Microbial Influenced Accelerated Low Water Corrosion (ALWC): Species Isolation and Identification

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***Abstract*—Accelerated Low-Water Corrosion (ALWC) leads to degradation of maritime steel structure. This study is performed to isolate, identification, and study the growth of the microbes that induced ALWC. The sample of corrosion was taken from the jetty in Port Klang, Selangor, Malaysia. The rust collected was spread on agar containing artificial sea water medium. After 72 hours, five types of colonies grew around the rusts. The colonies were then cultured in five different petri dish containing artificial sea water medium and were left for 72 hours at temperature 27ºC. The pure colonies were then gram stained and it is found that the creamy, creamy white and yellow colonies were gram negative bacteria meanwhile the dark pink and light pink colonies were gram positive bacteria. The gram negative and positive bacteria were then tested with API Kit to identify their species. It is found that the yellow, creamy, creamy white, dark pink, and light pink are *Pseudomonas aeruginosa, Ochrobactrum anthropic, Pseudomonas luteola, Sphingomonas paucimobilis,* and *Burkholderia cepacia* respectively.**

Keywords*—accelerated low water corrosion, *Burkholderia cepacia*, microbial induced corrosion, *Ochrobactrum anthropic, Pseudomonas aeruginosa, Pseudomonas luteola, Sphingomonas paucimobilis.

I. Introduction

Accelerated Low Water Corrosion (ALWC) is a corrosion phenomenon that typically occurs at or below low-water level, and in tidal and brackish waters on steel maritime structures. ALWC is associated with bacterial activity and it is in a form of microbial induced corrosion (MIC). In advanced stage, ALWC can cause the structure infected to collapse. Although there are some other mechanisms that can cause accelerated corrosion on steel maritime structures at low water, the characteristics of the pattern of damage on steel structures is most similar to ALWC (Breakell*, et al*, 2005).

ALWC phenomenon has been noted in literature as in the first half of the 20th century. Average rate of ALWC can be 0.3 to 1.0 mm/side/year or more over time to the point of complete perforation of steel plate. However, there are possibilities that the rates can be higher once ALWC has initiated on a structure. ALWC can lead to a loss of 33% to 66% of the asset value. The process will compromise the integrity of the corroded structures and lead to costly repair or replacement (Breakell, *et al*, 2005). If is left untreated, ALWC may cause pre mature weakening, perforation and soon the collapse of maritime structures.

Statistical analysis of results that is obtained during a course in large European study on the causes of ALWC of steel piling in European harbours identified the factors which could serve as indicators when evaluating ALWC risk. A range of parameter varied significantly between ALWC sites and non-affected sites within the same harbour including thickness and morphology of corrosion products, pH values underneath corrosion products, presence of algae and invertebrates, content of organic carbon, hydrogen and oxygen in fouling layers, and high viable numbers of sulphur-oxidising bacteria (SOB) in corrosion products combined with the presence of sulphate-reducing bacteria (SRB). Moreover, mean tidal range and total organic carbon of seawater both showed higher statistics value in harbours experiencing ALWC attack than in unaffected harbours were identified as useful ALWC risk indicators (Iwona, *et.al*, 2007).

It is recognised that in the case of Microbial Induced Corrosion (MIC), the rates of corrosion recorded in the field are difficult to reproduce in laboratory experiments and the rates measured under former conditions are higher. The necessity of using biofilm bacteria recovered from the surfaces of corroding metallic materials and the need to carry out electrochemical corrosion testing using condition as close as the marine environment (Abdolahi, *et al*, 2014).

II. Material and Methods

A. Materials

Sample of rust was taken from bottom of the jetty at Port Klang, Selangor Malaysia. All the chemicals needed were provided by the faculty of chemical engineering, Universiti Teknologi Mara. Bacteriological peptone, sodium chloride, yeast extract, sodium sulphate, potassium chlrodie, sodium bicarbonate, sodium silicate, ammonium nitrate, boric acid, agar, distiller water, crystal violet, iodine, 95% ethyl alcohol, safranin solution, sterilized 0.85% sodium chloride (saline solution), JAMES reagent (Ref. 70 542), NIT 1 + NIT 2 reagent (Ref. 70 442), zinc reagent (Ref. 70 380), mineral oil (Ref. 70 100), McFarland standard (Ref. 70 100).

B. Medium Preparation

Artificial sea water medium was prepared for cultivation of marine bacteria. Table 1 shows the ingredients of the artificial sea water medium. The ingredients were suspended in 1000 mL distilled water. The mixture was mixed thoroughly using glass rod. The pH of the mixture was adjusted within range 7.6±0.2. The mixture is buffered to the desired pH by using acid or base. After the pH of the mixture was stabilized, the mixture was heated using hotplate with frequent agitation by using magnetic stirrer for 1 minute to dissolve the powder. After the powder was completely dissolved, the medium was sterilized by autoclaving at 121°C for 20 minutes. After the medium was autoclaved, leave it at room temperature and wait for a few minutes until it cooled down. The agar was then poured into 20

petri dishes and the broth was poured into 20 bottles. The leftover was stored in the refrigerator. After the agar was solidified under aseptic condition and normal environment condition, all the petri dishes containing agar were stacked and wrapped using aluminium foil. All the bottles containing broth were placed in a large beaker and then wrapped using aluminium foil. The medium were stored into the refrigerator (ZoBell, *et al*, 1941).

Table 2.1: Medium composition for artificial sea water agar and broth

Ingredients (g) for 1000 mL dH_2O	**Agar (g)**	**Broth (g)**
Peptone	5.0000	5.0000
Sodium Chloride	19.450	19.4500
Yeast extract	1.0000	1.0000
Sodium Sulphate	3.2400	3.2400
Potassium Chloride	0.5500	0.5500
Sodium Bicarbonate	0.1600	0.1600
Sodium Silicate	0.0040	0.0040
Ammonium Nitrate	0.0016	0.0016
Boric Acid	0.022	0.022
Agar	15.00	-
Total	44.572	29.572

C. Isolation of Bacteria

The rusts of the sample were spread on the agar and into the broth prepared. The step was performed under fume hood to prevent agar and broth contaminated from surrounding. The petri dish and universal bottle containing the sample were placed in an open container in room at temperature 26°C. They were monitored daily until growth of bacteria can be seen.

D. Analyzing and Culturing Colonies

The different species of bacteria was observed and the colony of different species were cultured on agar. The colony was cultured under aseptic technique under fume hood. The wire of inoculum loop was sterilized by passed it through the flame of Bunsen burner until the entire length of the wire becomes glowing read from the heat. After that, the lid of the plate culture was partially lifted and it was opened just enough to insert the inoculation loop. The loop was touched to an area of the agar with no growth to cool down the loop to prevent from killing the bacteria. An isolated colony was chosen and scraped off a very small amount of culture with the loop. The lid of the plate was closed immediately once the colony has been picked up. The loop was streaked across the surface of the agar medium using pattern as shown in Figure 3.7.2.0. The loop was kept parallel to the agar surface and streaking started at “12:00 position” of the plate and streak side to side. The lid of the plate was closed and sealed with parafilm. The culture was incubated under temperature 26°C for a few days until visible growth can be seen.

E. Gram Stain

A drop of water was placed on a clean slide. The wire of inoculum loop was sterilized by passed it through the flame of Bunsen burner until the entire length of the wire becomes glowing read from the heat. After the wire of inoculum loop was cooled, the a small amount of culture was scraped off from the agar surface and touched several times onto the drop of water on the slide until it turns cloudy. After that, the inoculum loop was passed through the flame to kill the bacteria. After the inoculum loop was cooled, the loop was used to spread the suspension of water and bacteria over the surface of the slide to form a thin film. The slide was then heat fixed by passing it briefly over the flame. Five drops of crystal violet was added over the fixed culture and let it stand for approximately one minute. The excess stain was rinsed off the slide using tap water by picking the slide up and holding it at an angle over the sink. The excess stain and water was blotted off using filter paper but do not rub it. Five drops of iodine was added to the smear and let it stand for approximately one minute. The excess iodine was rinsed off the slide using tap water by picking the slide up and holding it at an angle over the sink. The excess stain and water was blotted off using filter paper but do not rub it. The slide was tipped and several drops of 95% ethyl alcohol were added to the upper end. The alcohol was allowed to flow over the smear. After 30 seconds, the slide was rinsed again. Five drops of safranin was added to the slide and let it stand for approximately one minute. The slide was rinsed, drained and blotted away from excess moisture. The slide was then examined under oil by using microscope under magnification 100x. The results were recorded (Aryal, 2015).

F. Determination of Bacteria Using API Kit

An incubation box (tray and lid) was prepared and distributed about 5 mL of distilled water into the honey-combed wells of the tray to create a humid atmosphere. The strip was removed from its packaging and placed in the incubation box.

a) API 20E Kit

By using an inoculum loop, 2-3 colonies of the species were taken and placed into a universal bottle containing 5 mL of saline solution (sterilized 0.85% sodium chloride). The suspension was homogenized and must be used immediately after preparation. By using a sterilized pipette, the tube and cupule of test CIT, VP and GEL was filled with the bacteria suspension. For other test, only the tube was filled. The tests ADH, LDC, ODC, H_2S and URE were overlaid with mineral oi to create anaerobiosis. The strip was incubated at 27ºC ± 2ºC for 18-24 hours. After incubation period, the strip was read by referring to the Reading Table provided in the manual. If 3 or more tests (GLU test + or -) were positive, the spontaneous reactions were recorded on the result sheets. If the tests positive were less than 3, the strip was reincubated for a further 24 hours without adding any reagents. For TDA Test, 1 drop of TDA reagent was added. For IND Test, 1 drop of JAMES reagent was added. For VP Test, 1 drop of VP 1 and VP 2 reagents were added and waited for at least 10 minutes for the reaction (API 20E REF 20100/201600).

b) API 20NE Kit

By using an inoculum loop, 2-3 colonies of the species were taken and placed into a universal bottle containing 2 mL of saline solution (sterilized 0.85% sodium chloride). A suspension with a turbidity equivalent to 0.5 McFarland was prepared. The suspension was homogenized and must be used immediately after preparation. The tests NO_3 to PNPG was performed by distributing the saline suspension into the tubes by using a sterilized pipette. An ampule of API AUX Medium was opened and 200 µL of remaining saline solution was added into the ampule. The mixture was homogenized well. The tubes and cupules of tests GLU to PAC was filled with the suspension. Mineral oil

was added to the cupules GLU, ADH and URE until a convex meniscus is formed. The incubation box was closed and incubated at 27ºC ± 2ºC for 24 hours. After incubation period, the strip was read by referring to the Reading Table provided in the manual. The spontaneous reactions of GLU, ADH, URE, ESC, GEL and PNPG were recorded. For NO_3 test, 1 drop of NIT 1 and NIT 2 reagents were added to the NO_3 cupule. After 5 minutes, a positive reaction to be recorded on the result sheet. If the result was negative, 2-3 mg of Zn reagent was added to the NO_3 cupule (Api 20 NE REF 20050).

After the tests were performed, all the result were the result was read by referring to the Reading Table. The result sheet was filled. On the result sheet, the values corresponding to positive reactions within each group were added. 7 digit profile number was obtained for the 20 tests of the API 20 E Strip. By using database (V4.0), the 7 digit profile number was entered manually and the database showed the name of the species.

G. *Growth Profile*

a) *Preparation of Cell Culture*

15 mL (10%) of inoculum was transferred to the main experiment media. The shake flask is capped with cotton plug and swabbed with 70% ethanol. It is then being incubated in a thermostated rotary shaker at required rotational speed and temperature for 24 hours.

b) *Sampling*

2 mL of each sample was withdrawn by using micro pipette every time sampling was done, where 1 mL to measure optical density and 1 mL to measure cell dry weight (biomass concentration: g/L). The sample was transferred into the sampling tube with interval time for every 12 hours.

c) *Absorbance Analysis (Optical Density)*

In order to analysis the absorbance for optical density (OD), 1 mL of sample was transferred into a cuvette and the optical density measurement was made using spectrophotometer with its wavelength at 600nm. The spectrophotometer was calibrated to zero by blank consists of 1 mL distilled water. Absorbance analysis is used to measure cell growth. Low transmittance causes high absorbance. High number of cells means high value of absorbance.

d) *Cell Dry Weight (Biomass Concentration)*

Biomass concentration can be determined by its cell dry weight. Firstly, empty centrifuge tubes were weighed and noted their initial mass. 1 mL of the sample was added to the weighted centrifuge tube. The sample was then centrifuged at 10 000 rpm and at 4°C temperature for 20 minutes. The supernatant was taken out. The washing with distilled water and centrifuging was repeated. The dried centrifuged tubes were left in desiccator. The centrifuge tube was weighed and noted as the final mass. Cell Dry Weigh can be determined by subtracting final mass with its initial mass.

III. Results and Discussion

A. *Isolation and identification microbes that induced Accelerated Low Water Corrosion.*

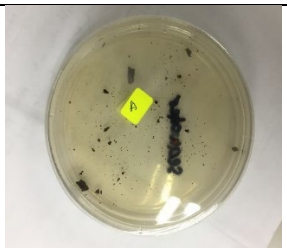
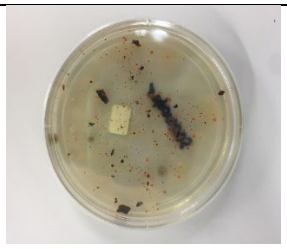
Accelerated low water corrosion (ALWC) has seven corrosion zones which are atmospheric zone, splash zone, tidal zone, low water zone, immersed zone, embedded zone, and concentrated corrosion in all zones (Accelerated Low Water Corrosion, 2005). Fig. 3.1 shows the sample of ALWC at Port Klang which occur in the tidal zone where metals are alternately submerged in seawater and exposed to splash zone as tide decrease. The sample was taken at the jetty in Port Klang where the changing of tides causing the metal structure of the jetty to submerged during high tides. Tides causes water rises and falls causing wetting and drying exposed metal structure of the jetty. Fig. 3.1 shows the metal structure of the jetty in Port Klang that were then taken as sample for the experiment.

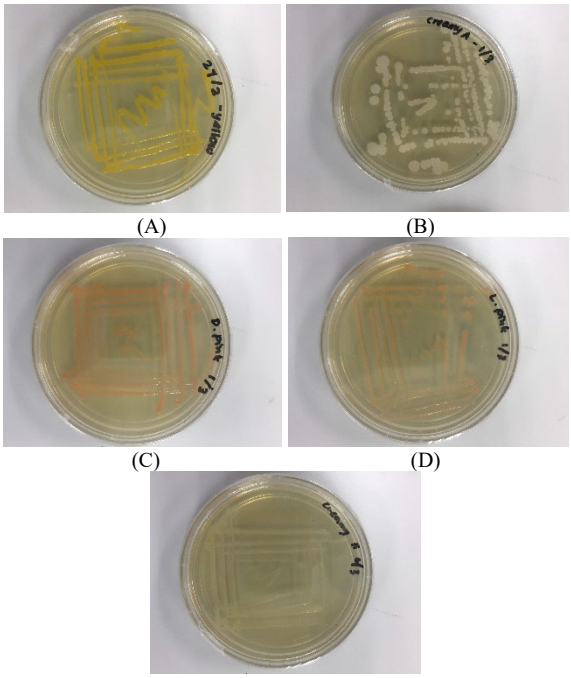
Fig. 3.1: Sample of Accelerated Low Water Corrosion (ALWC) at Port Klang

In the sea water, there are many bacteria that can harm the living things, the source for food for the aquatic animals, cause corrosion to the steel and many more. In this study, the main concern is the bacteria that can cause corrosion.

After incubation at 27ºC for 72 hours, the rusts from the jetty at Port Klang that has been spread on the agar plate showed bacteria growth as shown in Table 3.1. It can be seen that 5 different colour that represents 5 different colonies grow around the rusts. This proves that the bacteria grow around the steel and cause steel corrosion in the sea water. The 5 colonies were then cultivated into 5 different petri dishes with artificial sea water as their medium. After 72 hours, the colonies grew into bigger and brighter colonies. As shown in Fig. 3.2, the 5 types of colonies are bright yellow colony, creamy white colony, dark pink colony, light pink colony, and creamy colony. Since the name of the species were not known, the species were labelled based on their colour.

Table 3.1: Growth of ALWC promoting species before and after 72 hours of incubation

Before incubation	After incubation
	

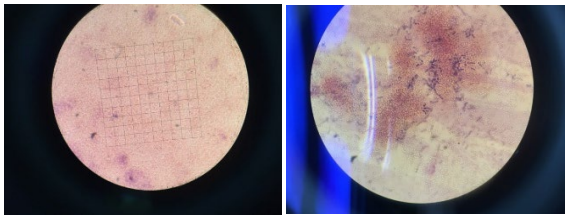


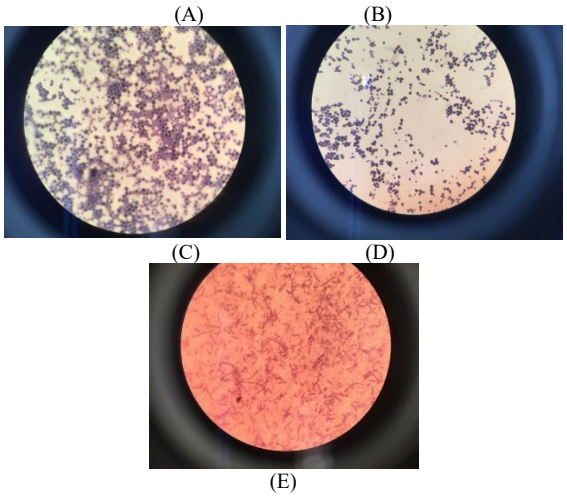
(A) (B) (C) (D) (E)

Fig. 3.2: Colonies of ALWC promoting bacteria. (A): Bright yellow colony. (B) Creamy white colony. (C) Dark pink colony. (D) Light pink colony. (E) Creamy colony

Each of the colonies was proceed for gram staining to differentiate the bacteria into gram positive or gram negative bacteria which then tested with API Kit to determine its species. The gram negative bacteria was tested with API Kit 20E meanwhile gram positive bacteria was tested with API Kit 20NE. When the bacteria was stained with crystal violet, some of the bacteria were able to retain the crystal violet and some were decolorized by alcohol. The cell walls of gram positive bacteria have a thick layer of peptidoglycan and content of lipid is low. Decolorizing the cell caused the thick cell wall to dehydrate and shrink which then closes the pores in the cell wall and prevent the stain from exiting the cell. Thus, ethanol cannot remove the crystal violet-iodine complex that was bound to the thick layer of peptidoglycan of the gram positive bacteria and appears blue in colour. Meanwhile for gram negative bacteria, the cell walls also take up crystal violet-iodine complex. However, because of the thin layer of peptidoglycan and thick outer lipid layer, crystal violet-iodine complex washed off. When they were exposed to alcohol, the decolourizer dissolved the lipids in the cell walls, allowing the crystal violet-iodine complex to exit out of the cells. When safranin was added, they took the stain and appears red in colour (Aryal, 2015).

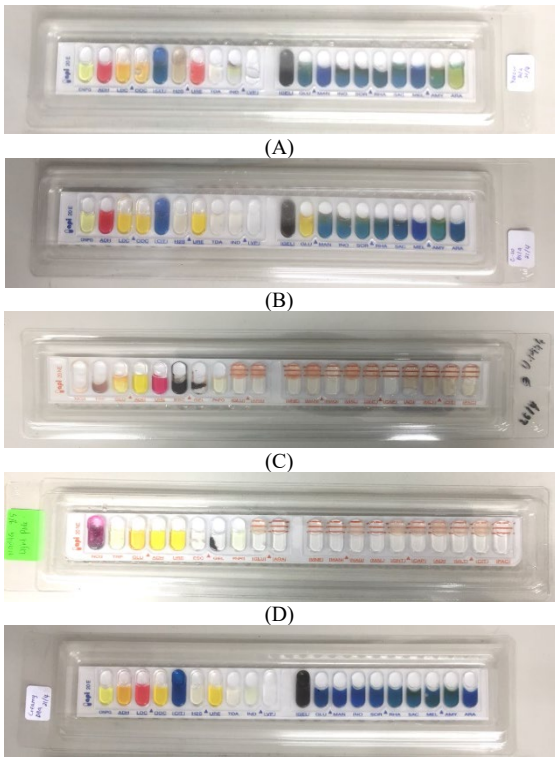
Based on the result of gram staining as shown in Fig. 3.3, the creamy, creamy white and yellow colonies appeared red in colour under the microscope indicate that the bacteria were gram negative bacteria. Meanwhile the dark pink and light pink colonies appeared purple in colour under the microscope indicate that the bacteria were gram positive bacteria.





(A) (B) (C) (D) (E)

Fig. 3.3: Gram staining of ALWC promoting bacteria. (A): Bright yellow colony. (B) Creamy white colony. (C) Dark pink colony. (D) Light pink colony. (E) Creamy colony



(A) (B) (C) (D) (E)

Fig. 3.4: API Test Slips of ALWC promoting bacteria. (A): Bright yellow colony. (B) Creamy white colony. (C) Dark pink colony. (D) Light pink colony. (E) Creamy colony

Fig. 3.4 shows the result for API Kit test. Based on the result, it is found that yellow colonies represent *Pseudomonas Aeruginosa*. *Pseudomonas* are a large group of free-living bacteria that live primarily in soil, seawater, and fresh water. Especially, *Pseudomonas aeruginosa* can be found in natural habitat such as in soil, seawater, sewage, and associated with some plants (Ugur, *et al*, 2012). *P. aeruginosa* is one of the aerobic slime-form-ing bacteria that are ubiquitous in marine environment that corrode steel structures. *P. aeruginosa* is a dominant bacterium in marine environments and one of the aerobic SFBs, which form a biofilm layer on steel surface The chemical reaction of the biofilm layer with steel and the formation of differential aeration cells create conditions on steel that initiate and accelerate the corrosion process. *P. aeruginosa*

can reduce ferric to ferrous iron, thus exposing steel to further oxidation since ferrous iron compounds are more soluble and the protective ferric iron layer is solubilized by this process This type of bacterium in its biofilm state could be detrimental to steels and cause severe corrosion damages. It forms a heterogeneous biofilm layer on the steel surface and causes the formation of differential aeration cells, which induce localized corrosion iron is an essential nutrient for *P. aeruginosa* (Abdolahi, *et al*, 2014).

Meanwhile, creamy colonies represent *Ochrobactrum anthropic.* Certain types of microbial biomass can retain high quantities of metals by biosorption, which depends on the affinity between the binding sites on the molecular structure of the cellular wall and the metallic species or its ionic forms (Manrique, Magana, Lopez, & Guzman, 2000). Biosorption is the ability of microbial biomass to collect heavy metals from wastewater through physico-chemical pathways of uptake or metabolically mediated (Ahalya, Ramachandra, & Kanamadi, 2003). Ochrobactrum anthropic able to bind and accumulate metal ions in the form of superficial mucilage layer. *O. anthropi* is a good adsorbing medium for metal ions and had high adsorption yields for the treatment of wastewater containing copper, cadmium, and chromium (Guven, *et al*, 2003).

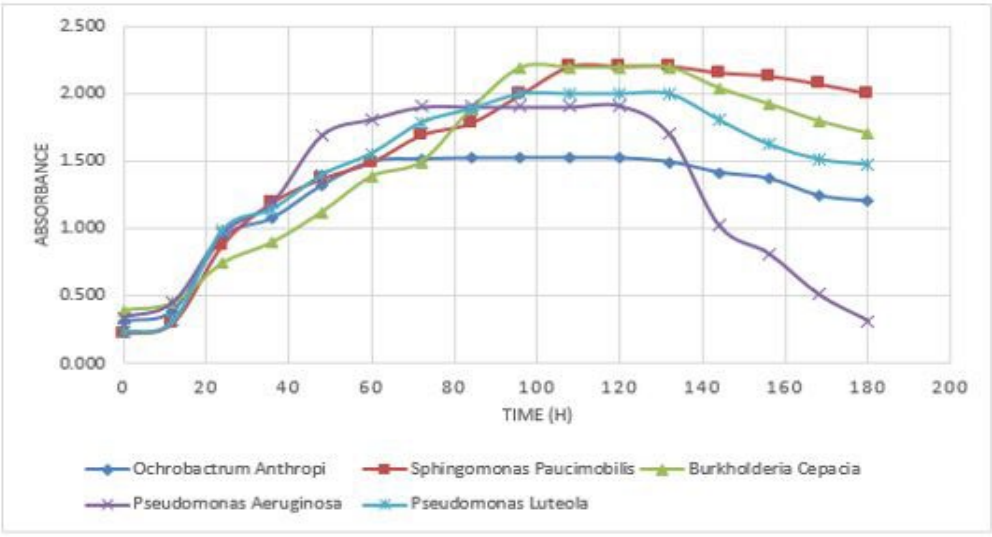
Creamy white colonies represent *Pseudomonas luteola*. *Pseudomonas luteola* can absorb certain heavy metals such as Cr(VI) and Al(III). Both ions are found in industrial wastewaters. These metals are specifically targeted by *P. luteola* strain TEM05. Under relatively acidic conditions (pH 4 for Cr(VI) and 5 for Al(III). Experiments indicated a maximum adsorption capacity of 55.2 mg g-1 for Al(III) and 3.0 mg g-1 for Cr(VI) (Ozdemir & Baysal, 2004). *P. luteola* strain TEM05 can also produce exopolysaccharide (EPS) that is utilized in the adsorption of nickel and copper. For the adsorption of Ni and Cu to occur, the strain must be immobilized in a calcium alginate beads. With this enhancement, maximum adsorption capacities range from 45.87-50.81 mg g-1 and 52.91-61.73 mg g-1, respectively (Ozdemir, *et al*, 2005).

Dark pink colonies represent *Sphingomonas paucimobilis.* *Sphingomonas paucimobilis* is non-fermentative, aerobic, and gram negative motile bacillus (Puca, *et al*, 2015). It is recovered from sea water, sea ice, water, river, and waste water. *Sphingomonas paucimobilis*'s distribution can be explained by their ability to survive and grow at low temperature, low nutrient concentration and in toxic chemical environments (Koskinen, *et al*., 2000). In the latter case, the presence of copper-tolerant film-forming *Sphingomonas paucimobilis*, resulted in a 20-fold increase in the corrosion rate of 90/10 Cu-Ni and Al-bronze alloys. *Sphingomonas paucimobilis* is also found grown on stainless steel. (John, 1999).

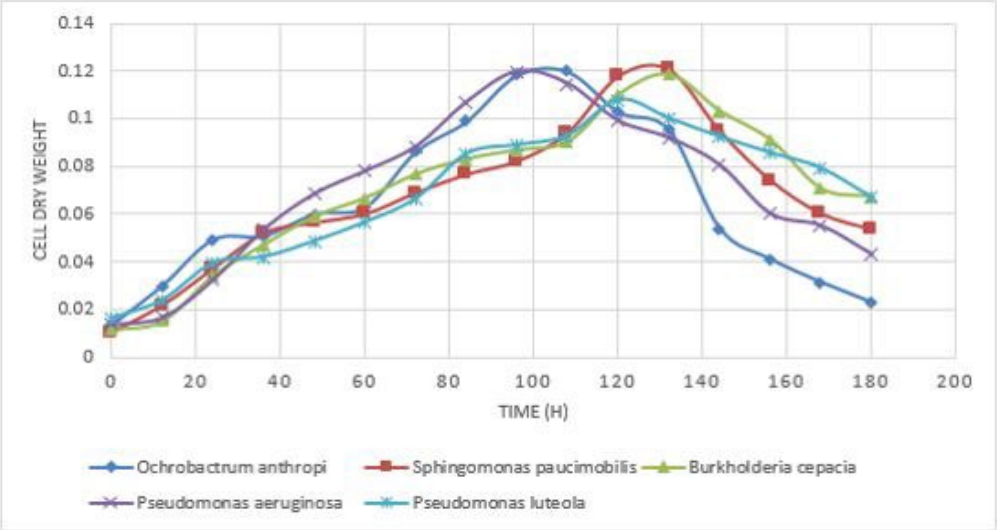
Light pink colonies represent *Burkholderia cepacia.* *Burkholderia cepacia* is a gram negative, motile, aerobic and catalase- and oxidase-positive bacteria (Hui, 2014). *B.cepacia* are closely related to *Pseudomonas* genus. *Pseudomonas* genus known to be the pioneer colonizers in biofilm formation causing corrosion that is found in marine and other aquatic environment. B.cepacia is one of the organism that is attached on the corroded galvanized steel (Dogruoz, Minnos, Illhan-Sungur, & Cotuk, 2009). *B.cepacia* form biofilms leading to negative outcomes such as corrosion pits, infections, and fouling depending on where they occur. Bacterial biofilms can only occur if the bacteria successfully attaches to a surface (Hui, 2014). The biofilms formation proves that *Burkholderia cepacia* attached to the metal surface.

B. Growth of the Accelerated Low Water Corrosion (ALWC) on artificial sea water.

The bacteria isolated from the sea water was then used to study the growth of the ALWC that was cultivated on artificial sea water agar. The reading was taken every 12 hours because the bacteria have slow growth rate. They grew within approximately 72 hours, thus the reading were taken for 5 days to observe the time taken for the bacteria to grow and died. Organism's growth is affected by nutritional and physical factors. Nutritional factors include the components or recipe of the growth medium meanwhile the physical factors are temperature and pH. The bacteria was cultivated on artificial sea water was to provide the bacteria the same environment as they grow in the sea water. The growth of bacteria can be observed by plotting the cell growth (absorbance) versus the time of incubation which is known as standard curve.



Graph 3.1: Absorbance of five species of bacteria vs time



Graph 3.2: Cell dry weight of five species of bacteria vs time

The absorbance was measured by using spectrophotometer. It measured the optical density (OD) which is the amount of light absorbed by the bacterial suspension (Bacterial Growth Curve). Thus, the optical density is related to the number of bacteria present. It is a convenient way to measure the cell growth rate of the bacteria. As the optical density increase, the microbial cell mas also increases. Standard growth curve has four phases which are lag phase, exponential phase, stationary phase and death phase. Graph 3.1 shows that all the five species of ALWC have all the four phases of the standard growth curve. Based on Graph 3.1, all the five species have similar length of lag phase in shorter time. This means the bacteria took shorter time to adapt with the new environment. This is because the medium that has high content of sodium chloride which was similar to their previous environment, which was the actual sea water. The bacteria started to synthesis the proteins, vitamins and co-enzyme that was needed for growth. Based on Graph 3.1, the time taken for the *Ochrobactrum anthropi* in the exponential phase was the shortest. This showed that *Ochrobactrum anthropic* has the greatest metabolic activity and it started to replicate the DNA by binary fission. The bacteria achieved its optimum growth rate and number of bacteria increases exponentially and the replications occur rapidly. It stop replicating at 60^{th} hour and undergoes stationary phase. The nutrients in the medium were used up by the bacteria as it continues to grow rapidly. This caused the accumulation of toxic metabolites, waste materials and the inhibitory compounds. This causes the conditions of the medium such as temperature and pH to change. Thus, this created unfavourable environment for the growth of bacteria. The rate of reproduction slowed down and the cells undergo replication is equal to the number of cell death. Finally, the microorganism stopped the replication. There is not increase in cell number and hence, the growth rate of microorganism is stabilized. *Ochrobactrum anthropic* undergo death phase at 120^{th} hour. The reducing of nutrients and increase in toxic metabolites, waste materials and the inhibitory compound in the medium caused the bacteria to die. As this happen, the bacteria completely loses its ability to replicate. It began to die and the death rate is rapid at constant rate. The number of dead cells exceeds the live cells.

IV. CONCLUSION

Accelerated Low-Water Corrosion (ALWC) leads to degradation of maritime steel structure. ALWC remove steel from maritime structures causing reduction in its value. It can be said that the main cause of the corrosion is the ALWC bacteria, which are *Pseudomonas aeruginosa, Ochrobactrum anthropic, Pseudomonas luteola, Sphingomonas paucimobilis,* and *Burkholderia cepacia*. However, a deeper study regarding their mechanism has to be done in order to prove that the corrosion is by the ALWC bacteria. It is highly recommended for owners and engineers to investigate and address the effects of ALWC and as well as to include ALWC corrosion protection for all maritime structures.

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