentrations:

i. 0.5 mg/l of monochloramine
ii. 0.5 mg/l of dichloramine
iii. 0.25 mg/l monochloramine plus 0.5 mg/l dichloramine

iv. 0.4 mg/l monochloramine plus 0.1 mg/l dichloramine

The bacteria consortium was exposed to the haloamines for 20 minutes before samples were removed for cell enumeration by the spread plate technique (Singleton 2009). It was observed that exposing the consortium to 0.5 mg/l monochloramine or 0.5 mg/l dichloramine resulted in decreased cell counts. The greatest decline in cell counts resulted when the consortium was exposed to a 4:1 ratio of monochloramine to dichloramine (Singleton 2009). Further investigation revealed that as the ratio of monochloramine to dichloramine changed, the relative efficacy also changed. The most effective ratio was found to be in the range of 9:1 to 2:1 monochloramine to dichloramine (Singleton 2009). However, monochloramine and dichloramine are oxidizing biocides, applied on suspended bacteria cells. Their synergy also lacks the complimentary action of a surface active agent. Hence, the needs to still test the efficacy of the combined action of an oxidizer and a surface active biocide against biofilm microorganisms – the objective of this work.

Also, a stable combination of hydrogen peroxide (H_2O_2) and silver (Ag) ions gives a multi-component oxidizing biocide called Accepta 8101. This combination generates a biocide twenty times more powerful than that of hydrogen peroxide alone (http://www.accepta.com, March 12, 2007). However, the demerits of this oxidizing biocide were not indicated. After preliminary confirmation of microbiologically influenced corrosion on a water system, Hurh *et al*. (1999) added two (2) non-oxidizing biocides (glutaraldehyde and quaternary ammonium compounds) to control the bacteria growth in the system. After draining and drying the system, the combined biocide was found to have sanitized the system such that no further treatment was carried out. However, these are non-oxidizind biocides, hence, the need to investigate the combined effects of an oxidizing biocide and a non-oxidizing biocide against biofilm microorganisms.

Spectrophotometer is an instrument that measures the fraction of the incident light transmitted through a solution. It consists of two parts: a Spectrometer for producing light of any selected colour (wavelength) and a photometer for measuring the intensity of light. It is used to measure the amount of light that passes through a sample material and, by comparison to the initial intensity of light reaching the sample, the amount of light absorbed by that sample is indirectly measured (Fankhauser 2009). Since different compounds absorb light at different wavelengths, a spectrophotometer can be used to distinguish compounds by analyzing the pattern of wavelengths absorbed by a given sample. The amount of light absorbed is directly proportional to the concentration of absorbing compounds in that sample, so a spectrophotometer can also be used to determine concentrations of compounds in solution (Fankhauser 2009). The relationship between concentration and absorbance is expressed by Lambert-Beer law, which is commonly known as Beer's law. The law states that the absorbance of a light absorbing material is proportional to its concentration in the solution (Fankhauser 2009) and is expressed mathematically as:

$$A = \varepsilon LC \quad (1)$$

ε = the extinction coefficient of the substance, has units of $M^{-1} * cm^{-1}$ (unique for each substance)

L = the sample path length measured in centimeters (i.e. the width of the cuvette – almost always 1 cm)

C = the molar concentration of the solution (expressed in terms of molarity).

2. Materials and Methods

2.1 Produced Water Sample

The produced water sample used for this experiment was collected directly from a sampling point of a high water-cut oil well in Ndokwa East Local Government Area of Delta State, (Niger Delta Region) Nigeria. Laboratory analysis indicated that the produced water sample contains different bacterial and fungal species. They include *Bacillus spp, Pseudomonas spp, Saccharomyces spp,* and *Penicillum spp.* Chemical and biological characteristics of the produced water sample are presented in Table 1 below.

Five litres (5 l) of the produced water sample was collected in a very clean and dried plastic container and transported to the laboratory the same day for immediate use. The produced water served as the aqueous medium for the growth of microorganisms and the development of biofilms.

Table 1. Chemical and Biological Characteristics of the Produced Water Sample

Parameter	**Value**
Chemical Oxygen Demand (COD), mg/l	1022
Biological Oxygen Demand (BOD), mg/l	961
Total Suspended Solids , mg/l	126
Total Dissolved Solids (TDS), mg/l	986
Total Organic Compound (TOC), mg/l	558
Total Kjeldahl Nitrogen (TKN), mg/l	79
Nitrate, mg/l	2.0
Nitrite, mg/l	0.038
Ammonia, mg/l	68
Total Phosphorus, mg/l	0.64
Dispersed Oil, mg/l	1.7
Conductivity, µmhos/cm	82673
Salinity, mg/l	1146
Temperature , ^{o}C	37
pH, SU	6.3
Total Microbial count, CFU/ml	$7.6*10^{4}$

2.2 Anti-microbial Agents (Biocides)

This study is concerned with the investigation of the synergistic effect of monochloramine, an oxidising biocide and glutaraldehyde, a non-oxidising biocide (surfactant), relative to the efficacy of the individual biocides on biofilm microorganisms. Monochloramine as an oxidising biocide was selected for this investigation based on the following criteria: (i) it penetrates biofilms very well and reacts specifically with biofilm microorganisms; (ii) it has higher residual effect due to lower reactivity with water ingredients and other chemicals; (iii) it is very stable in-situ, very easy to apply and monitor, and (iv) its operational capital costs are low, and it generates less toxic by-products (Videla 1996). Glutaraldehyde is a non-oxidising biocide and a surface active agent. It is a colourless liquid with a pungent odour. It is an oily liquid at room temperature and miscible with water, alcohol, and benzene. It is cheap, non corrosive and effective at low concentrations (Videla 1996). Its availability was also one of the reasons for selecting it for this study.

An overkill concentration of 1.5 mg/ml was adopted for the investigation. Chen and Stewart (1996) in one of their investigations treated a 526 µm thick biofilm with 18.6 mg/l chlorine and found that only 1.8 mg/l reacted with the substratum after 3 hours contact time. Chlorine concentration of 1.13 mg/l was also found to be effective (Chen and Stewart 1996). According to Videla (1996), if the initial microbial loading of the system is unknown (especially where a biofilm is likely to be present), it is difficult to judge the treatment necessary in terms of concentration, volume and contact time. It was therefore suggested that the wise thing to do in such case is to do an overkill treatment (Videla 1996). Hence, the choice of an overkill concentration of 1.5 mg/ml was based on facts from literature and preliminary investigations with different concentrations. The choice of a single "overkill" concentration was also informed by already established facts. According to Pereira *et al.* (2001), "it is interesting to note that the overall final mass of biofilm deposit is not affected by the increase in biocide concentration." It was concluded that doubling of biocide concentration did not bring about considerable increase in protein and polysaccharide removal (Pereira *et al.* 2001). The synergistic effect of monochloroamine and glutaraldehyde was investigated by dosing the biofilm medium with same concentration of 1.5 mg/ml each. Monochloramine was applied immediately after dosing with glutaraldehyde.

2.3 Preparation of Adhesive Slides

Polyvinyl chloride (PVC) material was obtained locally and cut into small slides, 5 cm long, 1.4 cm wide, and 0.1 cm thick (5 cm x 1.4 cm x 0.1 cm), with an average mass of 1251±5 mg. Each slide was perforated at one end to enable the passage of nylon thread used for suspending the adhesive slides in the batch reactors. The slides were further polished using silicon carbide (SiC) emery papers, degreased with acetone, washed with distilled water and air dried. Biofilms are consented to grow on the PVC slide surfaces under laboratory conditions using chocolate agar (enriched medium) as nutrient for microorganisms in produced water. Sixteen (16) adhesive slides were prepared and used for the experiment.

2.4 Experimental Setup

The laboratory experiment was carried out under hydrostatic and atmospheric conditions. Sixteen (16) conical flasks were used as batch reactors with sixteen (16) PVC adhesive slides. One thousand six hundred millilitres (I600 ml) of the produced water sample with initial microbial count of 7.6 $x10^4$ CFU/ml, was used as the aqueous medium for biofilm culture. 100 ml of produced water was poured into each batch reactor containing one prepared adhesive slide. 10 ml of chocolate agar enriched medium was added to each of the sixteen batch reactors. The enriched medium contains the nutrients required to support the growth of a wide variety of microorganisms, including some of the more fastidious ones (Fenollar and Raoult 2004). Chocolate agar is heated blood at 40-45°C, which turns brown and gives the medium the colour for which it is named.

The experimental setup was incubated at room temperature and atmospheric pressure for some days to achieve sessile microbial population. Preliminary inspection of the adhesive slide indicated microbial colonization of the slide after seven (7) days, with an average planktonic count of 8.7 $x10^8$ CFU/ml. Four (4) batch reactors were used for control test (the control reactor or sample contained everything found in the test reactors/sample except biocides), while the remaining twelve (12) batch reactors were used for testing the efficacy of the biocides. Four (4) reactors were used for testing the combined effects of monochloramine and glutaraldehyde, and four (4) reactors each for testing the individual effects of monochloramine and glutaraldehyde. The sets of reactors were labeled as:

R1-C: Reactor No. 1 for control

R2-C: Reactor No. 2 for control

R3-C: Reactor No.3 for control

R4-C: Reactor No. 4 for control

R1-S: Reactor No. 1 for the synergistic biocide

R2-S: Reactor No. 2 for the synergistic biocide

R3-S: Reactor No. 3 for the synergistic biocide

R4-S: Reactor No. 4 for the synergistic biocide

R1-M: Reactor No. 1 for monochloramine

R2-M: Reactor No. 2 for monochloramine

R3-M: Reactor No. 3 for monochloramine

R4-M: Reactor No. 4 for monochloramine

R1-G: Reactor No. 1 for monochloramine

R2-G: Reactor No.2 for glutaraldehyde

R3-G: Reactor No. 3 for glutaraldehyde

R4-G: Reactor No. 4 for glutaraldehyde

After a period of ten (10) days from the beginning of the experiments, 1.5 mg/ml of monochloramine and glutaraldehyde, respectively (the synergistic biocide), was applied into one of the designated reactor (R1-S). Also, 1.5 mg/ml of the each biocide was applied into one of their respective reactors (RI-M and RI-G, respectively). The biocides were allowed to react with the biofilms on the adhesive slides for a period of one (1) hour. One adhesive slide was removed from the control reactor (R1-C) simultaneously with one adhesive slide from each of the three sets of test reactors (R1-S, R1-M and R1-G) at the end of one hour contact time. The four slides were further conditioned for biomass measurement, protein and polysaccharide contents analysis. The results represent the efficacy of the synergistic and individual biocides on a 10 day old biofilm. This same

procedure was repeated under the same conditions after the periods of 15 days, 20 days and 25 days and the results recorded.

2.5 Biofilm Assessment Methods

Conventional methods available for measuring biofilm accumulated on industrial systems are classified as either direct or indirect measurement techniques. The direct techniques involve the measurement of biofilm thickness or biofilm mass. The indirect measurement techniques include methods for measuring specific biofilm constituents (e.g. protein and polysaccharide), and methods for determining microbial activity within the biofilm (Characklis *et al*., 1982). Biofilms generally consists of organic (biological) and non-biological components. Biocides are consented to act only on the biological constituents of the biofilms.

Measurement of biofilm mass and specific biofilm constituents (protein and polysaccharide contents) are the direct and indirect methods respectively, employed in this research to investigate the efficacy of the selected biocides (combined and/or individual) on biofilm of different maturity periods.

2.6 Biofilm Mass Assessments

The synergistic and individual effects of monochloramine and glutaraldehyde against biofilms of different maturity ages were investigated by quantification of the biofilm mass accumulated on the slides. At the start of the experiment, all the PVC slides were identified and weighed before being placed in their respective reactors. The slides were removed from each reactor after one hour contact time and washed in 0.9 % NaCl solution to remove biocide-detached biofilms. (Note that the biocides acted on only a fraction of the biofilm on the surface of the slides resulting in detached biofilms while the other fraction unaffected by the biocides remained attached to the slides. Hence, the washing of the slides in the NaCl solution was done gently to remove only biocide-detached biofilms. The entire biofilm mass was not removed either by scrapping or scrubbing at this stage). They were then air dried to a constant mass in similar conditions. The mass of the slides plus remaining attached biofilms were determined using a Mettler analytical mass balance (Model AE 260) and the results recorded. The efficacies of the biocides were assessed by comparing the biofilm mass of the control samples (without the addition of biocides) to those of the test samples. The organic constituents of the biofilm deposits on each slide were evaluated by spectrophotometric analysis of protein and polysaccharide contents.

2.7 Spectrophotometric Analysis of Biomass

A sterilized plastic spatula was used to scrape biofilms from the slides into sample tubes, and made up with a 1M phosphate buffer solution (PBS). The solutions were vigorously agitated for 3 minutes in a vortex to disrupt the biofilms. The suspensions were further centrifuged for 10 minutes at 10 ^{o}C. Aliquots of the suspensions were used to assess the biofilm contents in terms of total proteins and total polysaccharides. Total protein content of each slide was determined using the modified Lowry method with Bovine Serum Albumin (BSA) as protein standard and the total polysaccharide content by the phenol-sulphuric acid method of Dubois *et al*. (1956) using glucose standard.

Half-log dilution (a 3.162-fold serial dilution) of the BSA and glucose standards respectively, was made to cover the anticipated concentration of the unknown samples (control and test samples), using the test tube procedure. Subsequently, the absorbance of all the samples (standards, control and test) was measured with the aid of a spectrophotometer. The measure corrected absorbance ($\Delta\ A_{750\ nm}$ for BSA standard and $\Delta\ A_{630\ nm}$ for glucose standard) of each standard was tabulated against the known concentrations (serially diluted concentrations of BSA and glucose). Standard curves were generated by plotting $\Delta\ A_{750\ nm}$ against serially diluted concentrations of BSA and $\Delta\ A_{630\ nm}$ against serially diluted concentrations of glucose standards, respectively, using Microsoft Excel. The axes of the graphs were transposed by plotting concentration on the y – axis and $\Delta\ A_{750\ nm}$ standard or $\Delta\ A_{630\ nm}$ standard on the x – axis as the case may be. A quadratic equation of the form ($y = ax^2 + bx + c$) was generated from the BSA standard curve, while a linear equation ($y = ax + b$) was generated from the glucose standard curve, where,

y = dependent variable (concentration in mg/ml)

x = independent variable ($\Delta\ A_{750\ nm}$ or $\Delta\ A_{630\ nm}$)

a = constant

b = constant/intercept

c = intercept

The quadratic equation of the BSA standard curve was used to determine the protein concentration of the control and test samples, respectively using the absorbance values. The protein content of each unknown sample was determined by multiplying the concentration obtained from the standard curve by original sample volume (3.162 ml). That is,

mg protein = mg/ml protein from standard curve x 3.162 ml.

Similarly, the linear equation of the Glucose standard curve was used to determine the polysaccharide concentration of the control and test samples, respectively using their absorbance values. The polysaccharide content of each unknown sample was determined by multiplying the concentration obtained from the standard curve by original sample volume (3.162 ml). That is,

mg polysaccharide = mg/ml polysaccharide from standard curve x 3.162 ml.

The model used for this experiment is the Spectro-V11D spectrophotometer with the following specifications:

Wavelength range:	325 – 1000 nm
Wavelength accuracy:	+/- 2 nm
Wavelength setting:	manual
Photometric accuracy:	+/- 0.5 %T

2.8 Statistical Analysis

A paired comparison using t-test distribution based on differences was performed since all dependent variables in each assay are two related samples. This is to assess whether the difference in the deposit mass values obtained by the application of biocides and the one obtained in the corresponding control samples could be considered significant (Pereira *et al.*, 2001). The t-test was performed using Microsoft Excel at a probability of p = 0.05 and p = 0.01, respectively.

3. Results and Discussion

3.1 Effect of the Selected Biocides on Biofilm Mass

The synergistic effect of monochloramine and glutaradehyde, and the individual effects of monochloramine and glutaraldehyde on accumulated biofilm mass on the control slides and the test slides during the periods of the experiment are presented graphically in Figure 1. It may be seen from Figure 1 that, (i) biofilm mass of control samples increased with time, indicating a progressive growth of biofilm microorganisms in the absence of biocides; (ii) the amount of biofilm is always reduced when selected biocides are applied to the system; (iii) combined effect of monochloroamine and glutaraldehyde on the biofilm mass is greater than either of those biocides acting independently; (iv) the oxidizing biocide (monochloramine) is more effective on biofilm microorganisms than glutaraldehyde (non-oxidizing biocide); and (v) mature biofilms (older biofilms) are more resistant to applied biocides.

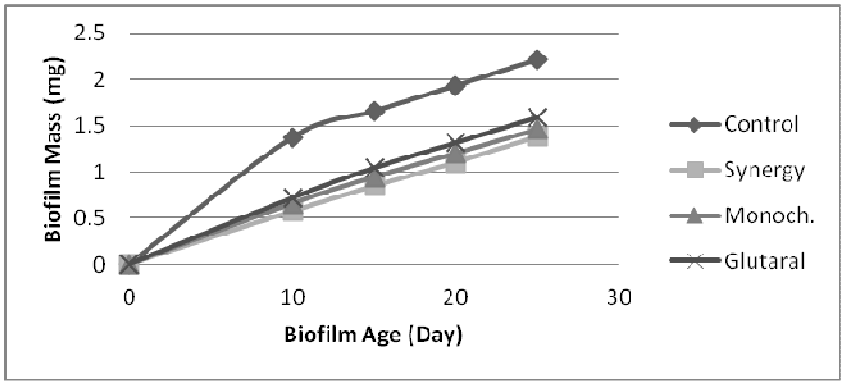

Figure 1. Comparison of the Effects of the Synergistic Biocide and the Individual Biocides on Biofilm

Statistical analysis demonstrate that the difference between the paired values (biofilm mass of control samples and those obtained by the addition of biocides) are statistically significant since confidence levels higher than

95% and 99% were obtained (P = 0.05 and P = 0.01), respectively. These values indicate that the biocides applied to the produced water affected the biofilm mass accumulated on the PVC slides in all test cases.

3.2 Biofilm Total Protein Content

The measured corrected absorbance (ΔA_{750nm}) and concentration of BSA standard are presented in Table 2. The BSA standard curve shown in Figure 2 was constructed by plotting (ΔA_{750nm}) BSA against serially diluted concentration of BSA. The axes of the curve were transposed as shown in Figure 3. The equation of the transposed curve in Figure 3 is quadratic in the form:

$$y = 3.3654x^2 + 0.7421x - 0.0106 \quad (2)$$

where,

y = protein concentration (mg/ml)

x = absorbance

The corresponding protein concentrations of the unknown samples are obtained by substituting the relative values of (ΔA_{750nm}) into equation (2) and solving for y.

Table 2. Absorbance at 750 nm for BSA standard curve Using Spetro V11D

Concentration (mg/ml)	Absorbance (ΔA_{750nm})
0.02	0.03
0.06	0.08
0.2	0.16
0.63	0.34

Working Range = 10-700 µg/ml

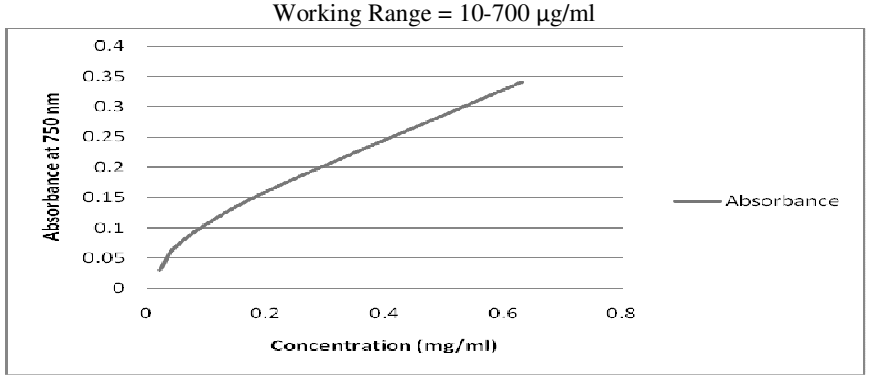

Figure 2. BSA (Protein) Standard Curve using Test Tube Procedure

Working Range = 10-700 µg/ml

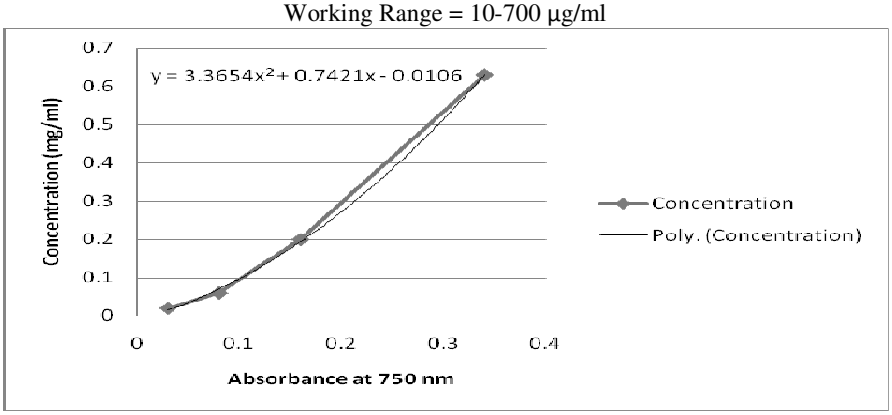

Figure 3. BSA (Protein) Standard Curve using Test Tube Procedure

The comparison of the effects of the selected biocides on biofilm total protein content presented in Figure 4 show that, (i) the protein contents of the control samples are greater than those obtained after the application of biocides, and increases with time (biofilm age). This suggests that increase in mass of biological constituents of the biofilm was partly responsible for the progressive increase in overall biofilm mass; (ii) total protein content is always reduced when selected biocides are applied. This shows that the selected biocides reacted specifically with biological constituents of biofilms; and (iii) the combined biocides caused greater reduction in total protein content of biofilms than if monochloramine or glutaraldehyde were applied independently. The percentage reduction in total protein content of the test samples relative to the control samples is presented in Figure 5.

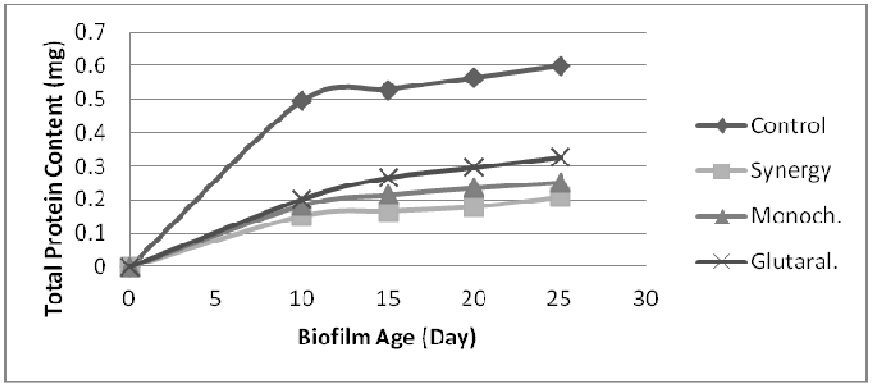

Figure 4. The Effect of the Biocides on the Total Protein Content of the Biofilms

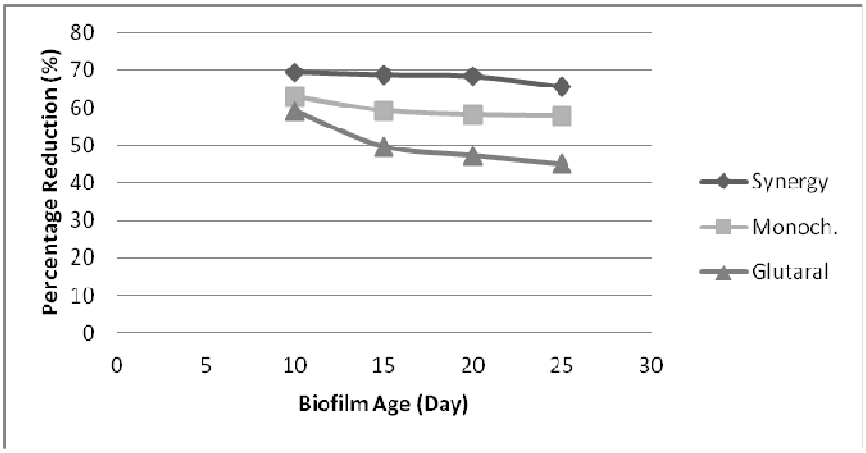

Figure 5. Percentage Reduction in Total Protein Content of the Test Samples Relative to the Control Samples after Treatment with Biocides

Figure 5 clearly shows greater percentage reduction in total protein contents of the biofilms (69.6 % reduction for a 10 day old biofilm) after the application of the combined biocides. It also indicates that monochloroamine which caused greater protein content reduction than glutaraldehyde is more effective against the investigated biofilm-microorganisms than glutaraldehyde.

3.3 Biofilms Total Polysaccharide Content

The measured corrected absorbance (ΔA_{630nm}) and concentration of glucose standard is presented in Table 3. The glucose standard curve shown in Figure 6 was constructed by plotting (ΔA_{630nm}) glucose against serially

diluted concentration of glucose standard. The axes of the curve were transposed as shown in Figure 7. The equation of the curve is linear in the form:

$$y = 4.9177x - 0.0009 \quad (3)$$

where,

y = glucose (polysaccharide) concentration (mg/ml)

x = absorbance

The corresponding polysaccharides concentrations of the unknown samples are obtained by substituting the relative values of (ΔA_{630nm}) into Equation (3) and solving for y.

Table 3. Absorbance at 630 nm for Glucose standard curve Using Spetro V11D

Concentration (mg/ml)	**Absorbance (ΔA_{630nm})**
0.05	0.0106
0.1582	0.0335
0.5	0.1
1.5813	0.32218

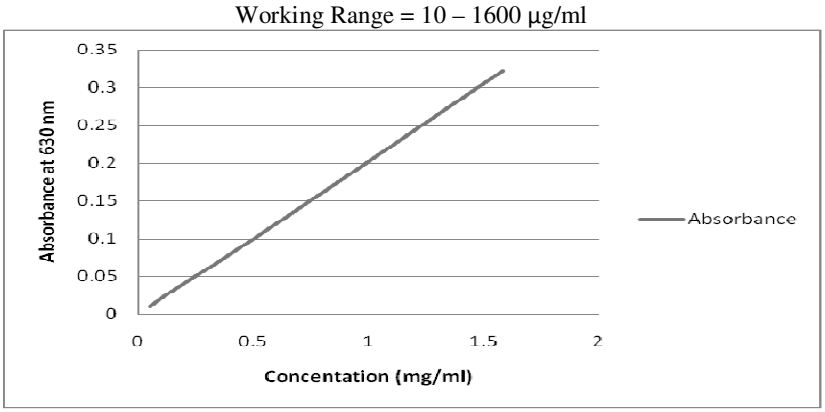

Figure 6 Glucose Standard Curve using Test Tube Procedure

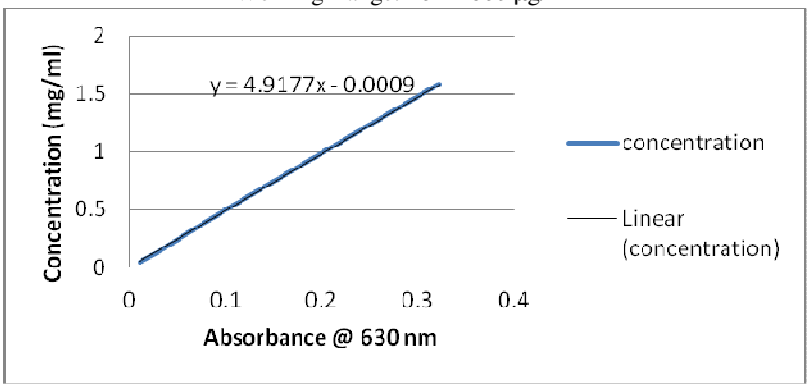

Figure 7. Transposed Glucose Standard Curve used for the Determination of Unknown Samples

The results of the biofilm total polysaccharide contents from the various experiments are presented in Figure 8. Figure 8 shows that: (i) the polysaccharide contents of the control samples are greater than those obtained after the application of biocides, and increased with time (biofilm age). This supports the fact that increase in mass of biological constituents of the biofilm was partly responsible for the progressive increase in overall biofilm mass; (ii) total polysaccharide contents is always reduced when selected biocides are applied. This shows again that the selected biocides reacted specifically with the biological constituents of biofilms; and (iii) the combined biocides caused greater reduction in total polysaccharide contents of biofilms than if monochloramine or glutaraldehyde were applied independently. The percentage reduction in total polysaccharide content of the test samples relative to the control samples are presented in Figure 9.

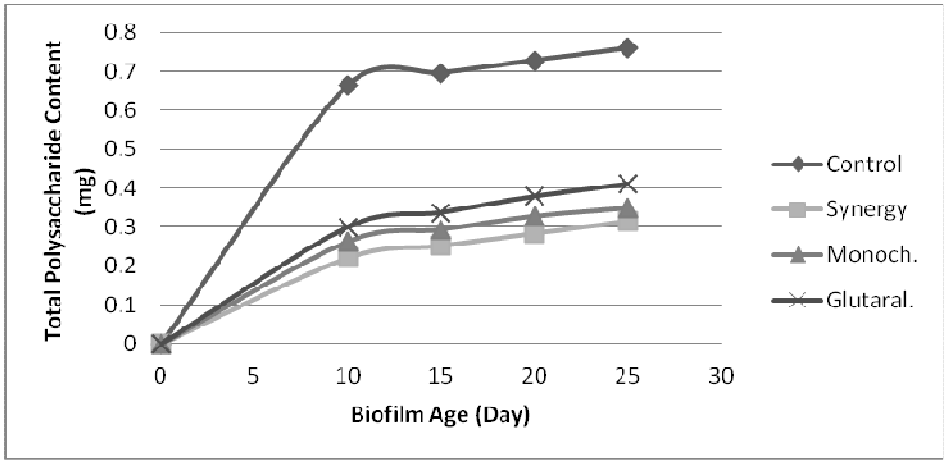

Figure 8. The Effect of the Biocides on the Total Polysaccharide Contents of the Biofilms

Figure 9 clearly shows greater percentage reduction in total polysaccharide contents of the biofilms (66.6 % reduction for a 10 day old biofilm) after the application of the synergistic biocides. Figure 9 also indicates that monochloroamine caused greater reduction in total polysaccharide content than glutaraldehyde. Hence, monochloramine is more effective against the investigated biofilm than glutaraldehyde.

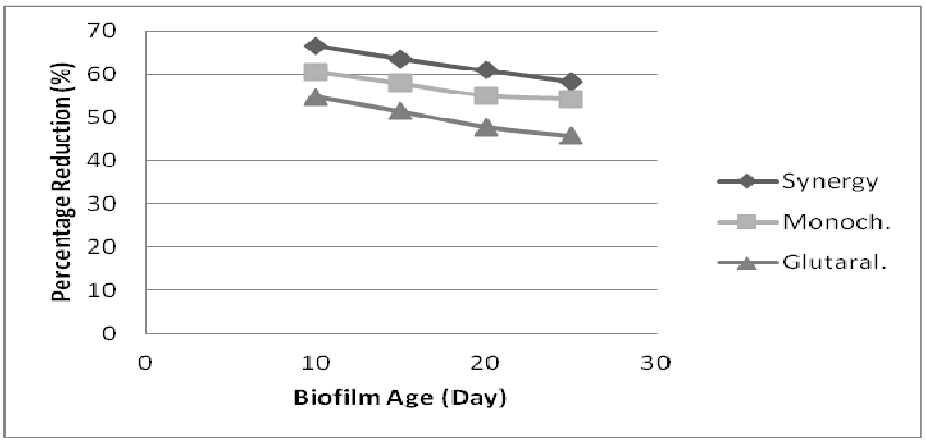

Figure 9. Percentage Reduction in Total Polysaccharide content of the Test Samples Relative to the Control Samples after Treatment with Biocides

4. Conclusions

Biofilm attachment on PVC slides was achieved with the aid of chocolate agar (enriched medium), in produced water. The biofilm mass of control samples and test samples were statistically significant since confidence levels higher than 95 % and 99 % were obtained. This shows that, the biocides applied to the produced water affected the accumulated biofilm on the PVC slides in all test cases. The synergy of monochloramine with glutaraldehyde caused greater reduction in the final biofilm mass than monochloramine or glutaraldehyde acting independently. Monochloramine proved to be more effective against biofilm-microorganisms than glutaraldehyde. Mature biofilms proved more resistant to applied biocides. The combined biocides caused greater reduction in total protein and polysaccharide contents of biofilms, respectively, than monochloramine or glutaraldehyde acting independently. Considering reduction in total protein contents and a 10 day old biofilm, synergistic biocide caused a 69.6 % reduction, monochloramine caused a 62.9 % reduction, and glutaraldehyde caused a 59.1 % reduction. In terms of reduction in total polysaccharide content and a 10 day old biofilm, synergistic biocide caused a 66.6 % reduction, monochloramine caused a 60.6 % reduction, and glutaraldehyde caused a 54.7 % reduction.

Therefore, if monochloramine and glutaraldehyde are combined, there effect on biofilm-microorganisms is greater than monochloramine and glutaraldehyde acting independently. The result indicates strong antimicrobial value for the combination of oxidizing and non-oxidizing biocides against biofilm-microorganisms.

5. Nomenclature and Abbreviations

5.1 Nomenclature

l	litre
ml	millilitre
µl	microlitre
µm	micrometre
mg	milligram
mg/l	milligram per litre
mg/ml	milligram per millilitre
µg/ml	microgram per millilitre
ε	Extinction Coefficient
nm	nanometre (unit of wavelength)

5.2 Abbreviations

EPS	Extracellular Polymer Substance or Exopolysaccharide Substance
PVC	Polyvinylchloride
UV	Ultra Violet
DOC	Dissolved Organic Compounds
MIC	Minimum Inhibitory Concentration or Microbiologically Influenced Corrosion
ND	Not Determined
NA	Not Achieved
K	Rate Constant

Acknowledgements

The authors acknowledge the moral and technical support provided by Dr. A. Rim-Rukeh. The financial support giving by High Chief Barrister Ezemonye N. Ezikiel-Amadi is also acknowledged. The authors also wish to acknowledge the support and contributions of some staff of Nigerian Agip Oil Company (Kwale/Okpai Gas Plant).

References

Biocides Disinfectant; Hydrogen Peroxide (H_20_2) and Silver (Ag), (n.d.). Retrieved from http://www.accepta.com. Accessed on March 12, 20013.

Characklis, W.G., Trulear, M.G., Bryers, J.D. and Zelver, N. (1982). Dynamics of Biofilm Processes: Methods, *Water Research Journal, Elsevier, 16* (7)*,* 1207-1216.

Coghlan, A. (1996). Slime City, *New Scientist 15,* 32-36.

Chen, X. and Stewart, P.S. (1996). Chlorine Penetration into Artificia Biofilm is limited by a Reaction-Diffusion Interaction, *Environ. Sci. Technol, 30,* 2078-2083.

Dreeszen, P.H., (2003). *Biofilm: The Key to Understanding and controlling Bacterial Growth in Automated Drinking Water System,* 2nd Ed., Edstrom Industries Inc. 2-17.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, S. (1956). Calorimetric Method for Determination of Sugar and Related Substances, *Analytical Chem., 28,* 350-356.

Fankhauser, D. B. (2009). Spectophotometer Use, University of Cincinnati Clermont College, Batavia. Lecture Material.

Fenollar, F. and Raoult D. (2004). Molecular Genetic Methods for the Diagnosis of Fastidious Microorganisms, *APMIS, 112* (11-12)*,* 785-807.

Hurh, P.G., Kubantseva, N.; and May, M.P. (1999). Micrbiologically Influenced Corrosion in the Fermilab Main Injector Magnet Low Conductivity Water System, Proceedings of the 1999 particle acceleration conference, New York, 1423-1425.

Pereira, M.O., Vieira, M.J., Beleza, V.M. and Melo, L.F. (2001). Comparison of Two Biocides – Carbonate and Glutaraldehyde – in the Control of Fouling in Pulp and Paper Industry, *Journal of Environmental Technology, 22,* 781-790.

Pietschmann, S., Hoffmann, K., Voget, M. and Pison, U. (2008). Synergistic Effects of Miconazole and Polymyxin B on Microbial Pathogens, *Vet Res Commun 33*, 489-505.

Singleton, F. L, Breen, A. W., and Mayer, M. J. "Method and Apparatus for Producing Synergistic Biocide Invention," *European Patent.Specification,* EP 1 928 784 B1, Oct. 14, 2009.

Videla, H. A. (1996). *Manual of Biocorrosion*. CRC Press, BOCA Raton, FL.

Videla, H.A. and Herrera, L.K. (2005): Microbiologically Influenced Corrosion: Looking to the Future. *International Microbiology, 8* (3), 3-11.