CHAPTER 5

SHIKONGO-NAMBABI MNNN, KACHIGUNDA B and VENTER SN (2010) **EVALUATION OF OXIDISING DISINFECTANTS TO CONTROL *VIBRIO* BIOFILMS IN TREATED SEAWATER USED FOR FISH PROCESSING**. *Water SA* **36** 215-220 3

5.1 ABSTRACT

Marine fish-processing plants often use sea-water during their operations. Chlorination and UV are commonly used for disinfection of this water but may not be effective in preventing biofilm formation within the water distribution network. These biofilms negatively impact on water quality and could lead to contamination of fish products. During a recent study *Vibrio alginolyticus* strains were detected on processed hake. The presence of most *Vibrio* spp. on fish products is of consumer safety concern and needs to be minimised. Water treatment strategies effective for sea-water disinfection but with minimal negative effect on fish quality are required. In this study the effectiveness of chlorine, ozone and hydrogen peroxide (H_2O_2) to inhibit mature biofilms or biofilm formation in natural sea-water was investigated. Two *V. alginolyticus* strains (V590 and V595) isolated from hake fish as well as the type strains of *V. alginolyticus* LMG4409 and *V. parahaemolyticus* LMG2850 were used. Chlorine was ineffective as experiments showed that strains V590, V595 and *V. parahaemolyticus* LMG2850 could form biofilms even in the presence of 4 mg/ℓ chlorine. When ozone was used, biofilm initiation and formation of only 2 strains of *V. alginolyticus*, i.e. LMG 4409 and V590, were completely inhibited at 1.6 mg/ℓ or 0.8 mg/ℓ ozone, respectively. Hydrogen peroxide performed the best of all the disinfectants evaluated in this study. Inhibition of biofilm formation was observed for all strains at 0.05% H_2O_2. The mature biofilms were more resistant to H_2O_2 but they were all eliminated at 0.2% concentrations. This study indicated that H_2O_2 is the most effective biocide to prevent biofilm formation in sea-water distribution networks and could potentially be used as an alternative or supplementary disinfectant of sea-water in marine fish-processing plants.

KEYWORDS: *V. alginolyticus*, *V. parahaemolyticus*, biofilms, H_2O_2, disinfection, sea-water

5.2 INTRODUCTION

The use of sea-water instead of freshwater during marine fish processing is an economical alternative especially in arid countries such as Namibia which are often faced with severe fresh water shortages. The water is typically used for activities such as cooling of the product or washing of surfaces and apparatus in the plant. Sea-water may, however, contain human pathogenic bacteria including *Vibrio* species such as *V. parahaemolyticus, V. cholera, V. vulnificus* and *V. alginolyticus* (Thompson et al., 2004; Wekell et al., 1994). The water, therefore, requires treatment and disinfection before being used for the processing of fresh fish. Currently chlorination and UV treatment are most commonly used to disinfect the water.

A recent study at a fish-processing plant showed that the quality of hake deteriorated along the processing line (Shikongo-Nambabi et al., 2010a). *Vibrio* species were isolated at the intermediate stages of processing. None of these species were isolated from the fish which had just been delivered to the factory for processing. From the study it was clear that *Vibrio alginolyticus* strains were introduced by the treated sea-water used during processing. Indications were that the major source of these bacteria was not inefficient treatment of the raw water but the subsequent formation of biofilms in the distribution network in spite of the presence of residual chlorine. *V. cholerae,* (Faruque et al., 2006; Mueller et al., 2007), *V. vulnificus* (Joseph and Wright, 2004). *V. alginolyticus* (Kogure et al., 1998) and *V. parahaemolyticus* (Enos-Berlage et al., 2005) are well known to form biofilms.

The presence of *Vibrio* spp. in the sea-water used during processing of the fish poses a potential health hazard to consumers and should be minimized and controlled. Chlorine, or chloramines are typically used to control bacterial biofilms in freshwater distribution systems (DeQueiroz and Day, 2007; Momba et al., 2002), but many studies have described the ineffectiveness of chlorine in controlling biofilm formation (Chu et al., 2003; Momba, 1998). There is, however, little information in the literature on alternative

disinfectant strategies to control biofilm formation in sea-water systems. The type and level of disinfectant to be used are, however, restricted as high concentrations of disinfectants have been shown to cause discolouration of the fish due to oxidation of the myoglobin (Kim et al., 2000).

The aim of this study was to evaluate 3 different oxidising disinfectants to control biofilm formation by selected *Vibrio* isolates in sea-water distribution systems. For this purpose chlorine, ozone and hydrogen peroxide were tested at a range of concentrations. Improvement in the treatment, disinfection and microbiological quality of the sea-water used for fish processing would help to ensure the safety of the final product and protect the consumers.

5.3 MATERIALS AND METHODS

5.3.1 Sampling and analysis of treated sea-water

Samples of sea-water used in a fish-processing facility, in Walvis Bay, Namibia, were collected to evaluate the current treatment system. Samples were taken from the raw water, after flocculation and chlorination, after UV treatment at 300 J/cm^2 at 254 nm (UV Pure, Cape Town) and at 2 points within the factory after distribution through the network. Thiosulphate (0.04% w/v) was added to all samples to neutralize the residual chlorine. Samples were refrigerated during transport and storage. After serial decimal dilutions samples were plated in duplicate onto nutrient agar (NA) (Oxoid), sea-water agar (SWA) (Atlas, 2006) and thiosulphate citrate bile salts sucrose agar (TCBS) (Farmer and Hickmann-Brenner 1991). NA and TCBS plates were incubated at 37^{o}C while SWA plates were incubated at 22^{o}C.

The studies to evaluate the ability of *Vibrio* strains to form biofilms and to determine the effect of selected disinfectants on the inhibition and control of biofilm formation were conducted using either natural or artificial sea-water. The artificial sea-water (ASW) consisted of water in which 0.4 M NaCl, 0.1 M $MgSO_4.7H_2O$, 0.02 M KCl and 0.02 M $CaCl_{2.}.2H_2O$ was dissolved (Farmer and Hickman-Brenner, 1991).

5.3.2 Bacterial cultures and inoculums

Four bacterial isolates were included in this study. Two of the isolates were *Vibrio alginolyticus* strains previously isolated from the processing plant. Isolate V590 was isolated from hake after filleting by the Baader machine and V595 from the finished product. These strains were identified using the API 20E (BioMérieux) test and amplification of the collagenase gene according to the method of Di Pinto et al. (2006). The type strains of *V. alginolyticus* (LMG 4409) and *V. parahaemolyticus* (LMG 2850)

were also included for comparative purposes. Both these strains were initially isolated from food- poisoning incidents related to seafood.

Strains were cultured individually on sea-water agar (SWA) by incubation overnight at 37^{o}C. This culture was then used to inoculate 250 mℓ sterile nutrient broth (NB) containing 3% NaCl and incubated unshaken at 37^{o}C for 24 h. The cell suspension (75 mℓ) was harvested by centrifugation at 4 000 r/min for 30 min and washed twice with 0.85% (w/v) NaCl. The pellet was re-suspended in 15 mℓ 0.85% NaCl to obtain an inoculum containing ±10^{7} cfu/mℓ.

5.3.3 Biofilm formation in artificial sea-water

The ability of the selected bacteria to form biofilms in sea-water was evaluated using a Pedersen's device (Pedersen, 1982). The device contained 20 clean microscope glass slides (7.6 × 2.6 cm) and was connected by means of silicone tubing via a peristaltic pump to a reservoir containing artificial sea-water. Sterile artificial sea-water (ASW) instead of natural sea-water was used for these experiments due to logistical constraints of transporting and storing large volumes of water. The ASW was supplemented with bacteriological peptone (Biolab) and nutrient broth (NB) (Biolab) at 1 g/ℓ each. The effluent was collected, disinfected and discarded. The system was disinfected by perfusing the systems for 24 h with tap water containing 2.5 g/ℓ residual chlorine at a rate of 500 mℓ/h. Afterwards the chlorine was neutralised by allowing 12 ℓ of sterile tap water containing 1.0 mg/ℓ sodium thiosulphate to run through the systems for another 24 h. The system was then rinsed with 10 ℓ of sterile tap water. Sterility was assessed by plating 0.1 mℓ of the effluent onto TCBS agar and incubating the plates at 37^{o}C for 24 h.

At the start of each experiment ASW was allowed to flow through the Pedersen's device for 1 h. After inoculation with 5 mℓ of the cell suspension the flow of ASW was maintained at 500mℓ/h. Slides were withdrawn at 24 h intervals up to 96 h. Each slide was washed in sterile water, transferred to a 100 mℓ screw-cap bottle containing 10 mℓ sterile 0.85% (w/v) NaCl solution supplemented with 0.01 mg/l sodium thiosulphate, and

sonicated (Integral systems) for 5 min. The suspension was diluted, plated in duplicate on TCBS agar plates and incubated at 37^{o}C for 24h after which the culturable counts were determined. The degree of biofilm formation was calculated as the density of bacteria per cm^{2} (Momba et al., 2002).

5.3.4 Disinfectants

The disinfectant concentrations used during this study corresponded to levels previously indicated as suitable for food processing. Previous studies showed that H_2O_2 could be used at a higher concentration than the other two disinfectants without negatively affecting the product quality (Kim et al., 2000). Chlorine concentrations were prepared by adding varying amounts of commercial sodium hypochlorite solution (3.5% m/v) to 250 mℓ NSW to give final concentrations of 0.2, 0.4, 0.6, 1.0, 1.5, 2.0, 2.5, 3.0 or 4.0 mg/ℓ free residual chlorine. The chlorine concentrations were measured using N, N-diethyl-p-phenelenediamine (DPD) Tablets No1 (The Tintometer (Ltd), England) and a Lovibond colour comparator. Ozone was generated using the Ozone Air and Water System, (Bulkmatech, Cape Town) to give ozone concentrations of 0.4, 0.8, 1.0, 1.6 and 2.0 mg/ℓ. Ozone concentrations were measured by the indigo colorimetric method (*Standard Methods*, 1998). H_2O_2 concentrations were prepared by adding varying amounts of 35% H_2O_2 (Merck) to 250 mℓ NSW for final concentrations of 0.05, 0.08, 0.1, 0.2, 0.3, 0.4 and 0.5%.

5.3.5 Inhibition of biofilm formation

Chlorine, ozone or H_2O_2 solutions were prepared at the concentrations described above in screw-top bottles containing 250 mℓ sterile NSW. Sea-water without any disinfectant added served as the control. Microscope slides were inserted in all the bottles. After inoculation with 1 mℓ of the test culture suspension, the bottles were kept at 22^{o}C. Slides were withdrawn after 72 h. All slides were washed in sterile ASW and examined for the presence of biofilms through culturable-count determination as described above.

5.3.6 Inhibition of mature biofilms

Chlorine, ozone or H_2O_2 were prepared at the concentrations described above in 250 mℓ sterile NSW. Sea-water without any disinfectant added was used as a control. Four slides containing mature (72h) monoculture biofilms of the selected strains were obtained from the Pedersen's device systems described above. The slides were rinsed in sterile ASW and then immersed into the bottles, swirled and allowed to stand at room temperature. A slide was withdrawn from each bottle after 1 h. Slides were examined for the presence of biofilms by determining the culturable counts using TSBC agar incubated at 37^{o}C for 24 h.

5.3.7 Statistical analysis

The experimental set-up was a randomised complete block design. The statistical analysis was conducted by ANOVA using Genstat Release 7.2. The mean, the least significant difference, and coefficient of variation were calculated to determine the significance in responses among treatments.

5.4 RESULTS

5.4.1 Microbial quality of treated sea-water

The aerobic heterotrophic, psychrotrophic and *Vibrio* culturable counts were determined for the treated sea-water used in the Namibian fish-processing plant and are presented in Table 5.1. The heterotrophic and psychrotrophic counts for the untreated sea-water (S1) were 2.0 $\times 10^2$ (cfu/mℓ) and 5.6 10^3 (cfu/mℓ) respectively. Only 10 cfu/mℓ presumptive *Vibrio* colonies were detected. Similar heterotrophic and psychrotrophic results were observed after dissolved air flotation (DAF) and chlorination. At this point of the treatment no *Vibrio* ssp. were detected. The water leaving the plant after UV disinfection had very low numbers of both heterotrophic (60 cfu/mℓ) and psychrotrophic bacteria (35 cfu/mℓ). No *Vibrio* spp. were detected in this sample. Inside the processing plant the value of both the heterotrophic bacteria and the psychrotrophic bacteria had risen sharply to the similar levels detected in the raw water. Growth of *Vibrio* spp. above the detection limit of 250 cfu/mℓ was also observed on the TSBC plates after 72 h of incubation (Table 5.1).

Table 5.1 Average bacterial counts (cfu/mℓ) of sea-water used in the processing plant after various treatments and distribution to the plant

Sample	Sampling point	Heterotrophic count (Nutrient agar)	Psychrotrophic count (Sea-water agar)	*Vibrio* spp. count
S1	Raw sea-water	2.0×10^2	5.6×10^3	10
S2	After DAF and chlorination	2.5×10^2	1.2×10^3	ND
S3	After UV disinfection	6.0×10^1	3.5×10^1	ND
S4	Distribution Line 1	9.8×10^3	6.4×10^3	@72 hrs +++[a]
S5	Distribution Line 2	1.7×10^2	9.7×10^3	@72 hrs +++

Abbreviations: a =Abundant growth with counts higher than 250cfu/mℓ which was the detection limit for this analysis. ND = Not detected, DAF = Dissolved air flotation

5.4.2 Ability of *Vibrio* isolates to form biofilms in sea-water

All *Vibrio* strains included in this study were able to form biofilms on glass slides in artificial sea-water (ASW) supplemented with bacteriological peptone and nutrient broth (Figure 5.1). The average bacterial densities in the biofilms peaked between $10^7$and 10^9 cfu/cm^2 after 72 h, changing only slightly over the next 24h.

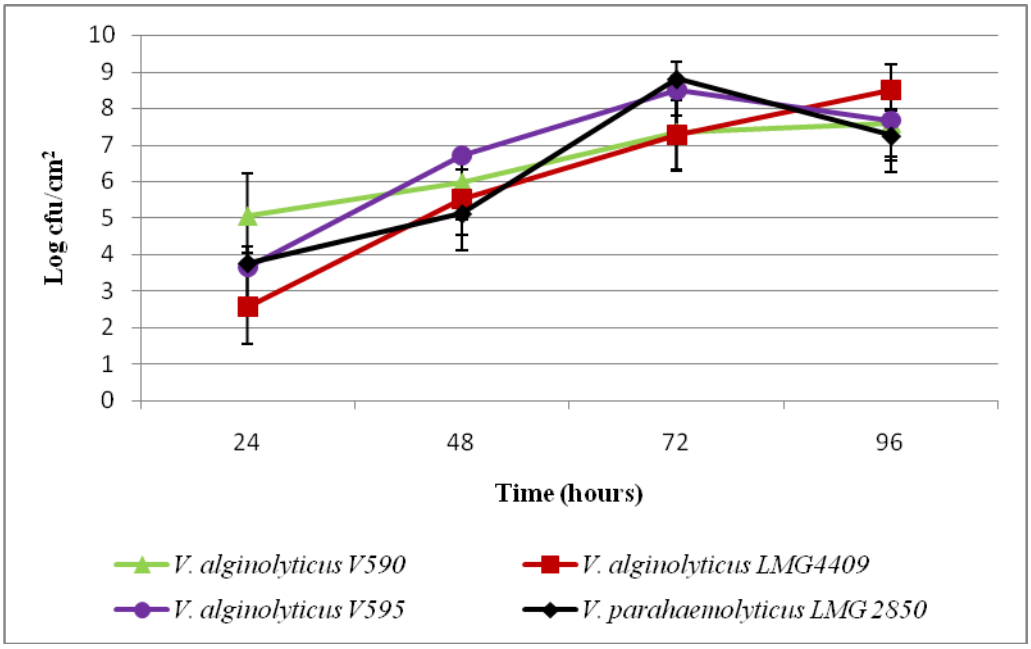


Figure 5.1 Biofilm formation of selected *Vibrio* isolates in artificial sea water. Counts based on 3 replicates.

5.4.3 Effect of disinfectant on biofilm formation

The development of biofilms in the presence of different pre-determined concentrations of disinfectant was measured. After 72 h of exposure to the chlorine concentrations biofilm formation was observed for almost all the strains (Fig. 5. 2a). At the lower chlorine concentrations (1.5 mg/ℓ and below) there was no significant reduction in bacterial densities, however, there was a significant difference in the mean bacterial

counts between the control without chlorine and the highest chlorine concentration (4 mg/ℓ) used (LSD=1.24) (Fig. 5.2a). The bacterial densities of strains V590, V595 and LMG 2850 dropped to log 3.73, 2.33 and 2.88, respectively. while *V. alginolyticus* type strain (LMG 4409) did not form biofilm at a chlorine concentration of 4mg/ℓ.

Biofilm formation varied substantially between the different *Vibrio* strains (Fig. 5.2b) at the different concentrations of ozone used. There was a significant interraction between *Vibrio* strains and the disinfectant ($p<0.001$). For the *V. alginolyticus* isolates, Strains V590 and LMG 4409, bioflim formation was completely inhibited at 0.8 mg/ℓ and 1.6 mg/ℓ, ozone respectively. Biofilm formation was, however, still observed for *V. parahaemolyticus* (LMG 2850) and one of the *V. alginolyticus* fish isolates (V595) after 72 h of exposure to 2.0 mg/ℓ ozone (Fig. 5.2b). Statistically the overall response of V595 was similar to that of *V. parahaemolyticus* LMG 2850. Their responses were, however, significantly different from that of *V. alginolyticus* LMG 4409 and V590. No formation of biofilms could be detected after 24 h at any of the H_2O_2 concentrations tested.

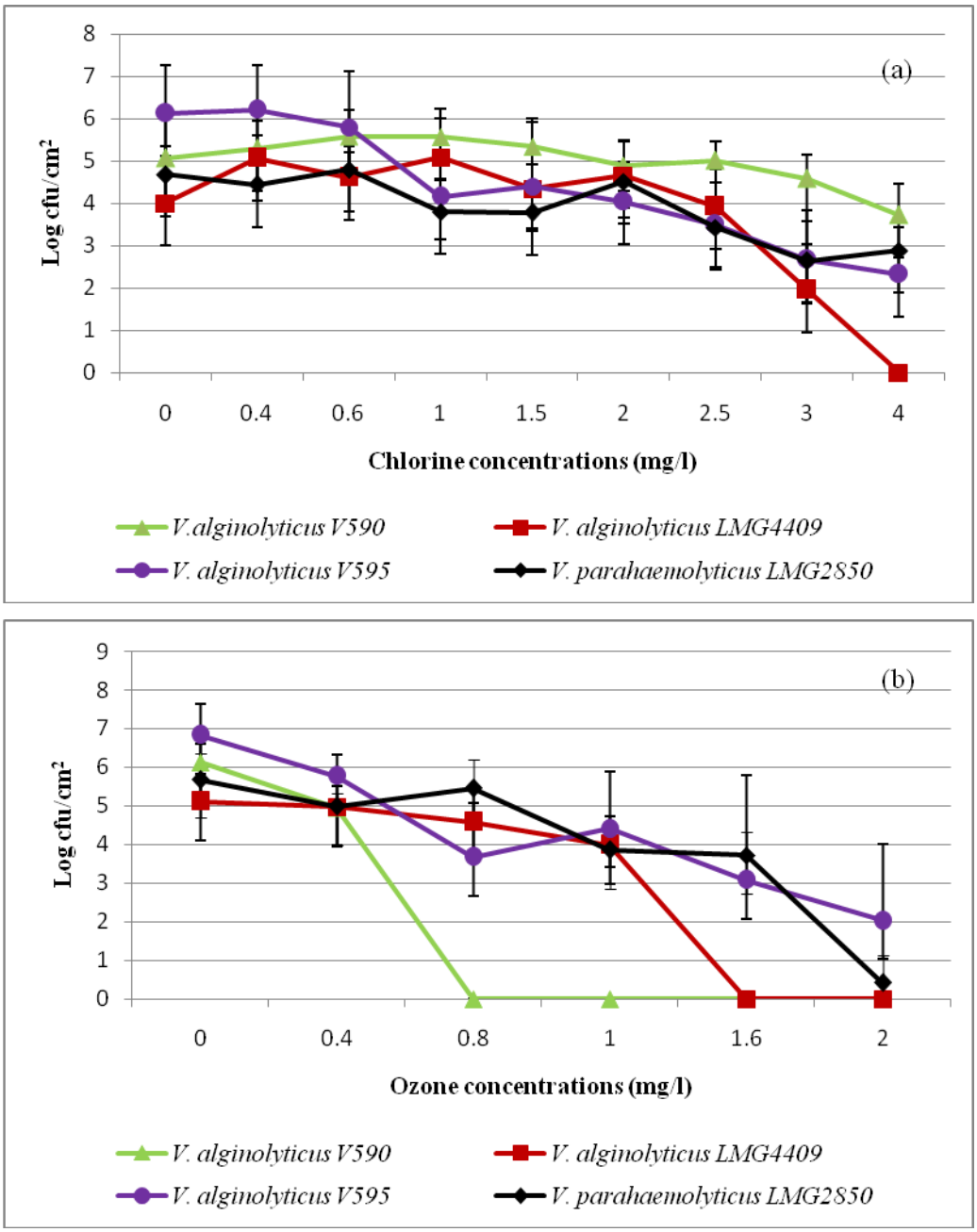
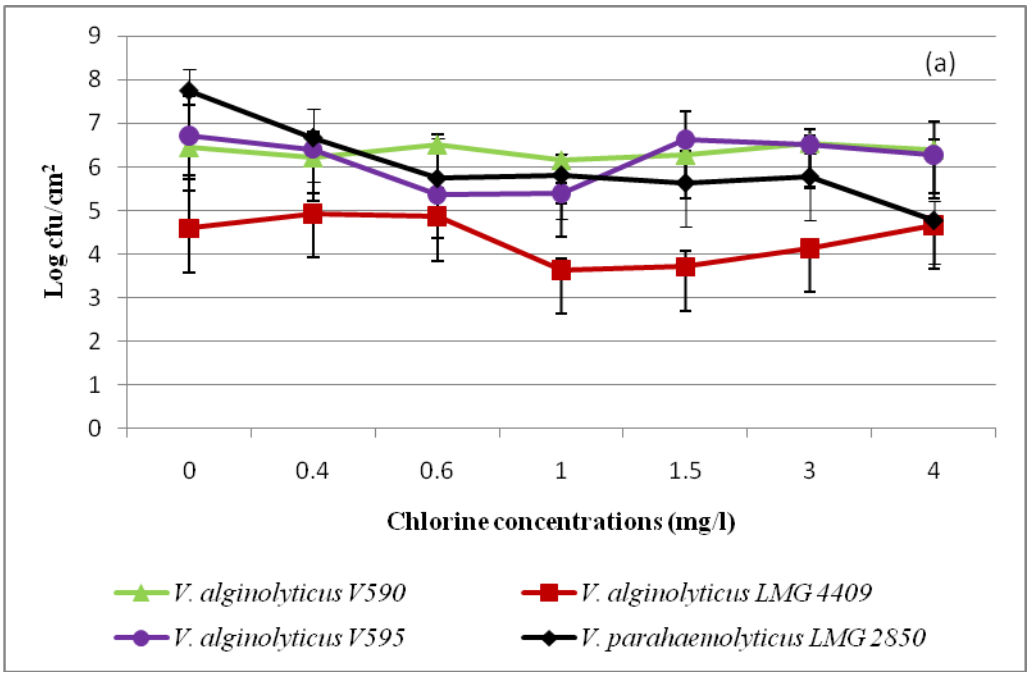


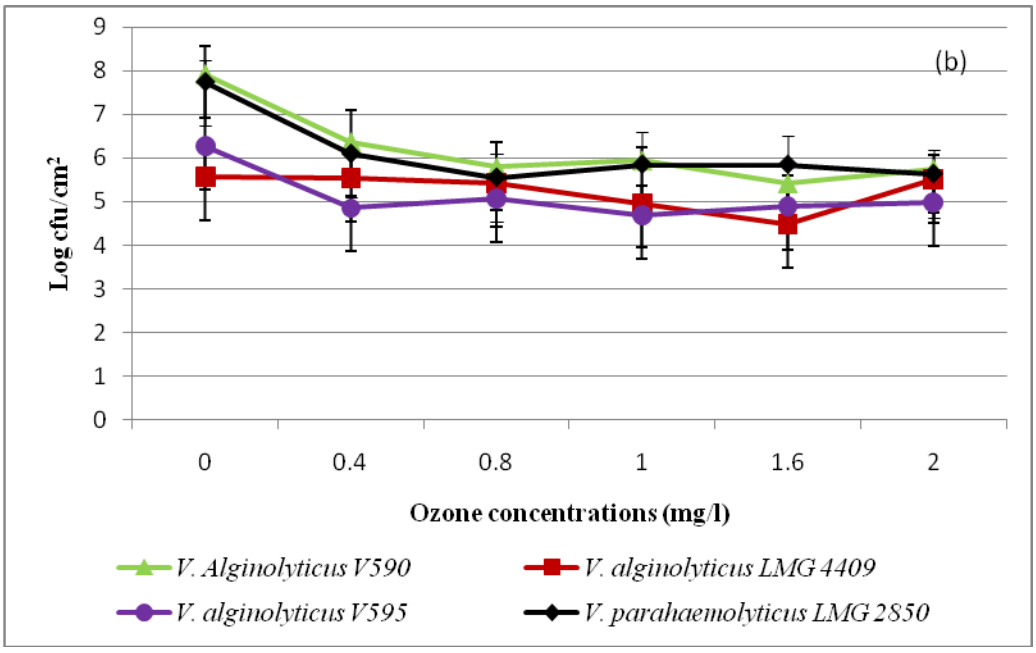
Figure 5.2 Biofilm densities of selected *Vibrios* after 72 h incubation in the presence of chlorine (a) and ozone (b). Values reflect the average of three independent experiments.

5.4.4 Effect of disinfectants on mature biofilms

Chlorine had a limited impact on the bacterial levels of all 3 *V. alginolyticus* biofilms after one hour of exposure (Fig. 5.3a) when compared to the control value. *V. parahaemolyticus* LMG 2850 was more sensitive to chlorine with a significant reduction in bacterial numbers. After 1h of exposure to 4 mg/ℓ the bacterial density dropped from 8.03×10^{7} to 9.43×10^{4} cfu/cm^2 (Fig. 5.3a). At 4 mg/ℓ the reactions of the type strains, *V. parahemolyticus* LMG2850 and *V. alginolyticus* LMG 4409 to chlorine were not significantly different, but it differed from those of the 2 fish isolates who were more resistant to chlorine. The ozone treatments resulted in a typical 1 log reduction in the bacterial levels, independent of the ozone concentration used (Fig. 5.3b) and these reductions were not statistically significant ($p=0.166$).

There was a significant reduction in bacterial counts of the mature biofilms of all the *Vibrio* isolates (Fig. 5.3c) as the H_2O_2 concentration increased ($p<0.001$). Of all the strains tested *V. parahaemolyticus* strain LMG 2850 was the most sensitive and the mature biofilm could be inhibited after 1 h of exposure to 0.08% H_2O_2. The *V. alginolyticus* type strain (LMG 4409) biofilms were inhibited at 0.1% H_2O_2. The two *V. alginolyticus* stains isolated from the facility (V590 and V595) were more difficult to remove and were only inhibited after one hour exposure to 0.2% H_2O_2 (Fig. 5.4c).





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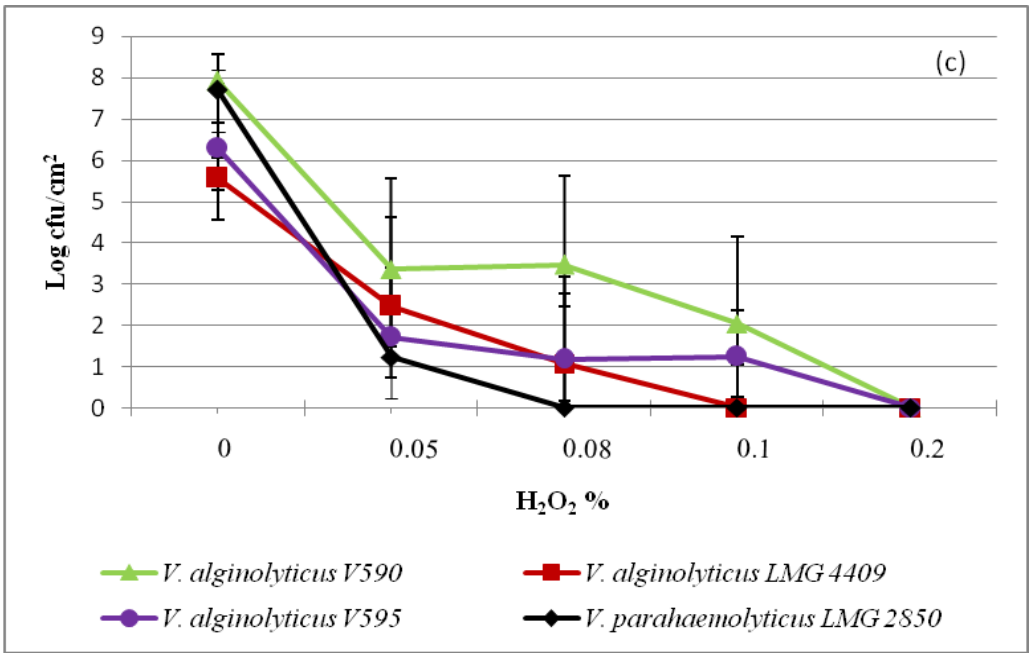

Figure 5.3 Survival of mature biofilms of selected *Vibrio* isolates when exposed to different levels of (a) chlorine, (b) ozone or (c) H_2O_2 as measured after one hour of exposure. Values reflect the average of three independent experiments.

5.5 DISCUSSION

For many marine fish-processing facilities the use of treated sea-water during operations is a viable economic alternative to the use of freshwater. The microbial quality of the water should be well managed as it may have a negative impact on the quality and safety of the final product. During an investigation into the deterioration of the microbial quality of hake during processing, *V. alginolyticus* strains most likely introduced by the treated sea-water used during processing were isolated (Shikongo-Nambabi et al., 2010b). Although certain strains of *V. alginolyticus* have been shown to be pathogenic, it is their close relationship to *V. parahaemolyticus,* a pathogen widely associated with food-borne infections and outbreaks linked to seafood (Thompson et al., 2004) that is of even greater concern. The ability of these *V. alginolyticus* strains to survive and grow in treated sea-water and the subsequent contamination of the hake may, therefore, be indicative of a similar behaviour of *V. parahaemolyticus*, should this pathogen be present. Control of this route of contamination is, therefore, of great importance for the efforts to minimize potential health hazards to consumers.

The 1st focus of the study was to survey the quality of the sea-water used at the facility and to investigate whether it could serve as a source of contamination. Results showed that chlorination combined with UV irradiation drastically reduced both the mesophilic and psychrotrophic bacteria in sea-water. Although the quality of the water directly after these treatments was very good it deteriorated during distribution and some *Vibrio* spp. could be detected. It was clear from these data that the residual chlorine was ineffective in inhibiting biofilm formation in the distribution network. This was not unexpected as biofilm formation is common in water distribution networks (September et al., 2007) and detachment of the biomass can lead to deterioration of the microbial quality of the water.

Under a defined set of conditions of temperature, pH and a limited supply of nutrients the 4 *Vibrio* isolates selected for this study were able to form monoculture biofilms on glass slides in the Pedersen's device. This was not unexpected as biofilm formation by *V. alginolyticus* has previously been demonstrated by Kogure et al. (1998). The biofilm

formation of the fish isolates (V595 and V590) was similar to that of the *V. parahaemolyticus* (LMG2850) and *V. alginolyticus* (LMG 4409) type strains. These results support the hypothesis that the *Vibrio* species detected on hake fish could have originated from bacteria released from biofilms that formed in the water distribution network after the initial treatment. It also showed that there was little difference in the overall behaviour of the *V. alginolyticus* strains isolated from the facility and the *V. parahaemolyticus* type strain. All isolates were quite similar in terms of their ability to form biofilms and their resistance to specific disinfectants.

Chlorine is not very effective against biofilms formed by either atypical *V. alginolyticus* isolates or the *V. parahaemolyticus* type strain LMG 2850 in sea-water. The effectiveness of chlorine against microorganisms in freshwater depends on a number of factors including the residual concentration, contact time, temperature, pH, and aggregation (Obi et al., 2008). Not much attention has, however, been given to the possible additional inhibitors of chlorine that might exist in water with high salt concentrations such as sea-water. From the historical data kept at the factory it is clear that a residual chlorine concentration of 0.2 mg/ℓ was constantly maintained in the system. These conditions might have selected for strains with an ability to tolerate high chlorine concentrations as was previously demonstrated by Ridgeway and Olson (1982). From the responses of the *V. parahaemolyticus* type strain it can be deducted that this bacterium will behave similarly in the sea-water distribution system and may, therefore, contaminate the final product whenever present.

Failure of chlorine to inhibit biofilm formation and mature biofilms was not due to the effect of pH. The pH of the NSW was 7.4, a level at which both HOCl and OCl^- exist in various proportions (LeChevallier and Au, 2004). In this study the CT (concentration × exposure time) value for the highest concentration used (4mg/ℓ) after 72 h exposure was 288, a value that was much higher than the 15 to 150 CT values recommended for drinking water (Debore and Von Gunten, 2008). The use of chlorine at concentrations higher than 4mg/l was not considered due to the potential effect that higher chlorine

levels might have on costs, acceptability of the final product, corrosion in the plant and the potential to generate possible carcinogens (Gopal et al., 2007; Wang et al., 2007).

The ability of ozone to inhibit biofilm formation varied between the strains tested. Again *V. alginolyticus* V595 was the most resistant to ozone and biofilm formation was not inhibited at 2 mg/ℓ ozone. Although biofilm formation could be inhibited for *V alginolyticus* V590 at an ozone concentration of 0.8 mg/ℓ, none of the mature biofilms were inhibited at the highest concentrations of ozone used (2mg/ℓ) in this study. Higher ozone concentrations to remove mature biofilms are, however not recommended. Reports have shown that at high concentrations ozone reacts with organic matter in water, generating nutrients that could stimulate bacterial attachment to surfaces and formation of biofilms (Clark et al., 1994). Ozone is also not stable for long periods and may not provide the level of residual disinfectant required to inhibit existing biofilms (Khadre et al., 2001; Guzel-Seydim et al., 2004)

Hydrogen peroxide (H_2O_2) was very effective in inhibiting biofilm formation at a concentration of 0.05 % (500mg/ℓ) H_2O_2. Mature biofilms of all four strains tested could be killed at concentrations between 0.08% and 0.2% H_2O_2. This suggested that H_2O_2 at higher concentrations could be used to remove existing mature biofilms from sea-water distribution systems during shock dose treatments. During this study $H_2O_2$was more effective in killing bacteria than during a study done by Kim et al. (2000) on channel catfish carried out in freshwater. The discrepancy found in H_2O_2 action between sea-water and freshwater could be ascribed to different disinfection environments implying that mineral ions present in sea-water are essential in maximising the action of H_2O_2 against bacteria. Pedahzur et al. (1995) found such a synergistic effect between silver ions and H_2O_2 when inactivating *E. coli* in phosphate buffer.

Part of the success of H_2O_2 was that it could be used at a higher concentration than the other two disinfectants without negatively affecting the product quality. Kim et al. (2000) investigated the effectiveness of 0.7% (7000 mg/ℓ) H_2O_2 on reducing bacterial counts on catfish fillets and found no significant differences between controls and H_2O_2 treated

fillets with regard to appearance, colour, and odour scores. The levels of H_2O_2 used by Kim et al. (2000) were 10 times higher than the concentrations used in this study.

5.6 CONCLUSION

This study has shown that although sea-water could be an alternative source of water for marine fish-processing plants, the treatment and the quality of the water needs to be carefully managed. The conditions in this seawater distribution network have selected for *V. alginolyticus* strains that can form biofilms in the presence of a residual chlorine concentration of 0.2 mg/ℓ. Once present in the system these bacteria may be released from the biofilm and would contaminate the fish during processing. The presence of *V. alginolyticus* on its own is not of a great health concern as it is rarely associated with cases of diarrhoea or gastroenteritis. *V. alginolyticus* is, however, closely related to the common food borne pathogen, *V. parahaemolyticus,* and may be predicative of the growth, behaviour and survival of this seafood pathogen in the water system. The current study has confirmed that this is the case as there was little difference between the behaviour of strains representing these 2 species in the experiments conducted. This finding also emphasised the need for control of biofilm growth in distribution systems even though it may not currently pose a significant health threat.

Evaluation of 3 oxidising disinfectant showed that chlorine and ozone are ineffective in preventing biofilm formation and in removing mature biofilms formed by *Vibrio* species in sea-water at their permissible concentrations. The only disinfectant that showed some promise was H_2O_2. Bench-scale experiments indicated that it would be possible to control biofilm formation at a concentration of 0.05 % (500 mg/ℓ) H_2O_2 and that existing biofilms could be removed by shock doses or 0.2%. This still needs to be investigated with larger-scale experiments run over a longer period of time. Results published by Kim et al., (2000) strongly support the notion that the proposed levels of H_2O_2 would not have any negative effect on the quality of the processed hake, but further studies would be required to confirm these conclusions.

5.7 ACKNOWLEDGEMENTS

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Microbiological quality of marine fish plays an important role in determining product shelf life and safety to end users. Controlling the quality and safety of the final processed fish is no easy feat as a number of bacteria present in marine waters are either pathogenic to humans or cause spoilage of stored fish. Many bacterial species are able to metabolise the fish proteins resulting in a reduction in shelf life through the generation of byproducts that lead to fish spoilage often associated with bad odour and taste, change in texture and discolouration (Gram et al., 1987; Huis in't Veld, 1996). Amongst the pathogenic bacteria, the *Vibrio* spp. are the most important pathogens associated with seafood and are often transmitted through the consumption of raw or improperly cooked produce. These pathogens cause diseases such as cholera, gastroenteritis and septicaemia. (Harwood et al., 2004; Hsueh et al, 2004; Oliver and Kaper, 1997).

Monitoring the levels of spoilage and pathogenic bacteria during fish processing is essential to ensure that bacterial contamination is kept to a minimum. For this purpose accurate diagnostic and identification tools are required as misidentification of pathogens may pose health risks to consumers or lead to loss of income through rejections of products. Monitoring also forms an integral part of quality assurance procedures such as HACCP and GMP implemented by seafood processing facilities to ensure consumer safety and compliance with microbiological criteria controlled by trade agreements. Little information with regard to the quality of fish harvested off the Namibian coast is, however, available.

This project has investigated microbiological contamination of fish during processing. A total of 120 fish samples were analysed for spoilage bacteria and potential human pathogens, focusing mainly on the human pathogenic *Vibrio* species. The quality of marine fish harvested off the Namibian coast was assessed by carrying out total counts of indicator organisms (total mesophilic counts and *Enterobacteriaceae* counts), total *Vibrio* species and total counts of mesophilic and psychrotrophic bacteria that would indicate potential spoilage of marine fish during storage.

This study has shown that typical psychrotrophic spoilage bacteria such as *Pseudomonas, Shewanella putrefaciens* and *Aeromonas*, already formed part of the microbial population of the incoming fish and that processing did not increase their levels dramatically. It was postulated that keeping fish on board the fishing vessels for several days before delivery to the factory for final processing may have increased the level of spoilage bacteria on the unprocessed fish. The study, however, revealed higher mesophilic counts in hake after filleting. Of particular interest was the sucrose fermenting *Vibrio* species that were not detected in the incoming (H&G) fish, but were detected in high numbers (ca. log 6.31 cfu/gram) at the various stages during processing.

Neither the biochemical tests nor the molecular biology methods used were 100% accurate in identifying *Vibrio* isolates to the species level. The 16S rRNA sequence comparison was accurate in differentiating the *Vibrio* species from other genera, but could not identify *Vibrio* isolates up to species level. Amongst the presumptive *Vibrio* isolates no human pathogens commonly associated with marine fish were present. *Vibrio alginolyticus* were the predominant *Vibrionaceae* bacteria isolated from hake as well as pilchards and horse mackerel. These isolates showed unusual pheno and genotypes and were difficult to distinguish from *V. parahaemolyticus.* Specific phenotypic traits combined with the *V. alginolyticus* collagenase gene specific PCR were used to make a final identification. In future improved methods such as DNA-DNA hybridisation (DDH) (Gomes-Gil et al., 2004) and MLSA employing other genes such as *recA* (Thompson et al., 2004) or multiple genes (Thompson et al., 2007) could be used to give better resolution in the identification of *Vibrio spp.* isolated from marine environments. The MSLA approach (Thompson et al., 2007) could supersede the polyphasic approach.

At the time of sampling, sea-water was used to wash the hake fillets before trimming and packaging and indications were that the sea water was the most likely source of contamination. To support this notion the source of *Vibrio alginolyticus* present on the processed hake was investigated. Water samples were analysed at various points within the treatment and distribution network. It was established that the levels of bacteria in water increased to values higher than the initial levels found before disinfection. The fact

that sucrose fermenting *Vibrio* species were not detected on the incoming hake fish also suggested that the water distribution system served as the source of *V. alginolyticus* to hake fish during processing. The factory used 0.2 mg/ℓ free chlorine as the residual sanitizer to prevent bacteria regrowth. Increases in bacterial counts after disinfection was a clear indication that the levels of chlorine used were ineffective in controlling bacterial attachment to surfaces in water pipes and subsequent multiplication thereafter. In addition the 300 J/cm^2 at 254 nm UV treatment given during water disinfection had no residual effect beyond the point of treatment. The problem of biofilms formation in the presence of residual chlorine is well known (Momba and Binda, 2002). In biofilms, bacteria are embedded in complex structures and protected from the adverse effect of sanitizers by the EPS (Hall-Stoodley and Stoodley, 2005).

This project has shown that chlorination and UV irradiation were unable to inhibit biofilms formed by *V. alginolyticus* prevalent in sea water and on processed marine fish. Although the fish processing plant used methods recommended for efficient treatment and biofilm control in fresh water distribution systems (chlorination and UV irradiation), these methods were ineffective for the same purpose in sea water. Methods specific for sea water therefore need to be extensively researched and defined. When studying the interactions between *Vibrio* biofilms and various disinfectants, it was shown that the *V. alginolyticus* isolates, obtained from the sea water system, formed biofilms in artificial sea water. In the laboratory scale experimental system it was demonstrated that chlorine could not prevent these strains from forming biofilms in natural sea water even at concentrations up to 4 mg/ℓ. The same level of chlorine was also not able to remove mature biofilms formed by the same bacteria.

Ozone and H_2O_2 were more effective in biofilm control formed by *Vibrio* species in sea water. Ozone has already been successfully used in the food industry to control bacterial contamination with minimal side effects to humans (Guzel-Seydim et al., 2004) and is recommended for use in slurry ice to extend the shelf life of marine fish (Campos et al., 2006). $H_2O_2$was the most effective and is therefore recommended for use in marine fish processing factories to inhibit biofilm formation in the distribution network. H_2O_2 has a

number of benefits including the lack of a residue in water as opposed to chlorine (Simpson, 2008). Further trials on the use of the two disinfectants at a larger scale over a longer time period is still required to substantiate these findings.

The presence of *V. alginolyticus* on its own is not of a great health concern as it is rarely associated with cases of diarrhoea or gastroenteritis. The current study has, however, shown that the *V. alginolyticus* isolates, obtained from the plant, were able to form biofilms in artificial sea water at rates similar to the type strain of *V. parahaemolyticus* (LMG 2850) The similarity in behaviour between the *V. alginolyticus* isolates and the pathogenic *V. parahaemolyticus* type strain may therefore indicate that *V. parahaemolyticus* stands a good chance of forming biofilms once present in sea water used in the factories. This finding emphasised the need for control of biofilm growth in distribution systems even though it may not currently pose a significant health threat. Sea-water could only be an alternative source of water for marine fish-processing plants if the treatment and the quality of the water are carefully managed.

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