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PREVALENCE OF GRAM NEGATIVE AND OXIDASE POSITIVE BACTERIA IN TROUT PROCESSING FACTORY

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Abstract

Many factors from catch to processing influence the quality, e.g. the natural condition of the fish when it is captured and the handling on board and in the processing plant.

The main objective of this work was to study the possible ways of contamination of aquaculture processing surfaces by bacteria spread in water ecosystems, which affect on quality and safety of fish products.

Influence of washing and disinfection procedures on growth and survival of bacteria typical for fresh water aquaculture has been studied. 50 samples of rainbow trout have been analyzed by swabbing method in 2012, during 6 mount period. Swabs have been taken from gills and skin of rainbow trout during harvesting and processing of fresh chilled fish. Quantitative assessment of contamination of fish processing surfaces by Gram negative oxidase positive bacteria has been carried out. Fish contact surfaces in gutting and packaging areas have been swabbed separately. Swabbing of working surfaces has been carried out during work process after appropriate time periods.

Three morphological types of Gram negative and oxidase positive bacteria have been isolated and identified form fish contact surfaces. They are characterized by high frequency of occurrence. For identification of bacterial species biochemical tests, chromogenic agar media and molecular identification techniques have been used. Bacteria from *Enterobacter, Klebsiella, Citrobacter, Serratia, Pseudomonas, Aeromonas, Alcaligenes, Vibrio* genera have been identified. Bacteria form genera *Aeromonas and Pseudomonas* have been found in prevailing quantities. *Aeromonas* species possess highest frequency of occurrence comparing with some genera from *Enterobacteriaceae* family, which are typical for water ecosystems. Many fish spoilage bacteria are able to attach food contact surfaces and remain viable even after cleaning and disinfection.

The choice of surface treatment of stainless steel is an important factor to have in mind when food processing equipment for open process is designed. Influence of washing and disinfection steps on growth and viability of Gram negative oxidase positive bacteria from genus *Aeromonas* has been studied in laboratory and industrial conditions.

***Key words**: Trout, Gram negative bacteria, Swabbing, Aeromonas, Food contact surface.*

1. Introduction

The safety and quality of fish is considerably depends on handling of the raw material and is dramatically decreased if measures for preventing contamination are unsatisfactory through the entire processing chain.

The main spoilage flora of fresh seafood products stored at low temperature is constituted of Gram negative bacteria like *Shewanella* spp., *Pseudomonas* spp. and *Photobacterium* spp. *Aeromonas* sp., *Morganella psychrotolerans*, *Hafnia alvei* and *Citrobacter freundii* [1].

Several species, such as *Aeromonas hydrophila*, *A. bestiarum*, *A. sorbia*, *A. veronii*, *A. salmonicida*, *A. jandaei*, and *A. allosaccharophila*, have been known to be associated with several diseases, in both warm and cold blooded animals as a result of their virulence and pathogenicity [2].

The formation of bacterial biofilm on the surface of fish processing equipment increases the threat of a cross-over contamination of the product [3]. This can have an effect on the quality and safety of the final product, especially if pathogenic bacteria or specific spoilage organisms (SSO) become dominant in the biofilm [4].

Microbiological sampling and enumeration of bacteria on seafood contact surfaces, non-contact surfaces, and

seafood products coupled with an auditing system, is of vital importance for HACCP systems to evaluate and record the microbiological condition of seafood and contact surfaces [5].

Several types of fish-contaminated-bacteria are found to be biofilm-forming, including *Vibrio cholerae* [6]. Many genera other than *Vibrio*, such as *L. monocytogenes*, *Salmonella* spp., *Bacillus* spp., *Aeromonas*, and *Pseudomonas* spp., are also known to be biofilm forming in fish and seafood processing [7].

Pseudomonas putida and *Pseudomonas fluorescens* were the main species of *Pseudomonas* spp. are considered as the causes of spoilage in fresh or chilled fish [1]. Some bacteria (e.g. *Pseudomonas* spp.) may have certain resistance mechanisms against commonly used disinfectants [8 and 9]. More importantly, it was noted that the presence of *Pseudomonas* spp. would significantly enhance the colonization of *L. monocytogenes* on stainless steel [10]. Other genera such as *Pseudomonas* spp., *Aeromonas* spp., and so on are also found to be significant biofilm producers and their presence would enhance the biofilm formation of other genus [11].

In order to control the risk of biofilm-induced microbiological contamination of fresh fish and processing line cleaning and disinfection procedures of fish contact surfaces must be provided. There are factors that strongly influence the cleaning and disinfection such as the physiological conditions, types and numbers of the organisms that contaminate the seafood environment, microbiological response to cleaning and disinfection, and the type and amount of soil present [5].

According to [1] microbiological breakdown of tissues is one factor that decrease quality and is unavoidable but can be minimized by incorporating standard hygiene protocols, especially in the early handling and in processing plants.

The main objective of this work was to study the possible ways of contamination of aquaculture processing surfaces by bacteria spread in water ecosystems, which affect on quality and safety of fish products.

2. Materials and Methods

2.1 Sampling

Sampling of fish and fish contact surfaces (25 cm^2) at all processing steps have been performed by swabbing method. HiCultureTM Transport Swabs (w/Amies Medium w/Charcoal, MS651, HiMedia, India) have been used for transporting the isolated bacterial cultures to the laboratory.

2.2 Isolation and identification of bacteria

Coliform bacteria

For enumeration of coliform bacteria transport swabs have been transferred on to Endo Agar (M029, HiMedia Laboratories, India) surface. Plates were incubated at 37 ^{0}C for 24 hours. Hi25™ Enterobacteriaceae Identification and HiIMViC™ Biochemical Test Kits (HiMedia Laboratories) have been used for confirmation of detected colonies.

Aeromonas spp.

For enumeration of *Aeromonas* species transport swabs have been inoculated on to Aeromonas Isolation Medium Base (M884, HiMedia Laboratories, India). Plates were incubated at 30 ^{0}C for 48 hours. Suspected colonies have been transferred onto Furunculosis Agar (M432, HiMedia Laboratories, India).

Pseudomonas spp.

For enumeration and detection of bacteria from genus *Pseudomonas* Cetrimide agar base was used (M024, HiMedia Laboratories, Mumbai, India). Plates with Cetrimide agar were incubated at 30 ^{0}C for 48 hours. For identification of *Pseudomonas* species HiFluoro Pseudomonas Agar Base (M1469, HiMedia Laboratories, Mumbai, India) has been used.

Vibrio spp.

For enumeration and initial identification of bacteria from genus Vibrio, TCBS Agar (M189, HiMedia Laboratories, India) has been used. After 24 hours of incubation at 37 ^{0}C, yellow and bluish green colonies has been transferred onto HiCrome Vibrio Agar (M1682, HiMedia Laboratories, India). For the final confirmation and identification of *Vibrio* species HiVibrio™ Identification Kit (KB007, HiMedia Laboratories, India) has been used.

2.3 Washing and disinfection

Disinfecting agents

In this study commercial solutions of hydrogen peroxide and peroxyacetic acid have been used both in processing and laboratory conditions. Working solutions of disinfectants at concentration of 1% and 3% were made using sterile water.

Processing (in place) test

For the evaluation of disinfection activity of peroxyacetic acid (1%) against fish spoilage psychrotrophic bacteria tables with stainless steel surfaces in eviscerating area have been swabbed every 30 minutes including working, washing and disinfection steps and then transferred onto selective media mentioned above.

2.4 Laboratory tests

Surface test

For testing of survival of *Aeromonas and Pseudomonas* on fish processing surfaces, stainless steel coupons (5 cm x 5 cm) were used. 200 µl of bacterial suspension was inoculated onto sterile coupons. After drying the coupons were treated with disinfectant solutions as well as cold and hot water. After 10, 60 and 180 min. of contact time coupons were swabbed with cotton swabs (previously immersed into neutralizer solution/1% Na_2SO_3). Then serial dilution was performed and diluted samples were plated onto Aeromonas Isolation Medium and Cetrimide Agar and incubated at 30 ^{0}C for 48 hours.

Well-diffusion test

Antibacterial activity of mentioned disinfecting agents has been carried out by well-diffusion method [12]. Diameters of inhibition zones in Petri dishes were measured by special microbiological ruler.

3. Results and Discussion

Eleven species of Gram-negative bacteria have been isolated and identified from 50 analysed samples of rainbow trout. Contamination of skin and gills of rainbow trout by Gram-negative and oxidase-positive bacteria at receiving and processing stages of fresh trout production has been studied. The results are shown in Table 1.

Table 1. Bacterial species isolated from skin and gills of fresh fish

Bacterial species	*n=50	
	Skin	Gills
	Frequency of occurrence (%)	
A. hydrophilla	100	96
A. sobria	24	10
A. caviae	40	24
A. salmonicida	24	4
Alcaligenes spp.	70	16
P. aeruginosa	80	60
P. statzeri	18	-
P. putida	54	30
P. fluorescens	90	76
V. mimicus	28	-
V. alginoliticus	80	40
C. diversus	40	30
K. pneumonia	32	10
S. marcescense	20	-

*number of swabs taken from gills and skin

The highest frequency of occurrence has been shown by species from *Pseudomonas* and *Aeromonas* genera. Mentioned species were also found in large amounts in ground water used for aquaculture. At ambient temperature, the microflora at the point of spoilage is dominated by mesophilic *Vibrionaceae* and, particularly if the fish are caught in polluted waters, *Enterobacteriaceae* [13]. *Aeromonas* species are widely distributed in the aquatic environment, including raw and processed drinking water [14], and have been frequently isolated from various food products such as fish and shellfish, raw meat, vegetables and raw milk [15]. Motile aeromonads are considered as emerging food-borne pathogens because it was shown that some *Aeromonas* food isolates can produce different virulence factors, not only at optimal growth temperature, but also at refrigeration temperatures [16].

In current study quantitative relation between coliform bacteria, *Aeromonas* spp. and *Pseudomonas* spp. in chilled fish processing chain is presented. Influence of washing, pre-chilling, packaging and chilling processes on quantitative distribution of mentioned bacteria on fresh fish skin is shown in Figure 1. After washing stage 1log reduction of coliform bacteria has been occurred.

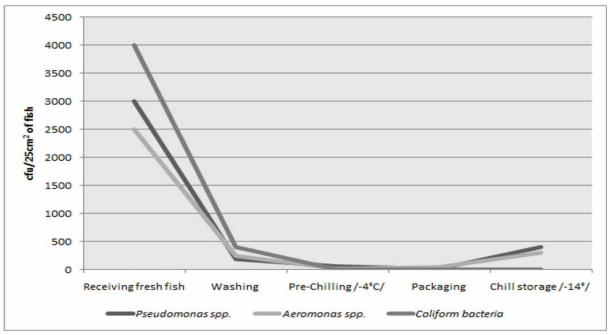

Figure 1. Growth dynamics of *Pseudomonas* spp., *Aeromonas* spp. and coliform bacteria at all stages of fresh fish processing

After 12 hour of chilled storage of gutted fish at -4 ^{0}C dramatic decrease of coliform bacteria almost to 0 cfu/50cm^2 was occurred. The same picture was observed after subsequent chilling at -13 to -15 ^{0}C . After 24 hours of chilled storage the number of bacteria from genus *Aeromonas* increased from 65 to 300 cfu/50 cm^2. Gram-negative and oxidase positive bacteria possessed more resistance to used temperatures.

In accordance with [13] bacteria on fish caught in temperate waters will enter the exponential growth phase almost immediately after the fish have died. This is also true when the fish are iced, probably because the microflora is already adapted to the chill temperatures. During ice storage, the bacteria will grow with a doubling time of approximately 1 day and will, after 2 - 3 weeks, reach numbers of 10^8 - 10^9 cfu/g flesh or cm^2 skin. During ambient storage, a slightly lower level of 10^7 - 10^8 cfu/g is reached in 24 hours. The composi-

tion of the microflora also changes quite dramatically during storage. Thus, under aerobic iced storage, the flora is composed almost exclusively of *Pseudomonas* spp. and S. *putrefaciens* after 1-2 weeks. This is believed to be due to their relatively short generation time at chill temperatures [17, 18] and is true for all studies carried out whether on tropical or temperate-water fish. Simultaneously, comparative assessment of contamination of working surfaces (tables, boxes and refrigerators) in processing conditions by Gram-negative oxidase positive bacteria has been carried out (Figure 2).

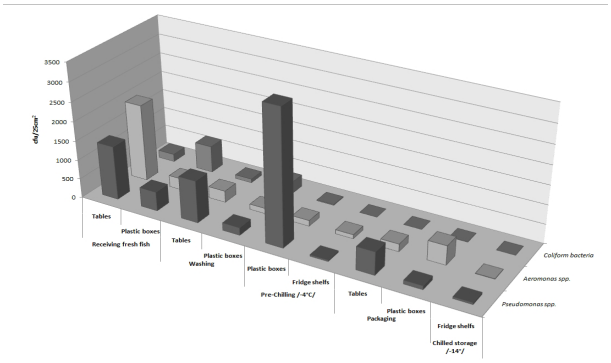

Figure 2. Prevalence of fish contact surfaces by *Pseudomonas* spp., *Aeromonoas* spp. and coliform bacteria at all stages of fresh fish processing

Aeromonas spp. and *Pseudomonas* spp. were the main contaminants of plastic boxes and working surfaces made from stainless steel. During the production of fresh fish plastic and stainless steel surfaces, which come in close contact with fish, serves as good substrate for biofilm formation and consequently causes recontamination of fish and fish contact surfaces (e.g. shelves of refrigerator) by fish spoilage psychrotrophic bacteria at packaging and storage stage. Microbiological analysis of biofilms revealed four different types of bacterial colony to be predominant and the easiest bacteria to isolate, viz. *Vibrio*, *Flexibacter*, *Pseudomonas* and *Aeromonas* [19]. Contamination of refrigerator shelves by *A. hydrophylla* at -13 to -15 ^{0}C shown in Figure 3.

Figure 3. Growth of *Aeromonas hydrophila,* isolated from shelves of refrigerators, on (a) Endo and (b) Aeromonas Isolation agar media

Assessment of microorganisms on surfaces is important in order to determine the most effective cleaning and sanitizing protocols [20]. In this work special attention has been drawn to study of influence of washing and disinfection processes on prevalence of Gram-negative oxidase positive bacteria on working surfaces with stainless steel finish in fresh fish evisceration area. The results are shown in Figure 4.

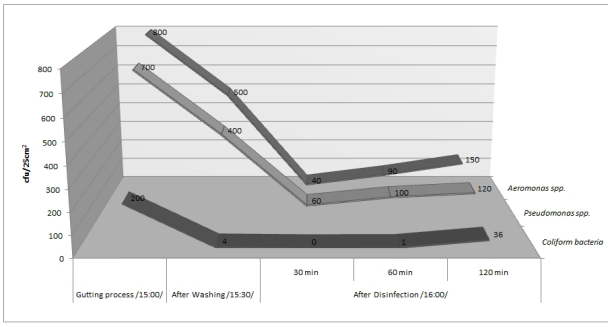

Figure 4. Effect of washing and disinfection on *Pseudomonas* spp., *Aeromonoas* spp. and coliform bacteria contaminated table surfaces in eviscerating area

Peroxiacetic acid which has been used as a disinfecting agent for sanitizing of fish contact surfaces showed high inhibition activity against coliform bacteria. The quantity of bacteria from *Aeromonas* and *Pseudomonas* genera considerably decreased on 30th minute after treatment of surface with disinfecting agent. However, on 60th minute after disinfection increase of number of *Aeromonas* and *Pseudomonas* up to 120 and 150 cfu/50 cm^2 occurred. Gram-negative and oxidase positive psychrotrophic spoilage bacteria have shown higher resistance to peroxyacetic in comparison with coliform bacteria. Peroxyacetic acid had temporary inhibition activity against psychrotrophic bacteria causing spoilage of chilled fish.

According to [21], peracetic acid eliminated approximately 98% and 99% of viable *S. aureus* and *P. aeruginosa,* respectively, with only 1 min of contact time but not the biofilm matrix. However, there are also several other studies which showed that peracetic acid is inefficient or less effective than other disinfectants against biofilms [22]. Moreover, *Pseudomonas* spp. may have certain resistance mechanisms against antibacterial components commonly used in disinfectants such as quaternary ammonium compounds.

The dominance of *Aeromonas* spp. was established under the conditions prepared for the biofilm formation. The bacterial identification of the biofilm shows that the washing protocol was effective in removing a large number of different species from the surfaces. All the species in the biofilm originate from fish and can therefore be expected to play a role in biofilm formation in processing plants [23]. Influence of cold, hot washing and disinfection with 3% of hydrogen peroxide on survival and growth of psychrotrophic fish spoilage bacteria (SSO), *A. hydrophylla and P. aeruginosa* on stainless steel surfaces has been studied (Figure 5). Washing of stainless steel surfaces with hot water promote more significant and stable bacterial reduction. 3% solution of hydrogen peroxide actively inhibited growth of psy-

chrotrophic bacteria, which presents significant problem for fresh fish processing.

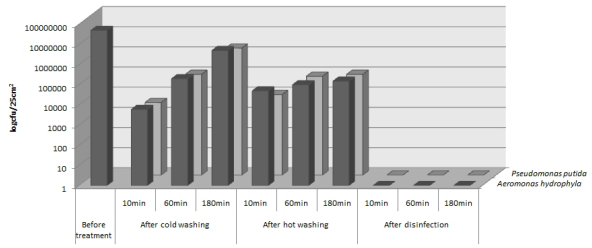

Figure 5. Effect of cold, hot washing and disinfection on survival of *Pseudomonas* spp., *Aeromonoas* spp. and coliform bacteria on stainless steel coupons after certain period of time

Evaluation of inhibition activity of 1% peroxyacetic acid and 1% and 3% solutions of hydrogen peroxide against *A. hydrophilla*, *P. aeruginosa* and *E. coli* in laboratory conditions have been done by well-diffusion test (Figure 6).

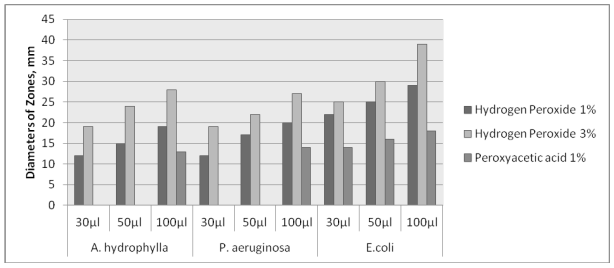

Figure 6. Determination of antibacterial activity of 1% and 3% solutions of hydrogen peroxide and 1% solution of peroxyacetic acid against *A. hydrophylla, P. aeroginosa* and *E. coli* by well-diffusion method

3% aqueous solution of hydrogen peroxide possessed higher inhibition activity. Diameters of inhibition zones were 27 - 28 mm and 39 mm in case of *A. hydrophila* and *E. coli* respectively. Mentioned bacteria were more resistant to peroxyacetic acid in comparison with hydrogen peroxide.

4. Conclusions

- The main sources of contamination of chilled fish by Gram-negative, oxidase positive bacteria have been determined. *Aeromonas spp. and Pseudomonas spp.* possessed high resistance and viability on fish and working surfaces during processing and storage of fresh fish at temperatures varied from -4 to -13 ^{0}C.
- In laboratory conditions 3% solution of hydrogen peroxide was more active against Gram-negative, oxidase positive bacteria comparing with 1% solution of peroxyacetic acid.
- More detailed studies on modeling of growth and biofilm formation by psychrotrophic Gram-negative, oxidase positive bacteria as well as their control by washing and disinfection both in fish processing and laboratory conditions should be performed.

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5. References

[1] Gram L., and Huss H H. (1996). *Microbiological spoilage of fish and fish products*. International Journal of Food Microbiology 33, pp. 121 - 137.

[2] Gosling P. J. (1995). *Aeromonas species in disease of animals*. In: Austin B., Altwegg M., Gosling P. J., and Joseph S. W. (ed.), The Genus Aeromonas. John Wiley & Sons Ltd. Chichester, pp. 175 - 196.

[3] Kusumaningrum H. D., Riboldi G., Hazeleger W. C., Beumer R. R. (2003). *Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods*. International Journal of Food Microbiology, 85, pp. 227 - 236.

[4] Gudbjornsdottir B., Suihko M. L. P., Gustavsson P., Thorkelsson G., Salo S., Sjoberg A. M., Niclasen O. and Bredholt S. (2004). *The incidence of Listeria monocytogenes in meat, poultry and seafood plants in the Nordic countries*. Food Microbiology, 21, pp. 217 - 225.

[5] Suvveb N. V. (2011). *Efficiency of cleaning and disinfection on fish contact surfaces.* Final Project, Reykjavik.

[6] Faruque S. M., Biswas K., Udden S. M. N., Ahmad Q. S., Sack D. A., Nair G. B. (2006). *Transmissibility of cholera: in vivo-formed biofilms and their relationship to infectivity and persistence in the environment.* Proceedings of the National Academy of Sciences of the United States of America 103(16), pp. 6350 - 6355.

[7] Rajkowski K. T. (2009). *Biofilms in fish processing*. In: Fratamico P. M., Annous B. A., and Gunther N. W. (ed.), Biofilms in the food and beverage industries, Woodhead Publishing, pp. 499 - 516.

[8] Langsrud S., Sundheim G. and Borgmann-Strahsen R. (2003). *Intrinsic and acquired resistance quaternary ammonium compounds in food-related Pseudomonas spp.* Journal of Applied Microbiology 95, pp. 874 - 882.

[9] Sundheim G., Langsrud S., Heir E., and Holck A. L. (1998). *Bacterial resistance to disinfectants quaternary ammonium compounds.* International Biodeterioration & Biodegradation, 41, pp. 235 - 239.

[10] Gudbjörnsdóttir B., Suihko M. L., Gustavsson P., Thorkelsson G., Salo S., Sjöberg A. M., Niclasen O., Bredholt S. (2004). *The incidence of Listeria monocytogenes and other Listeria species in meat, poultry and seafood factories in the Nordic countries*. Food Microbiology, 21, pp. 217 - 225.

[11] Srey S., Jahid I. K., Ha S. D.(2013). *Biofilm formation in food industries: A food safety concern.* Food Control, 31(2), pp. 572 - 585.

[12] National Committee for Clinical Laboratory Standards. (2002). *Performance Standards for antimicrobial susceptibility testing*. 8th Informational Supplement. M100 S12. National Committee for Clinical Laboratory Standards.

[13] Huss H. H. (1995). *Quality and quality changes in fresh fish*. FAO Fisheries Technical paper - 348, FAO, Denmark.

[14] Holmes P., Niccolls L. M. and Sartory D. P. (1996). The *ecology of mesophilic Aeromonas in the aquatic environment*. In: Austin B., Altwegg M., Gosling P.J. and Joseph S.W. (ed.), The Genus Aeromonas. John Wiley & Sons Ltd. Chichester, pp. 127 - 150.

[15] Palumbo S. A. (1996). *The Aeromonas hydrophila group in food*. In: Austin B., Altwegg M., Gosling P. J. and Joseph S. W. (ed.), The Genus Aeromonas. John Wiley & Sons Ltd. Chichester, pp. 287 - 310.

[16] Merino S., Rubires X., Knochel S. and Thomas J. (1995). *Emerging pathogens: Aeromonas spp.* International Journal of Food Microbiology, 28, pp. 157 - 168.

[17] Morita R. Y. (1975). *Psychrophilic bacteria*. Bacteriology Reviews, 39, pp. 144 - 167.

[18] Devaraju A. N. and Setty T. M. R. (1985). *Comparative study of fish bacteria from tropical and cold/temperate marine waters*. In: Reilly A. (ed.), Spoilage of tropical fish and product development. FAO Fish. Rep. pp. 97 - 107.

[19] Sriyutha Murthy P. (2004). *Biofilm control for plate heat exchangers using surface seawater from the open ocean for the OTEC power plant*. International Biodeterioration & Biodegradation, 53, pp.133 - 140.

[20] Verran J. and Whitehead K. A. (2006a). *Assessment of organic materials and microbial components on hygienic surfaces*. Food and Bioproducts Processing, 84, pp. 260 - 264.

[21] Toté K., Horemans T., Vanden Berghe D., Maes L., and Cos P. (2010). *Inhibitory effect of biocides on the viable masses and matrices of Staphylococcus aureus and Pseudomonas aeruginosa biofilms*. Applied and Environmental Microbiology, 76(10), pp. 3135 - 3142.

[22] Królasik J., Zakowska Z., Krepska M., and Klimek L. (2010). *Resistance of bacterial biofilms formed on stainless steel surface to disinfecting agent*. Polish Journal of Microbiology, 59(4), pp. 281 - 287.

[23] Reynisson E. (2009). *Decontamination Efficiency of Fish Bacterial Flora*. Food Technology and Biotechnology, 47 (1), pp. 75 - 82.