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Better washing practises in fish processing plants

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Icelandic Fisheries Laboratories Report Summary

Titill / Title	**Better washing practises in fish processing plants -** **The effect of detergents and disinfection agents on adhesion, analysed by molecular methods and cultivation.**		
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Ágrip á íslensku:	Í þessu verkefni var leitað leiða til að kanna hvort mögulegt sé að nota lægri styrki af þvottaefnum en mælt er með við þrif á fiskvinnslubúnaði. Lækkun á styrk þvottaefna gæti leitt af sér fjárhagslega hagræðingu fyrir fiskvinnslufyrirtækin og ekki síst umhverfisvænni vinnubrögð. Til að komast að þessu var sett upp tilraunaröð þar sem kannaðir voru nokkrir þættir í almennu þrifaferli en þeir voru: Gerð yfirborða (ryðfrítt stál og plast), hitastig skolvatns (8 eða 28°C), tvær gerðir þvottaefna og styrkur þvottaefna (2% og 4,5%). Til að meta þessar breytur var notast við nýuppsetta þvottastöð á Rannsóknastofnun fiskiðnaðarins sem gerir kleift að staðla þrifin betur en áður þekkist. Bakteríutalningar voru notaðar til að meta áhrif breytanna í þvottaferlinu. Niðurstöðurnar sýndu að mögulegt er að nota lægri styrk þvottaefna en mælt er með og náð sambærilegum árangri. Önnur hlið á þessu verkefni var greining á náttúrulegri bakteríuflóru í örveruþekjunni og bera saman aðferðir sem byggjast á ræktun og aðferð sem byggist á mögnun erfðaefnisins með sameindalíffræðilegum aðferðum. Nokkuð sambærilegar niðurstöður fengust með hvorri aðferðinni fyrir sig.		
Lykilorð á íslensku:	*Fiskvinnsla, þrif, bakteríur, þvottaefni, viðloðun*		
Summary in English:	In this project, ways to explore the possibilities of using more dilute detergents than recommended in fish processing surfaces were performed. Less concentrated detergents can lead to economical advantage for producers and more importantly, more environmental safe work practises. To investigate this, a series of experiments were carried out were some general parameters in a typical washing protocol were tested. Two surface materials (plastic and stainless steel), water temperature (8 and 28°C), two types of detergents and detergent concentration (2 and 4,5%) were compared. A new semi-automated washing station at the Icelandic fisheries laboratories which enables standardised washing was used to evaluate the affect of these parameters. Bacterial count was used to determine washing efficiency. The results show that it is possible to use less dilute detergents than recommended with comparable success. Another output of this project was the analysis of the bacterial flora of the biofilm. Two independent approaches were compared for this analysis: Cultivation method and molecular amplification of genetic material. Both of these methods showed a reasonable analogous result.		
English keywords:	*Fish processing, hygiene, bacteria, detergents, adhesion*		

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1. INTRODUCTION

The quality of fish products is critical to ensure a high economical value of the catch on markets. 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. 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 [1]. The formation of bacterial biofilm on the surface of fish processing equipment increases the threat of a cross-over contamination of the product [2]. 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 [3]. The shelf life and quality of fish products is greatly dependent on the handling of the catch and is severely diminished if measures for preventing contamination are unsatisfactory through the entire processing chain [4, 5].
Surface finishing is considered to affect bacterial adhesion [6]. Electro-polished surface of stainless steel has been shown to reduce bacterial growth in comparison to untreated, sandblasted and sanded steel [6]. Other studies, however, reveal that glass beaded or polished finishing of stainless steel does not reduce hygienic properties compared to untreated and smooth steel and it is concluded that smooth surfaces do not necessarily provide hygiene benefits over rougher surfaces [7]. Effective hygienic protocols are essential to minimise the formation of biofilms and to prevent contamination of the products. However, it must also be noted that the use of detergents and disinfection agents in great quantity, such as in food processing plants, must be used with care and precaution because of environmental issues, health issues and governmental regulations [8]. Moreover, some bacteria (e.g. *Pseudomonas* spp.) may have certain resistance mechanisms against commonly used disinfectants [9, 10].

The aim of this study was to monitor the natural bacterial community in a biofilm through a semi-automated washing protocol, applying the same washing parameters as used in a typical fish processing plant. The effect of the washing was monitored by cultivation and molecular amplification of the 16S rRNA gene.

2. MATERIAL & METHODS

2.1 Biofilm formation

A biofilm was prepared by natural bacterial flora of fish fillets on glass beaded stainless steel (GSS) AISI-304-2B and polyethylene plastic (PEP) surfaces as follows: A fresh fish product (local retail shop) was kept at 4°C overnight, minced with a sterile mincer and split into eight portions which were used separately for the following eight experiments. This was done in order to have the same original material to minimise sample variation between experiments. The minced portions in the main experiment were kept at -80°C until the biofilm was prepared. When forming the biofilms, 175g of mince was mixed with 350g of sterile water in a stomacher bag with a lateral filter and mixed for 30 seconds in a Stomacher (Labsystem 400, Seward Medical, England). Thirty ml of the fish juice was placed in a sterile glass tube containing a sterile stainless steel or plastic coupon and was kept agitated (75 rpm) at 19-21°C for 48 hours.
At the beginning of this study, the microbiological adhesion in cod-, shrimp- and herring juice was compared. This was done to simulate the processing conditions and according to these results, the cod juice was used in main experiment.

2.2 Washing protocol

After the incubation, the coupons went through a stepwise washing protocol consisting of rinsing, washing, rinsing, disinfection and rinsing (Figure 1). After each washing step, coupons were collected in triplicate from the washing station. A semi-automated washing station was used for the washing, which minimised variations between experiments and for the imitation of fish processing environment (Figure 2). The station consisted of pump/blender (Hygiene System from ECOLAB) and a washing chamber (designed and produced by the company Marel and IFL). The pump enabled mixing of detergents and the intake of air to form foam. The pump injected the water/soap into the chamber through a hose where the coupons had been placed. The temperature and water pressure was monitored throughout all experiments. The detergent concentration was measured with a kit supplied by

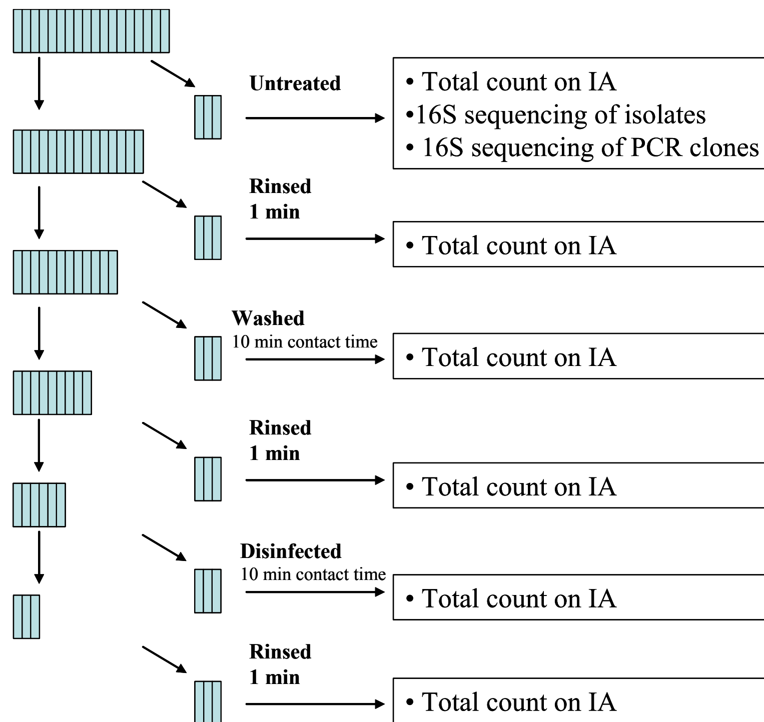

Figure 1. The experimental layout of the study. After each washing step, coupon were collected in triplicate and analysed.

Tandur hf. and the disinfection agent concentration was measured using the JDK06 Active cationic products test kit (Johnson Diversey, Nottingamshire, UK). The following washing parameters were compared: Two surfaces (GSS and PEP), two detergents (manufactured by Tandur, Iceland), two concentrations of detergent (2% and 4,5%) and two washing temperatures (8°C and 28°C). GSS is commonly used in fish processing equipment and PEP is commonly used in fish tubs. Detergent 1 (Det1) and detergent 2 (Det2) are commercially available for cleaning of food- and fish processing environments. Det2 is based on thixofoam technology which enables the detergent to stick better to vertical surfaces. The disinfection agent concentration was kept constant at 0,25%, the minimum recommended concentration (0,25-0,5% is recommended). It is based on quaternary ammonium compounds. All these chemicals have been approved by the Environmental Agency in Iceland for use in the food industry.

Figure 2. The semi-automated washing station enabled a controlled washing protocol to be executed. Detergent concentration, temperature, water pressure and distance from sample plate was all standardised and monitored between experiments.

2.3 Culturing

Bacteria from the biofilm were isolated from the coupons by rubbing a cotton swab tightly on the surface. The swab was dipped in D/E-neutraliser (Difco, Franklin Lakes, NJ USA) before capturing the bacteria to neutralise the detergents leftovers. The swab was released into 5 ml of Maximum Recovery Diluent (MRD) buffer (Oxoid, Mampshire, UK) and shaken vigorously. Serial dilutions were prepared for each sample and plated onto Iron Agar (IA) [11]. The plates were incubated for 15°C for 7 days. H_2S producing bacteria form black colonies on IA and were counted separately. Hundred isolates were selected for 16S rRNA taxonomic identification by transferring single colonies to a plate count agar (PCA) with added 0.5% NaCl (Difco, Franklin Lakes, NJ USA) and incubated at 15°C for 48 hours before DNA isolation was carried out.

2.4 DNA isolation

DNA from bacterial isolates cultured on PCA plates was extracted by suspending a loopfull of bacteria in 200 µl 5% Chelex solution by vortexing. The suspension was

incubated at 55°C for 15 min and then vortexed again, boiled for 10 minutes and then placed on ice for 3 min. The samples were centrifuged at 11.000 x g for 7 minutes and the supernatant, containing the DNA was recovered.

DNA isolated directly from the biofilm on the GSS and PEP coupons was done as follows: One ml of the MRD buffer containing the swab was centrifuged at 11.000 x g for 7 minutes to form a pellet. The supernatant was discarded and DNA was recovered from the pellet using the ChargeSwitch gDNA Mini Bacteria kit (Invitrogen, Paisley, UK), according to the manufacturers instructions with minor modifications: In order to maximise DNA recovery from both Gram+ and Gram– bacteria, the samples were incubated with lysis buffer and proteinase K at 10 minutes at 55°C following incubation at 80°C for 1 hour.

2.5 Real-time PCR

PCR reaction for taxonomic identification of isolates was done by amplifying the 16S rRNA gene with 9F and 1510R primers (5'-GAGTTTGATCCTGGCTCAG-3 and ´5-GGTTACCTTGTTACGACTT-3´ respectively). The reaction volume was 18 µl which contained Teg polymerase (Prokaria, Reykjavík, Iceland) at 0,05 U/µl, 0,2mM MgCl, 300 nM primers, x 1 dilution of SYBRTM DNA gel stain (Invitrogen Molecular Probes, Eugene, Oregon, USA) and 1 µl of tenfold diluted gDNA (genomic DNA). Thermal program was as follows: 5 min at 95°C, 40 cycles for 25 sec at 95°C, 30 sec at 57°C, 105 sec at 72°C and a final extension step at 72°C for 5 min. For the determination of satisfactory amplification for sequencing of the PCR product, a dissociation curve was performed on the PCR product after amplification. PCR reactions were carried out in Mx3000P real time PCR instrument (Stratagene, La Jolla, CA, USA).

Real time PCR quantification was used only in the preliminary experiments. Quantitative PCR of total bacterial load in the biofilm was done by using the same parameters as above, except for a 25 µl reaction volume and 2 µl of undiluted DNA. Absolute quantification was done using a tenfold dilution series of genomic DNA (gDNA) from *Escherichia coli* (DSM 30083). The DNA concentration was measured in a ND-1000 Nanodrop spectrometer (Nanodrop Tehcnologies, DE, USA). The gDNA copy number was calculated from the genome size of *E.coli*. One genome of *E. coli* contains seven

copies of 16S rRNA which was taken into account for quantitative analysis. Quantitative data are represented as 16S rRNA copies per sample.

2.6 Construction of 16S rRNA cDNA library

The cDNA library was constructed from a 16S rRNA PCR product, amplified from an untreated biofilm. The PCR product was cloned with TOPO TA Cloning kit (Invitrogen, Paisley, UK) and transformed into competent One Shot TOP 10 cells. The cells were then plated on LB medium (1% tryptone, 0,5% yeast extract, 1% NaCl, 1,5% agar) with ampicillin at 50 µg/ml for the selection of transformed cells and incubated at 37°C overnight. Hundred colonies were picked for analysis and sequencing of the insert.

The bacterial flora of untreated biofilm was analysed by sequencing of 16S rRNA gene of selected isolates (above) cultured on IA medium and sequencing of 16S rRNA from a cDNA library. Subsequently, a comparison between cultivate and molecular method became possible.
Sequences were edited and assembled with 98% minimum match percentage in Sequencer (Version 4.0.5.). The assembled sequences were then blasted on the NCBI server to find the closest relative in the database. Multiple alignments were carried out using ClustalW and subsequent phylogenetic dendrogram of the 16S rRNA was plotted with the neighbour-joining software.

3. RESULTS

3.1 Preliminary experiments

3.1.1. Selection of fish species

Preliminary experiments were performed in order to develop a fish juice to be used in the main experiment and to verify the methodology to be used. Flesh from shrimp, cod and herring was used and juice of each species was made and added to sterile glass containing a glass beaded stainless steel (GSS) coupon. After 48 hours of agitating the biofilm was

analysed by culturing and by qPCR (Figure 3). All three juices were usable for the main experiment, but the cod juice was chosen due to a higher count and it proved to be a convenient working material.

A comparison between counting and qPCR quantification showed a good correlation (Figure 3). The results from qPCR are displayed as 16S copies per reaction. Since bacteria species contain multiple 16S rRNA operons in their genome it makes quantification on cell level difficult. Different species can contain from 2 up to 11 16S rRNA operons.

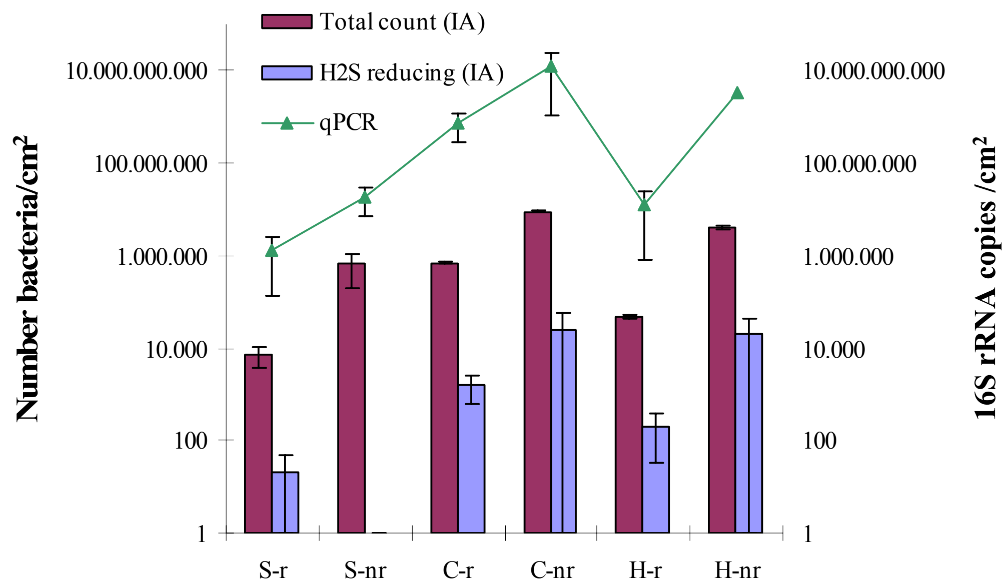

Figure 3. Microbiological growth on stainless steel coupon quantified by counting of colonies on IA medium and by qPCR. The left axis contains values representing the count on IA (columns) and the axis on the right represents qPCR results (line). The columns indicate quantification of microorganisms from shrimp (S), cod (C) and herring (H) mince which had been (-r) or had not been (-nr) rinsed with water.

3.1.2. Trial run using the semi-automated wash station

To ensure smooth washing and analysis process in the main experiment, a trial run was conducted. As before, the biofilm was formed in a cod juice on GSS and the coupons were then washed with a typical washing protocol in the station. After the rinsing step, the colony count decreased by 5 log units (Figure 4) and after the washing with a detergent and disinfection few colonies were counted in 1 or 2 replicates of 3.

The qPCR results showed a much higher count, especially in the samples after the rinsing.

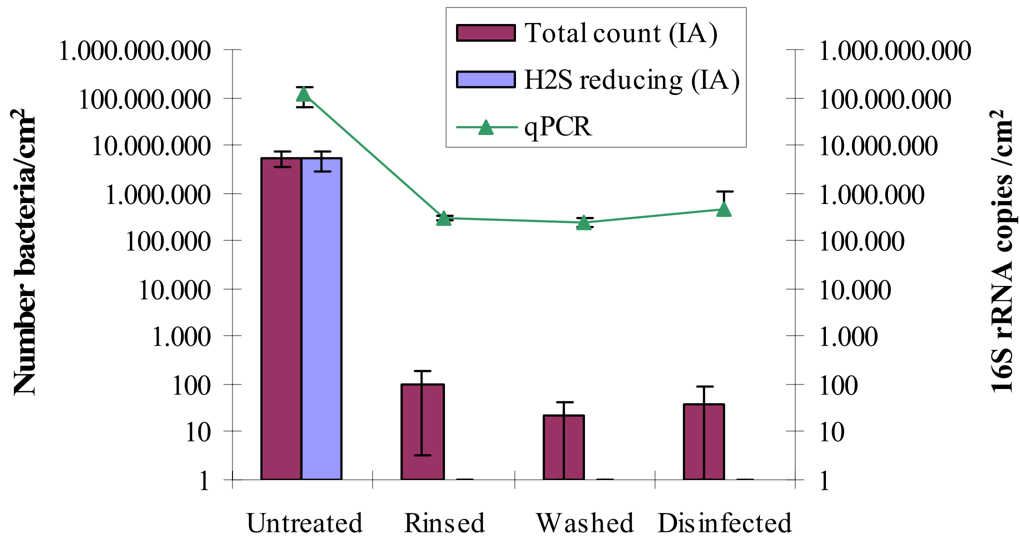

Figure 4. Microbiological growth on stainless steel coupon quantified by counting of colonies on IA medium and by qPCR. The left axis contains values representing the count on IA (columns) and the axis on the right represents qPCR results (line). The columns indicate quantification of microorganisms in an untreated biofilm, after rinsing, washing with detergent and after disinfection.

3.2. Main experiment

A full scale experiment with the aim of comparing different variables in a typical washing protocol was performed. Two temperatures (8°C and 28°C), two surfaces (GSS and PEP), two concentrations of detergent (2% and 4,5%) and two types of detergents were tested.

To compare all combinations of these variables, the experiment had to be split up into eight sub-experiments or protocols (Table 1) due to limiting sample number which could be processed at the same time. Eight protocols (A-H) were then set up and different variables were tested. All the variables were carefully monitored throughout all the experiments (Table 1). The efficiency of removing the biofilm from the surfaces was estimated by counting cultivable bacteria on IA. Generally, all the different combinations of the variables tested on the biofilm proved to be successful in removing or destroying the viability in the biofilm (Figure 5). However, as expected, protocols using the lower temperature and the lower detergent concentration generally proved to be less efficient, although on some occasions, only a few colonies or none at all were able to grow after the final rinsing step.

Table 1. Temperature, water pressure, detergent- and disinfection agent were monitored in the eight separate experiments (A-H).

	Det1 and Det2[1]				Det1				Det2			
	Surface	Temp. before exp. (°C)	Temp. after exp. (°C)	Water pressure (mBar)	Concentration (%)	pH	Disinfection concentration (%)	Disinfection pH	Concentration (%)	pH	Disinfection concentration (%)	Disinfection pH
Protocol A	Steel	8,4	-	17,9	2,0	12,3	0,30	7,75	2,9	12,5	0,20	11,60
Protocol B	Steel	23,3	29,0	-	-	-	0,25	9,09	2,3	12,3	0,20	9,72
Protocol C	Steel	7,6	7,7	17,0	3,7	12,6	-	-	4,9	12,1	0,25	8,16
Protocol D	Steel	25,5	29,5	17,0	3,5	12,1	0,25	8,32	5,4	11,7	0,25	8,40
Protocol E	Plastic	9,1	7,5	17,0	1,7	12,1	0,25	8,67	2,6	-	0,20	9,64
Protocol F	Plastic	25,8	27,5	17,0	1,7	12,1	0,20	9,03	2,6	12,3	0,20	9,15
Protocol G	Plastic	7,7	8,2	17,0	3,3	13,1	0,20	8,41	4,9	11,6	0,25	7,87
Protocol H	Plastic	21,8	28,4	17,0	3,7	12,5	0,30	9,10	5,0	12,6	0,30	9,15
		High temp before exp. (B,D,F,H)	High temp. after exp. (B,D,F,H)		Det1 high conc. (C,D,G,H)				Det2 high conc. (C,D,G,H)			
Mean		24,1	28,6	17,2	3,5	12,4	0,24	8,5	5,0	12,1	0,22	9,2
SD		1,90	0,86	0,37	0,16	0,39	0,04	0,50	0,27	0,35	0,03	1,27
		Low temp. befor exp. (A,C,E,G)	Low temp. after exp. (A,C,E,G)		Det1 low conc. (A,B,E,F)				Det2 low conc. (A,B,E,F)			
Mean		8,2	7,8		1,8				2,6			
SD		0,70	0,36		0,19				0,23			

[1] In each experiment (A-H) both DET1 and Det2 detergents were tested and thus the temperature and water pressure measurements apply for them both.
SD, standard deviation from mean.

When comparing the surfaces tested, the occurrence of bacterial growth after the final rinsing step was more frequent with PEP surfaces than GSS (Figure 5). In seven out of eight experiments, where PEP was used, bacterial growth was observed after the final rinsing step but only in two out of eight experiments where GSS was used. However, the high bacterial count after washing in protocol A has to be taken with a certain reservation because of the high pH of the disinfection agent (Table 1), which can decrease the disinfection activity. This was the first protocol tested and the reason for the high pH could be due to poor rinsing of the hoses in the washing station, which most likely led to some detergent residues in the hose which had a pH value of 12.5.

Removal of the H_2S producing bacteria, which are generally regarded as the potential spoilage organisms in fish, was satisfactory in almost all protocols. Det1 failed to remove all of them from a plastic surface at low temperature (protocol E and G) and Det 2 failed to remove them sufficiently in protocol F and G, although a growth was observed in only one replicate of three in both cases (Figure 5).

During the project period, a visit was paid to a local fish processing plant and detergent concentration was measured on 4 different places along the processing chain. The detergent which was used there is the same as Det1. The average detergent concentration was 5,4 % ± 0,2.

3.3. Species composition in untreated biofilm

The species composition of an untreated biofilm was analysed by 16S rRNA sequencing of isolates on the IA plates (culture) and by amplifying the 16S rRNA directly from the biofilm (PCR), cloning it in a TOPO vector and sequencing the clones. By comparing these two methods a comparison between cultivable cells on IA and a method based only on molecular methodology can be established.

3.3.1 Species composition of isolates on IA (culture)

About 100 colonies were isolated from the IA plates, both black and white colonies from a biofilm formed on metal- and on plastic surfaces. Amplification and sequencing of the

16S rRNA gene was successful with 91 isolates. Phylogenetic analysis on 460 bp sequence was carried out and evolutionary dentrogram was constructed (Figure 6).

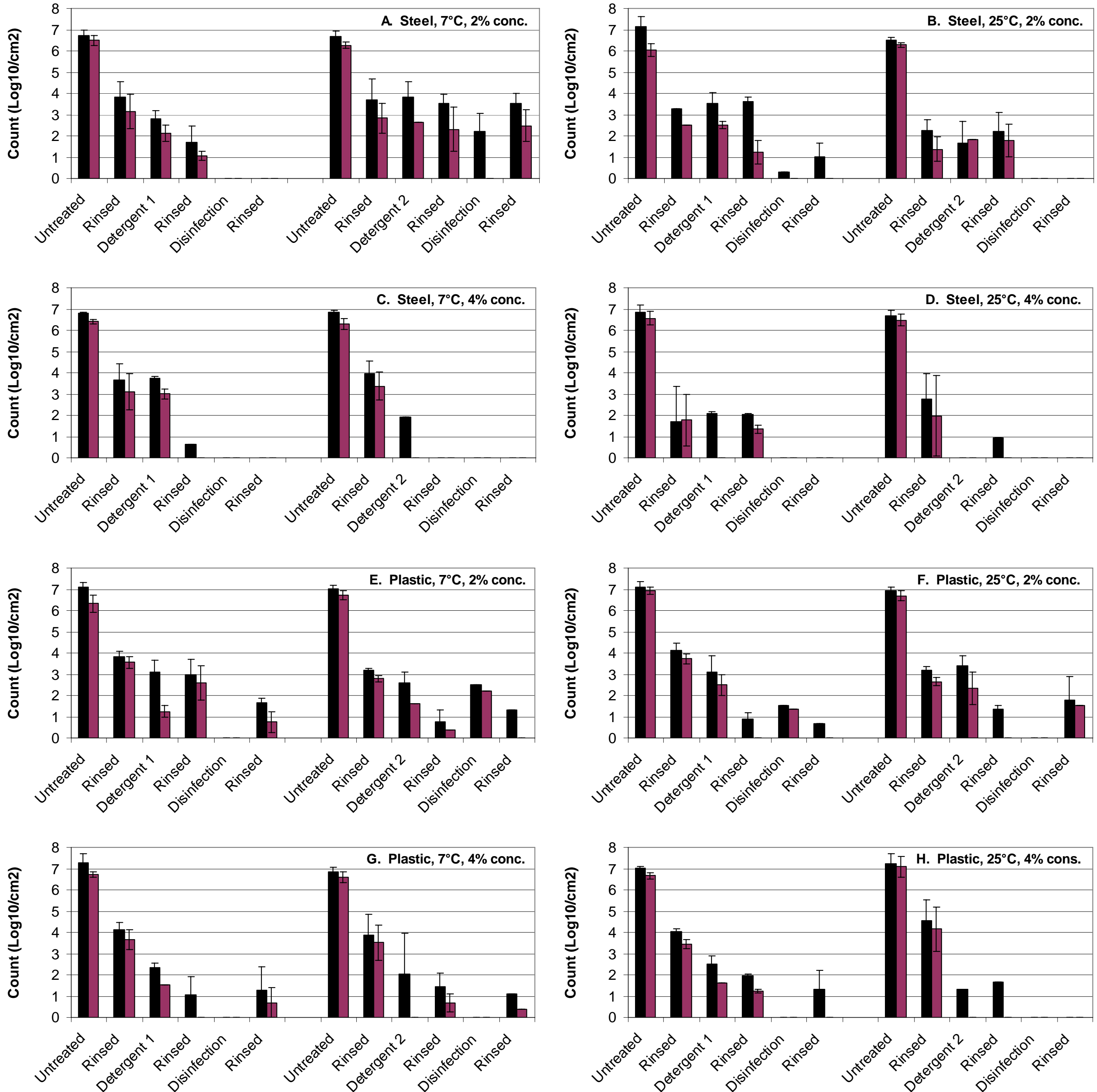

Figure 5. Results from the eight protocols (A-H) tested in the main experiment. Black columns indicate total count on IA and the gray columns indicate the count of black colonies on IA (H_2S producers).

The results indicate a high dominance of the genus *Aeromonas* (Table 2) in an untreated biofilm harvesting 27,4% of the total population. *Aeromonas* sp. belongs to the class Gamma-proteobacteria which dominated the population (87,9%), but organisms belonging to other classes were more recessive in the biofilm (Table 2).

Shewanella putrefaciens and *Pseudomans* species were detected in the biofilm, but they have been characterised before as the main specific spoilage organisms in fish (SSO) [1]. These species and other potential spoilage organisms form a black precipitation on the IA agar because of their H_2S production. In addition to the SSO, *Aeromonas* sp., *Morganella psychrotolerans*, *Hafnia alvei* and *Citrobacter freundii* were also able to produce H_2S and form black colonies (Figure 6, Table 2).

Table 2. Composition of cultured isolates from an untreated bacterial biofilm on steel- and plastic surfaces. H_2S producing bacteria, which form a black precipitation on IA medium, are represented with a whole circle (●) and none H_2S producing bacteria with open circle (○).

Identity (%)	Closest database match (%)	Colony color	Number of isolates/contic	Percentage of population (%)	GenBank gi number	Class
100	*Aeromonas salmonicida smithia*	●/○	25	27,5	gi11127589	γ-Proteobacteria
99	*Serratia sp./Serratia proteamaculans*	○	9	9,9	gi53680558/gi4582259	γ-Proteobacteria
100	*Shewanella baltica*	●	8	8,8	gi60458802	γ-Proteobacteria
100	*Shewanella putrefaciens*	●	7	7,7	gi23345133	γ-Proteobacteria
100	*Acenetobacter sp.*	○	5	5,5	gi829098	γ-Proteobacteria
100	*Uncultured bacterium/Comamonas sp*	○	5	5,5	gi45772011/gi49617309	β-Proteobacteria
100	*Pseudomonas sp.*	●/○	4	4,4	gi64500530	γ-Proteobacteria
99	*Klebsiella ornithinolytica*	○	3	3,3	gi3282036	γ-Proteobacteria
100	*Morganella psychrotolerans*	●/○	3	3,3	gi86451976	γ-Proteobacteria
99	*Carnobacterium maltaromaticum*	○	2	2,2	gi47155909	Firmicutes
99	*Acinetobacter sp.*	○	2	2,2	gi12049704	γ-Proteobacteria
100	*Hafnia alvei*	●	2	2,2	gi30230473	γ-Proteobacteria
99	*Serratia sp*	○	2	2,2	gi51339856	γ-Proteobacteria
99	*Shewanella baltica*	●	2	2,2	gi2764470	γ-Proteobacteria
97	*Unidentified bacterium/Myroides sp.*	○	1	1,1	gi2791654/gi86277057	Bacteroidetes
97	*Wautersiella falsenii*	○	1	1,1	gi113207006	Bacteroidetes
100	*Citrobacter freundii*	●	1	1,1	gi94983682	γ-Proteobacteria
98	*Unidentified bacterium/Myroides odoratimimus*	○	1	1,1	gi2791951/gi55956965	Bacteroidetes
100	*Uncultured bacterium/Acinetobacter sp.*	○	1	1,1	gi81238422/gi12049702	γ-Proteobacteria
99	*Shewanella putrefaciens*	●	1	1,1	gi60458797	γ-Proteobacteria
99	*Acinetobacter sp.*	○	1	1,1	gi112148861	γ-Proteobacteria
100	*Buttiauxella agrestis*	○	1	1,1	gi90655953	γ-Proteobacteria
99	*Delftia tsuruhatensis*	○	1	1,1	gi52788430	β-Proteobacteria
99	*Pseudomonas sp.*	○	1	1,1	gi37704575	γ-Proteobacteria
99	*Serratia sp.*	●	1	1,1	gi53680558	γ-Proteobacteria
99	*Pseudomonas fragi*	-	1	1,1	gi62465826	γ-Proteobacteria
	Total isolates sequenced		**91**	**100**		

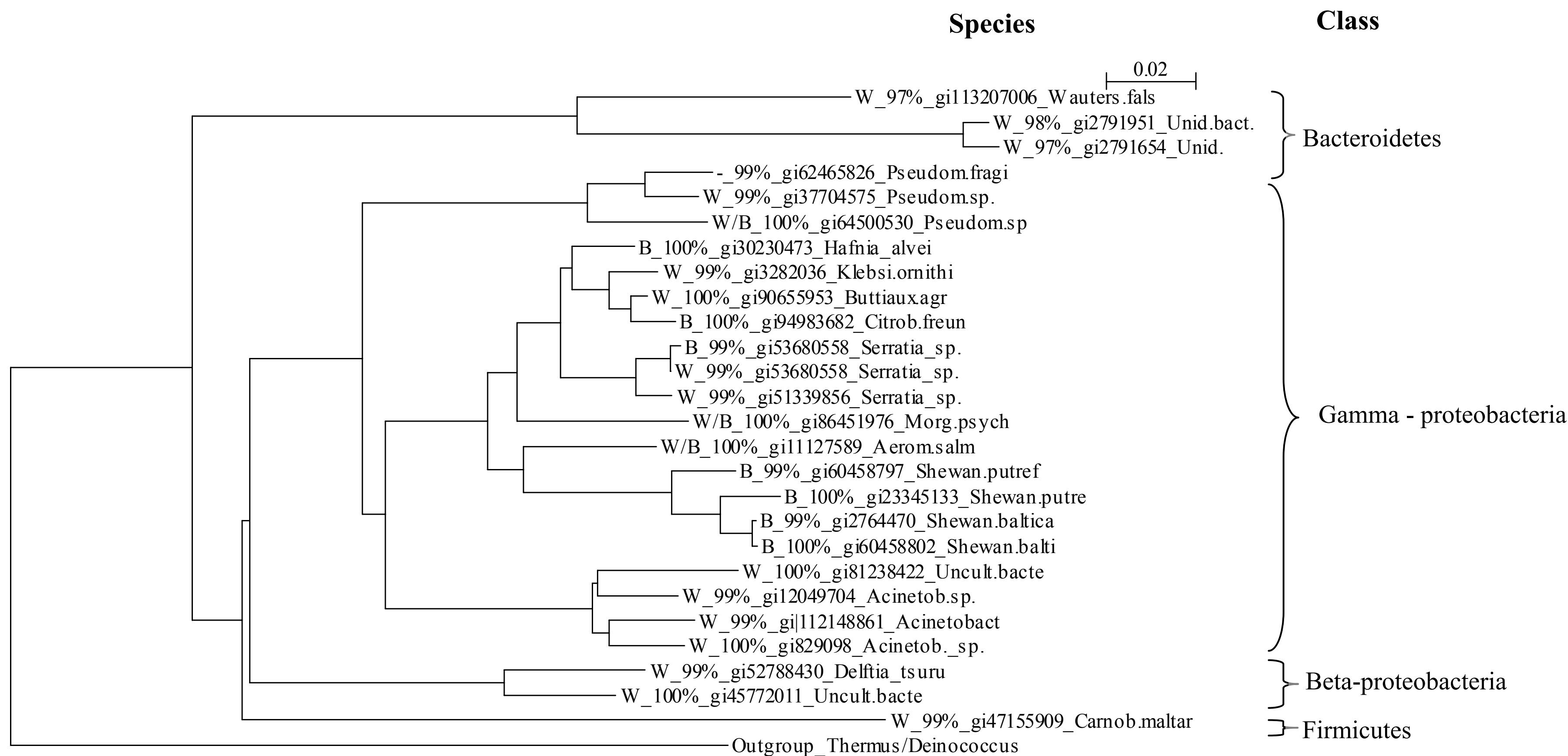

Figure 6. Evolutionary neighbour-joining phylogenetic dendrogram of the 16S rRNA partial sequencing data from isolates on IA medium cultured from an untreated biofilm on steel- and plastic surfaces. The biofilm was formed from a natural bacterial flora of cod fillets. The letter W represents white colony and B black colony. The closest match in Genbank is presented with the identity score and the gi number of corresponding hit. Thermus Deinococcus was used as an outgroup.

3.3.2 Species composition by a direct molecular amplification (PCR)

A sample taken from a biofilm formed on GSS surface was placed directly in nucleic acid purification. PCR amplification with universal primers was then performed on the sample and a clone library was constructed. This enabled the species composition to be analysed by sequencing the cloned 16S rRNA products.

Phylogenetic analysis on 360 bp sequence was carried out and evolutionary dentrogram was constructed (Figure 7). As with the cultivation, a high dominance of the genus *Aeromonas* was detected (total of 53,8%) with molecular amplification. Likewise, most of the organisms detected, or 75,1%, belonged to the class Gamma-proteobacteria (Table 4). Using this method, no occurrence of the SSO *Shewanella* was observed and only one representative of *Pseudomonas* sp. was detected.

Table 3. Composition PCR amplified and cloned sequences derived from an untreated bacterial biofilm on steel surface.

Identity (%)	Closest database match (%)	Number per contic	Percentage of population (%)	GenBank gi number	Class
99%	*Aeromonas salmonicida atypical*	23	35,4	6683059	γ-Proteobacteria
99%	*Aeromonas sobria*	10	15,4	39005	γ-Proteobacteria
99%	*Serratia proteamaculans*	4	6,2	4582259	γ-Proteobacteria
95%	*Vagococcus carniphilus*	4	6,2	76782242	Firmicutes
99%	*Chryseobacterium sp.*	3	4,6	88999740	Bacteroidetes
97%	*Myroides sp.86277057*	2	3,1	86277057	Bacteroidetes
97%	*Uncultured Acinetobacter sp. clone NMT sF6*	2	3,1	67082912	γ-Proteobacteria
98%	*Brochotrix thermosphacta*	2	3,1	47155903	Firmicutes
94%	*Unidentified bacterium*	1	1,5	2791951	Bacteroidetes
93%	*Aeromonas salmonicida subsp. Achromogenes*	1	1,5	62547921	γ-Proteobacteria
96%	*Comamonas sp.*	1	1,5	111380772	β-Proteobacteria
98%	*Pantoea sp*	1	1,5	7110410	γ-Proteobacteria
92%	*Serratia proteamaculans*	1	1,5	85677238	γ-Proteobacteria
97%	*Serratia liquefaciens*	1	1,5	30230475	γ-Proteobacteria
99%	*Klebsiella ornithinolytica strain ATCC 31898*	1	1,5	6693807	γ-Proteobacteria
98%	*Acinetobacter sp.*	1	1,5	11414595	γ-Proteobacteria
97%	*Carnobacterium maltaromaticum isolate MF 82*	1	1,5	47155909	Firmicutes
95%	*Serratia proteamaculans*	1	1,5	4582259	γ-Proteobacteria
91%	*Aeromonas sp. ydcc-5-1 16S*	1	1,5	112361491	γ-Proteobacteria
98%	*Pseudomonas gessardii*	1	1,5	3309635	γ-Proteobacteria
98%	*Acinetobacter sp. LUH3790*	1	1,5	13275213	γ-Proteobacteria
94%	*Wautersiella falsenii subsp. genomovar 1*	1	1,5	113207010	Bacteroidetes
97%	*Wautersiella falsenii subsp. genomovar 2*	1	1,5	113206996	Bacteroidetes
	Total clones sequenced	**65**	**100**		

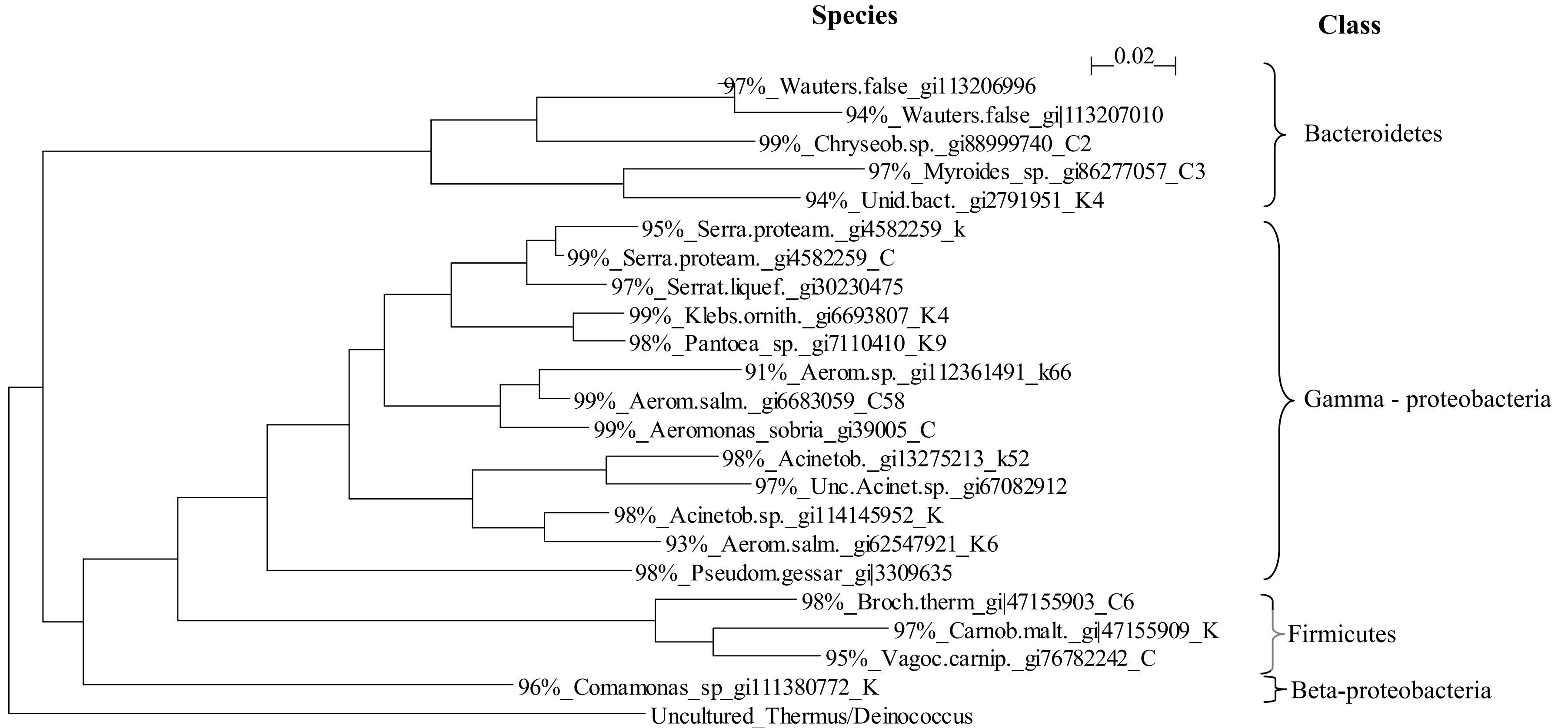

Figure 7. Evolutionary neighbour-joining phylogenetic dendrogram of the 16S rRNA partial sequencing data from 16S rRNA clone sequences amplified from untreated biofilm on steel and plastic surfaces. The biofilm was formed from a natural bacterial flora of cod fillets. The closest match in Genbank is presented with the identity score and the gi number of corresponding hit. Thermus Deinococcus was used as an outgroup.

Table 4. Class composition from an untreated bacterial biofilm on steel- and plastic surfaces analysing 16S rRNA sequences from cultivated isolates and from PCR amplified sequences.

Class	Isolates		PCR amplification	
	Number of matches	Ratio of total (%)	Number of matches	Ratio of total (%)
γ-Proteobacteria	80	87,9	49	75,4
β-Proteobacteria	6	6,6	1	1,5
Bacteroidetes	3	3,3	8	12,3
Firmicutes	2	2,2	7	10,8

4. DISCUSSION & CONCLUSIONS

This project has basically two outputs, a practical one and a theoretical one. Important factor affecting both the practical and theoretical outputs of this project is that the biofilm used was a natural bacterial flora from fish. Most studies on biofilm adhesion or resistance concentrate on only one or few representatives of the fish bacterial flora [12-14].

The practical output covers optimal washing conditions for fish processing plants and the appropriate usage of detergents. The results of testing critical factors in a typical washing protocol can have economical and environmental significance for fish processing companies, which often use detergents and disinfection agents in excess. In the case of the processing plant visited, which was using a detergent concentration of 5,4%, it is evident that it can reduce the detergent quantity by 36% simply by using a 3,4% dilution of detergent instead of 5,4%. This could have a considerable economical and environmental benefit. It would be interesting to carry out a survey in different fish processing plants to get an overview of detergent usage, in order to make recommendations for improved washing protocols to the industry.

The detergent manufacturers recommend 1-4% dilution of Det1 and 4-10 % dilution of Det2. In Iceland, where hot water is easily accessible a more diluted detergent with warmer water can have a similar effect as a highly concentrated detergent. When comparing the efficiency of the two detergents it has to be considered that the actual concentration was not the same, where Det2 tended to be more concentrated than Det1. Nevertheless, Det1 showed an equal or better efficiency than Det2 in destroying viable

cells in the biofilm. Det2 has gel-like physical properties which enable the detergent to stick better to vertical surfaces. This property did not seem to give Det2 advantage over Det1 in the present study.

In the protocols that were not able to destroy all the viability in the biofilm a low bacterial count was observed. Generally, on the GSS coupons the bacterial viability was completely removed after the washing protocol, but on the PEP coupons the washing was less efficient. On the PEP coupons in some incidents, it was noticed that after the disinfectant step, no bacterial count was observed but after the final rinsing step, viable cells were detected. It is quite possible that the D/E neutraliser is not as efficient in neutralising the disinfection agent on PEP surface as on GSS surface and therefore retained some of its activity while processing the PEP coupon.

In the protocols that were not able to destroy all the viability in the biofilm, the number of bacteria left on surface was from 5 - 400 cfu/cm^2, often only in one replicate out of three. The limit used for clean food contact surface is 5-10 cfu/ cm^2 (IFL-guidelines), so detecting just a few bacteria is acceptable. If the number reaches high value, as it was in one case where it was 400 cfu/ cm^2 then it is likely that something has gone wrong during the washing process and the cleaning procedure must be checked and improved. Exceptions from this are in protocol A which has been explained.

As expected, the bacterial number on PEP surface was higher than on GSS surface. This is in accordance to previous studies [3].

No direct evidence suggests that one detergent is more efficient than the other. The concentration of the Det2 was generally higher than that of DET1 (Table 1), but that did not make any difference in its ability to decrease microbial survival.

The other output of this project is the scientific significance which can help to characterise and identify important bacteria species in biofilm formation on fish processing surfaces.

Real time PCR is an increasingly popular method for quantitative analysis of bacterial load in environmental samples [15-18]. The present study shows a high correlation between colony counting and real time PCR quantification. However, the PCR method detected about two log higher loads than colony counting. Many reasons could cause these results. First of all, with PCR, dead cells can contribute to the measurement since it

is based on the presence of specific nucleic acids but not viable cells. The quantitative analysis is also based on number of 16S rRNA copies present in a sample and each bacteria species can contain from 2-11 copies of this gene in their genome [19]. These factors are currently used for revision of the real-time PCR method and the standards used for quantification.

Phylogenetic diversity analysis of the biofilm was compared by 16S rRNA sequence data from cultured isolates and data derived from a direct 16S rRNA molecular amplification of the sample, cloning and subsequent sequencing of the 16S rRNA insert in the clones. The composition was similar in many ways where the most abundant class was the γ-proteobacteria and the most abundant genus was *Aeromonas* sp (Figure 7 and Table 3). In this ecosystem, a high degree of dominance of *Aeromonas* sp. is evident. This dominance had been acquired through 48 hours incubation of the natural flora at isothermal temperature.

The genera *Shewanella putrefaciens* and *Pseudomans* spp. in the biofilm have been identified as the main specific spoilage organisms in fish (SSO) [20, 21]. When comparing the data from the conventional and molecular methods it can be seen that *Shewanella putrefaciens* was not detected by PCR. The reason is not due to a lack of detection ability of this species by PCR, since amplification of the pure *Shewanella putrefaciens* isolate was not problematic. More likely reasons could be that the samples used for the separate techniques were not from the same specific sample. The sample used in the molecular study is only from one coupon (steel), but the data from the cultures were from several coupons (both plastic and steel). Colonies cultured on IA medium were picked up from various coupons and sequenced. The sample for PCR amplification was taken from one of these coupons (steel) and this particular sample did not contain any *Shewanella putrefaciens* when cultured on IA medium. This discrepancy could perhaps have been avoided if the isolated DNA from several surfaces were pooled prior to PCR amplification.

In conclusion, more economically and environmentally friendly approaches in cleaning fish processing equipment are possible. Further optimisation and determination of

minimum concentration of detergent chemicals used in the washing protocols would be necessary for composition of washing guidelines for the industry.

Comparison of phylogenetic diversity analysed by cultivation or molecular amplification give similar results. *Aeromonas* sp. is a typical species found in many fish material and showed a high degree of dominance in the present study, both with cultivation and molecular amplification.

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