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Decontamination Efficiency of Fish Bacterial Flora from Processing Surfaces

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Summary

There are numerous parameters that can influence bacterial decontamination during washing of machinery and equipment in a food processing establishment. Incomplete decontamination of bacteria will increase the risk of biofilm formation and consequently increase the risk of pathogen contamination or prevalence of other undesirable microorganisms such as spoilage bacteria in the processing line. The efficiency of a typical washing protocol has been determined by testing three critical parameters and their effects on bacterial decontamination. Two surface materials (plastic and stainless steel), water temperatures (7 and 25 °C) and detergent concentrations (2 and 4 %) were used for this purpose in combination with two types of detergents. Biofilm was prepared on the surfaces with undefined bacterial flora obtained from minced cod fillets. The bacterial flora of the biofilm was characterised by cultivation and molecular analysis of 16S rRNA genes. All different combinations of washing protocols tested were able to remove more than 99.9 % of the bacteria in the biofilm and reduce the cell number from 7 to 0 or 2 log units of bacteria/cm^2. The results show that it is possible to use less diluted detergents than recommended with comparable success, and it is easier to clean surface material made of stainless steel compared to polyethylene plastic.

Key words: biofilm, fish processing, washing, bacteria, molecular methods

Introduction

The quality of fish products is critical to ensure a high economic value of the catch on markets. Many factors, from catch to processing, influence the quality and safety of the fish, *e.g.* natural conditions when it is captured, handling on board and in the processing plant. Microbiological breakdown of tissues is one factor that decreases the quality. It is unavoidable, but can be minimized by incorporating standard hygiene protocols, especially in the early handling and in processing plants (*1*). The formation of a microbial biofilm on the surface of fish processing equipment increases the threat of a crossover contamination of the product (*2*). This can have influence on the quality and safety of the final product, especially if specific spoilage organisms (SSO) or pathogenic bacteria become dominant in the biofilm (*3*). The shelf life and quality of fish products are greatly dependent on the handling of the catch and are severely diminished if measures for preventing contamination are unsatisfactory through the entire processing chain (*4,5*).

Surface finishing on working surfaces is considered to affect bacterial adhesion (*6*). Significantly fewer bacterial cells and less biofilm formation were observed on

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electropolished surface of stainless steel compared 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 has been concluded that smooth surfaces do not necessarily provide hygiene benefits over rougher surfaces (*7*). Effective hygienic protocols are essential to minimize the formation of biofilms and to prevent contamination of the products (*8*). 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 taken with care and precaution because of environmental purposes, health issues and governmental regulations (*9*). Moreover, some bacteria (*e.g. Pseudomonas* spp.) may have certain resistance mechanisms against antibacterial components commonly used in disinfectants such as quaternary ammonium compounds (*10,11*).

Different fish processing establishments have different ways of cleaning and washing their equipment. Water temperature, water hardness, acidity, surface material of the equipment, type of detergent or disinfectant and their concentrations are examples of variables that are likely to be different in each plant and between countries. Generation of persistent microflora in food processing, most commonly *Pseudomonas*, is well known and studies have been made on decontamination efficiency of bacteria from various surfaces, but most of them use only one or few model organisms commonly found in food processing environments (*7,12–14*).

The aim of this study is to investigate the washing efficiency of a typical washing protocol on a naturally occurring biofilm by analysing bacterial survival during processing and after cleaning. Two different surfaces in combination with common washing protocols in fish processing plants were used for this purpose and *in situ* bacterial flora from cod was used for biofilm formation. For characterisation of the *in situ* biofilm, a 16S rRNA microbiological analysis was performed and the effect of washing on survival and compositional changes in the microflora was monitored by cultivation and terminal restriction fragment length polymorphism (TRFLP) (*15,16*).

Material and Methods

Biofilm preparation

A biofilm was developed from a bacterial flora of fish fillets on glass beaded stainless steel (SS) AISI 304 2B and polyethylene plastic (PEP) surfaces as follows: a fresh fish fillet was kept at 4 °C overnight and then minced and split into eight portions. The minced portions were kept at –80 °C in order to minimize sample variation between experiments. About 175 g of the minced fillet were mixed with 350 g of deionised water in a Stomacher bag with a lateral filter and mixed for 30 s in a Stomacher® (Lab System 400, Seward Medical, UK). A volume of 30 mL of fish juice was placed in a sterile glass tube containing a sterile stainless steel or plastic coupon and was kept agitated at 75 rpm and 19–21 °C for 48 h.

Washing protocol

After the biofilm formation, the coupons went through a stepwise washing protocol consisting of rinsing, washing, rinsing, disinfecting and rinsing (Fig. 1). After each washing step, coupons taken in triplicate were collected from the washing station. A semi-automated washing station was used for washing, which minimized variations between experiments and imitated fish processing washing procedures. The station consisted of a pump/blender (Hygiene System, Ecolab Inc., MN, USA) and a washing chamber (Marel Food Systems, Reykjavík, Iceland). The pump enabled mixing the 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 were monitored throughout all experiments. The detergent mass fraction was measured with a kit supplied by Tandur H.F. (Reykjavík, Iceland) and the disinfection agent concentration was measured using the JDK06 active cationic products test kit (Johnson Diversey Inc., Nottingamshire, UK).

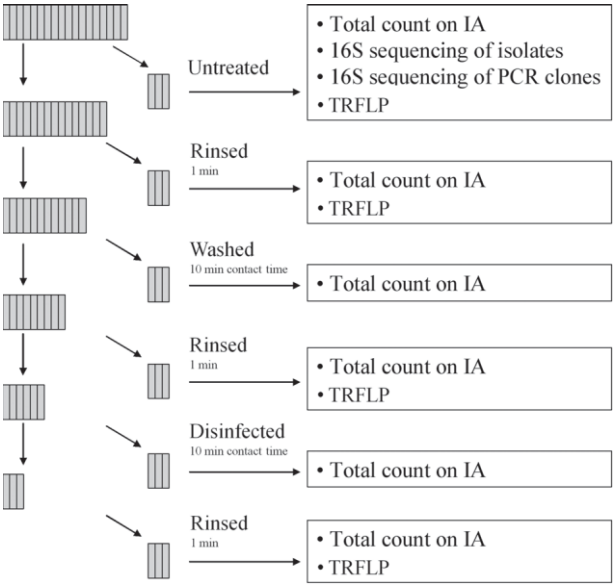

Fig. 1. The experimental layout of the study. After each washing step, coupons were taken in triplicate and analyzed

The following washing parameters were used to investigate the cleaning efficiency: two surfaces (SS and PEP), two mass fractions of detergent (2 and 4 %) and two washing temperatures (7 and 25 °C). Two different types of detergent were used for this investigation (Det1 and Det2, manufactured by Tandur H.F., Reykjavík, Iceland). Both of them are alkaline-based detergents. Quaternary ammonium-based disinfection agent (Tandur H.F., Reykjavík, Iceland) was used and kept constant at 0.25 % concentration. Both surface materials are commonly used as a contact material in fish industry, SS is used in fish processing equipment and PEP in fish tubs. Actual concentration of Det1 in a fish processing plant was measured using the same kit as in the laboratory experiment.

Cultivation

Bacteria attached to the surface were removed and isolated from the coupons by rubbing a cotton swab tightly on the surface. The swab was dipped in Day and Engley (D/E) neutralizer (Difco, Franklin Lakes, NJ, USA) to neutralize the disinfectant leftovers before capturing the bacteria. The swab was released into 5 mL of maximum recovery diluent (MRD) buffer (Oxoid, Hampshire, UK) and shaken vigorously. Serial dilutions were prepared for each sample and plated onto iron agar (IA) (*17*). The plates were incubated at 15 °C for 7 days. Sulphite-producing bacteria form black colonies on IA and were counted separately. Colonies were picked from different samples for 16S rRNA taxonomic identification.

DNA isolation

DNA from bacterial isolates was extracted by suspending a loopful of bacteria in 200 μL of 5 % Chelex solution by vortexing. The suspension was incubated at 55 °C for 15 min and then vortexed again, boiled for 10 min and then placed on ice for 3 min. The samples were centrifuged at 11 000×*g* for 7 min and the supernatant was recovered.

DNA was isolated directly from the biofilm on the SS and PEP coupons for direct molecular analysis as follows: 1 mL of the MRD buffer containing the swab was centrifuged at 11 000×*g* for 7 min 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 manufacturer's instructions.

PCR amplification and 16S rRNA sequencing

Since the washing experiments were performed on undefined bacteria, the species composition of the biofilm was characterized. It was done using two approaches; sequencing of the 16S rRNA gene of the isolates, and a method without cultivation where the 16S rRNA gene was amplified directly from the biofilm, cloned and sequenced (clones). PCR reaction for taxonomic identification of isolates was done by amplifying the 16S rRNA gene with universal primers, 9F and 1510R (5′-GAGTTTGATCCTGGCTCAG-3 and ´5-GGTTACCTTGTTACGACTT-3´, respectively). PCR conditions and sequencing were done according to Marteinsson *et al.* (*18*). The species coverage by the 16S analysis was estimated using the equation:

$$C=1-(n_1/N_t) \qquad /1/$$

where C is coverage, n_1 is the number of unpaired sequences and N_t is the number of total clones analyzed.

Terminal restriction fragment length polymorphism (TRFLP)

The TRFLP analysis was done on samples derived from cleaning protocols (Fig. 1). Samples were taken in triplicate and PCR was done on each replicate. The PCR products of each replicate were pooled together prior to restriction and TRFLP analysis. For each sample, two separate PCRs were carried out (two primer sets) and two restriction enzymes were used in order to enhance the discriminatory power of the assay. The first PCR was with FAM-labelled forward primer from position 515 in the *E. coli* genome (´5-GTGCCAGCMGCCGCGGTAA-3´) and nonlabelled reverse primer 1510R (see above). The second PCR was with FAM-labelled forward primer 27F (´5-AGAGTTTGATCCTGGCTCAG-3´) and HEX-labelled reverse primer 805R (´5-GACTACCCGGGTATCTAATCC-3´). The labelled PCR products were digested with *Hae*III and *Alu*I (Fermentas, Hanover, MD, USA) in a 10-μL reaction volume for 3 h. The digested PCR product was diluted 1:10 and 2 μL were added to 8 μL of GeneScan™ 500 LIZ® internal size standard (Applied Biosystems, Warrington, UK) in formamide. The fragment analysis was carried out in ABI3730 DNA analyzer. Data analysis was carried out on the GeneMapper® software v. 4.0 using the AFLP analysis method. Peaks in the TRFLP profile that had a representative in the 16S rRNA analysis could be assigned to bacterial species or groups. Comparison of 16S analysis and TRFLP was done by estimating the relative abundance of each species using either the number of clones (16S rRNA sequencing) or peak area (TRFLP).

Results

Washing efficiency

The experiments were split up into eight subexperiments or protocols due to the limiting sample number which could be processed at the same time. Temperature, water pressure, concentrations of chemicals and pH were monitored throughout all the experiments and showed acceptable consistency (Table 1). Generally, all the different combinations of the variables tested on the biofilm proved to be efficient in removing or destroying the viability of the biofilm, reducing the CFUs from about 7 log units down to below 10 CFU/cm^2 after disinfection or the final rinsing step, which is generally regarded as a clean surface (Fig. 2). Lower temperature and lower detergent concentration generally proved to be less efficient, although only a few colonies or none at all were able to grow after the final rinsing step. All washing protocols removed more than 99.99 % of the total bacterial load and reduced the number of bacteria in

Table 1. Monitoring of parameters applied in the washing experiment

Parameters	Average	SD
Lower washing temperature/°C	7.0	0.58
Higher washing temperature/°C	23.4	2.76
Water pressure/mbar	17.1	0.34
Det1 lower concentration/%	1.8	0.19
Det1 higher concentration/%	3.5	0.16
Det 1 pH	12.4	0.36
Det2 lower concentration/%	2.6	0.23
Det2 higher concentration/%	5.0	0.27
Det2 pH	12.2	0.36
Disinfectant concentration/%	0.24	0.04
Disinfectant pH	8.38	0.95

SD=standard deviation

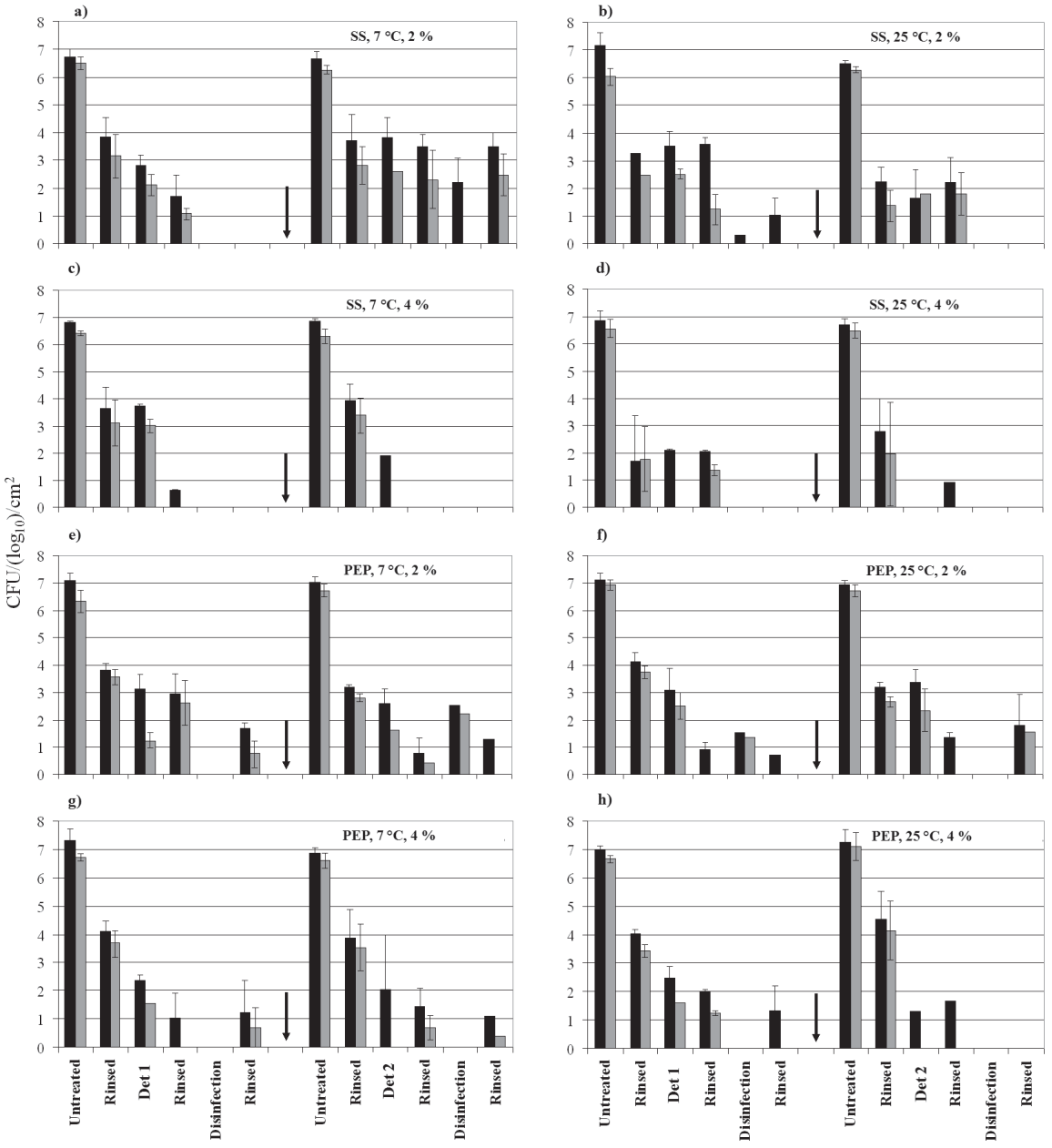

Fig. 2. Results from the decontamination experiment. Black columns indicate total count on IA and gray columns indicate the count of black colonies on IA (H_2S producers). Arrow divides experiments conducted with Det1 and Det2. Surface type, temperature and detergent mass fraction are indicated in each plot

the biofilm from 7 down to 0 log units and in some occasions down to 1 or 2 log units (Fig. 2). The concentration of Det1 was 5.4 % on average in an actual processing plant.

The survival of bacteria after the final rinsing step was more frequent on PEP surfaces than on SS surfaces (Fig. 2). In seven out of eight experiments where PEP was tested, bacterial growth was observed after the final rinsing step, but only in two out of eight experiments where SS was tested. High bacterial count was observed after washing with Det2 on one occasion (Fig. 2a), which could be explained by high pH of the disinfection agent in that experiment (data not shown).

Removal of the H_2S-producing bacteria was satisfactory in almost all protocols. Det1 failed to remove all of them from the plastic surface at low temperature at both detergent mass fractions tested. Det2 failed to remove them sufficiently in two PEP protocols as well, although the growth was observed in only one replicate of three in both cases (Fig. 2).

Characterization of bacterial species in biofilm

Amplification and sequencing of the 16S rRNA gene was successful with 94 isolates and 60 clones. The total species coverage was calculated to be 88 % for clone analysis and 92 % for the isolate analysis.

Gammaproteobacteria dominated the population with 87.9 % dominance (Table 2), where the genus *Aeromonas* showed the highest abundance in an untreated biofilm, having 27 % dominance with cultivation, and 50 % do-

Table 2. Class composition of an untreated bacterial biofilm from the surfaces analysed by 16S sequencing from cultivated isolates and uncultivated clones

Class	Isolates		Clones	
	Number of hits	Total ratio %	Number of hits	Total ratio %
Gammaproteobacteria	80	87.9	49	75.4
Betaproteobacteria	6	6.6	1	1.5
Bacteroidetes	3	3.3	8	12.3
Firmicutes	2	2.2	7	10.8

minance with cloning analysis (Table 3). *Shewanella putrefaciens* and *Pseudomonas* spp. were detected in the biofilm, but they had been characterized before as the main specific spoilage organisms (SSO) in fish (*1*). In addition to the SSO, *Aeromonas sobria*, *Morganella psychrotolerans*, *Hafnia alvei* and *Citrobacter freundii* were also able to produce H_2S and form black colonies (Table 3), which may indicate their spoilage potential.

TRFLP profile analysis

A clear correlation was established between the TRFLP profile and the expected profile indicated by the sequence information of the clone library and isolates (Table 4). Five terminal fragments (TF) of the size 119, 220, 228, 380 and 412 bp are common with all methodologies (Table 4). Four peaks in the TRFLP profile could not be correlated with species since the representative sequences were not found with the clone library. The most abundant TF was 220 bp in size and had 29 % relative abundance of the total peak area in the profile. This peak size correlates with *Aeromonas* sp., which showed 72 and 68 % relative abundance using cultivation and 16S rRNA, respectively (Table 4). Clear changes in the bacterial species combination between different washing steps were not detected, whatever primer restriction combination, although peak heights showed slight differences (data not shown). In some cases PCR amplification was not successful after the final rinsing step because of the low amount of target DNA.

Discussion

This paper describes a study where different parameters were tested to evaluate the decontamination efficiency of fish bacterial flora from two of the most common working surfaces. Most studies on biofilm adhesion or disinfectant resistance focus only on one or few bacterial representatives (*12,14,19*) which might not repre-

Table 3. Species composition of cultured isolates and clones from the untreated bacterial biofilm surfaces

Group label	Closest database match %	H_2S production	Isolates %	Clones %	Homology %	Accession number
G124	*Aeromonas salmonicida*	+/–	24	35	100	AM296510
G129	*Shewanella baltica*	+	19	0	100	CP000563
G125	*Serratia* spp.	–	13	10	100	AY745744
G157	*Comamonas* spp.	–	5	2	99	DQ851179
G130	*Acinetobacter* spp.	–	5	0	100	AM401576
G133	*Morganella psychrotolerans*	+/–	4	0	100	DQ358142
G146	*Aeromonas sobria*	+	3	15	100	DQ133179
G140	*Pseudomonas* spp.	+/–	3	2	100	AY689025
G142	*Klebsiella* spp.	–	3	2	99	DQ277701
G191	*Pseudomonas fluorescens*	–	3	0	99	AM183964
G150	*Carnobacterium maltaromaticum*	–	2	2	100	EF204311
G153	*Acinetobacter* spp.	–	2	2	100	AJ301676
G188	*Buttiauxella agrestis*	+/–	2	2	99	DQ440549
G144	*Hafnia alvei*	+	2	0	100	AY253922
G183	*Myroides* spp.	–	2	4	98	DQ357029
G134	*Wautersiella falsenii*	–	1	3	99	AM238684
G189	*Acinetobacter johnsonii*	–	1	3	99	EF204266
G163	*Vagococcus carniphilus*	nd	0	7	99	DQ166854
G176	*Chryseobacterium* spp.	nd	0	5	99	AY468476
G192	*Aeromonas sobria*	nd	0	5	99	DQ133179
G180	*Brochothrix thermosphacta*	nd	0	3	98	AY543029
Str 18	*Citrobacter freundii*	+	1	–	100	DQ481481
Str 40	*Acinetobacter* spp.	–	1	–	100	AJ301674
Str 42	*Shewanella putrefaciens*	+	1	–	100	AB205575
Str 60	*Delftia tsuruhatensis*	–	1	–	99	DQ864991

nd=not determined

Table 4. Species abundance and diversity profile determined with TRFLP compared with bacterial diversity of sequencing of isolates and clones

TF[a]	TRFLP[b]	Clones[c]	Isolates[c]	Bacterial species[d]
bp	%	%	%	
74	3	1	0	*Acinetobacter* spp.
119	6	2	3	*Myroides* spp.
208	4	0	0	nd
217	0	5	2	*Comamonas* spp.
220	29	72	68	*Aeromonas, Shewanella, Serratia* and others[e]
228	23	9	5	*Acinetobacter* spp. and *Acinetobacter johnsonii*
254	3	0	0	nd
325	2	0	0	nd
380	15	1	0	*Delftia tsuruhatensis*
381	0	6	2	*Pseudomonas* spp. and *Pseudomonas fluorescens*
382	0	2	8	*Carnobacterium maltaromaticum* and *Vagococcus carniphilus*
411	2	0	0	nd
412	4	1	8	*Wautersiella falsenii* and *Chryseobacterium* spp.
416	8	0	3	*Brochothrix thermosphacta*

[a]Size of terminal fragment in bp
[b]Values indicate proportional peak size area
[c]Values indicate proportional number of analysed sequences
[d]Bacterial species identification was done by BLASTing of sequences derived from 16S rRNA analysis
[e]Bacterial species in TF 220 were: *Aeromonas salmonicida, Aeromonas sobria, Shewanella baltica, Shewanella putrefaciens, Serratia* spp., *Morganella psychrotolerans, Klebsiella* spp., *Buttiauxella agrestis* and *Hafnia alvei*
nd=species representative not detected in a 16S rRNA analysis

sent the actual flora during fish processing. To our knowledge, this is the first paper where 16S rRNA sequencing on both cultivable and uncultivable species is used for the evaluation of biofilm decontamination.

In recent years, a better understanding has been established through research on biofilm microbial populations relating to their spatial structure, their community structures and their dependence on physicochemical parameters (*20*). The formation and persistence of a biofilm is not only dependent on the surface type, but also on the soiling status of the surface (*21*). Although this study has focused on the decontamination of bacteria with different physicochemical parameters, the presence of nutrients, exopolysaccharides and other substances from the environment also influence the primary attachment of bacteria (*21,22*). Monitoring of polymeric substances during washing could therefore also be used as an indicator of cleaning efficiency. This study was limited to the testing of new steel and plastic coupons, but in reality the surfaces can be scratched, worn or corroded, which reduces cleanability. Moreover, the equipment in food processing plants has joints, welds and crevices, which may be more difficult to clean than the coupons used in this study.

Food producers around the world work in different environments and have to deal with decontamination according to their resources. Water supply, proper cleaning agents and equipment can vary among industries and countries and therefore, information on washing efficiency and robustness of washing protocols can be of significant value for food producers.

In this study, these issues are addressed with regard to the bacterial decontamination efficiency. When comparing the effects of the two temperatures used during the washing procedure, our results indicate that using less concentrated detergent in combination with higher washing temperatures, similar decontamination efficiency can be expected. This was also valid at lower washing temperatures. Recommended concentration of Det1 is 2–4 %, but by measuring the actual concentration of the detergent in a processing plant, it was shown to be 5.4 % on average. Having the concentration at recommended values can therefore reduce the detergent usage by 26–63 % with economical and environmental benefit.

The biofilm on both SS and PEP coupons was successfully removed after washing, but in some cases, bacteria were detected after the final rinsing step, especially on PEP surfaces, even though no bacteria were detected after the disinfection step before it (Fig. 2). This might suggest contamination from the washing cabinet during rinsing. Contamination of this sort is more likely to be observed on PEP than SS due to its higher hydrophobic properties (*20*). The bacterial number on PEP surface was higher than on SS surface, which is in accordance with previous studies (*3,20*).

The analysis of the bacterial flora was performed by cultivation and molecular methods to study and compare the diversity of cultivated and uncultivated microorganisms. Both methods revealed similar species composition in the biofilm. The most abundant class was the Gammaproteobacteria and the most abundant genus in the class was *Aeromonas* spp. (Tables 3 and 4). The dominance of *Aeromonas* spp. was established under the con-

ditions 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. *Aeromonas salmonicida* is a fish pathogen (*23*) and was the most abundant species in the biofilm. *Pseudomonas*, *Shewanella baltica* and *S. putrefaciens* have all been correlated with the spoilage process of fish (*24*,*25*) and are therefore important for decontamination.

Sulphite-producing bacteria have a spoilage potential in fish and are recognised as black colonies on iron agar. These bacteria are generally regarded as *Shewanella putrefaciens (17*), but our results show that *Aeromonas salmonicida*, *A. sobria*, *Morganella psychrotolerans* and *Hafnia alvei* are also able to produce sulphite and can therefore contribute to the spoilage of fish if they are present.

TRFLP analysis was in agreement with the cultivation and PCR cloning, but it did not reveal the species or groups exceptionally tolerant of washing, which indicates even spatial distribution of the bacterial species in the biofilm.

Conclusions

We have shown that it is possible to reduce the mass fraction of detergents considerably in a laboratory scale testing of cleaning efficiency. In general, the washing efficiency proved to be acceptable using less concentrated detergents or lower water temperature. Such reduction could be financially and ecologically favourable for the fish industry and its surroundings. However, reduction in detergent mass fractions below advised limits is not recommended. In this study, *in situ* bacterial flora from fish were used for the evaluation of decontamination efficiency with regards to bacterial survival. This approach is more likely to reflect the total decontamination efficiency of washing in fish processing plants than using one or few representative strains. Molecular characterization of the biofilm revealed a diverse bacterial community, which proved to be cleanable from processing surfaces using appropriate washing protocols.

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