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Bacterial diversity in the processing environment of fish products

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Öryggi og umhverfi

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Report summary

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Titill / Title	**Bacterial diversity in the processing environment of fish products /** Fjölbreytileiki bakteríusamfélaga í vinnsluumhverfi fiskafurða		
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Ágrip á íslensku:

Í skýrslunni er leitað svara við fjölbreytileika og tegundasamsetningu örvera í fiskvinnsluumhverfi. Rannsóknarvinnan hófst með uppsetningu og þróun aðferða til að skanna örverusamsetningu með sameindalíffræðilegum aðferðum og svo á seinni stigum var hafist handa við að skoða valin umhverfi úr fiskiðnaðinum. Tvær fiskvinnslur voru heimsóttar, hvor um sig í tvígang þar sem úttekt var gerð á vinnslunni og u.þ.b. 20 sýni tekin í hverri ferð. Í ljós kom fjölbreytt samfélag baktería þar sem þekktar skemmdarbakteríur voru í jafnan í háu hlutfalli ásamt ýmsum öðrum tegundum. Örverutalningar sýndu fram á hátt magn baktería á yfirborðum vinnslulína á meðan á vinnslu stendur með fáa bakteríuhópa í yfirmagni en einnig fjölmargar aðrar tegundir í minna magni. Helstu hópar baktería sem fundust tilheyra *Photobacterium phosphoreum*, sem var í hæsta hlutfallslegu magni heilt yfir í rannsókninni, ásamt *Flavobacterium*, *Psychrobacter*, *Chryseobacter*, *Acinetobacter* og *Pseudoalteromonas*. Allar þessar tegundir eru þekktar fiskibakteríur sem lifa í roði og þörmum lifandi fiska. Þetta er fyrsta verkefnið sem vitað er um þar sem sameindalíffræðilegar aðferðir eru notaðar til að skanna bakteríuvistkerfi fiskvinnsluhúsa. Hér hefur því verið lagður þekkingargrunnur að bakteríuvistkerfum við mismunandi aðstæður í fiskvinnslum sem mun nýtast til frambúðar við rannsóknir og þróun á bættum vinnsluferlum og geymsluaðferðum á fiski.

Lykilorð á íslensku: *Sjávarafurðir, fiskvinnslur, bakteríur, fjölbreytileiki, skemmdarörverur, öryggi, gæði,*

Summary in English:

In this report we seek answers on diversity and species composition of bacteria in fish processing environment. The study initiated method development to screen microbial systems using molecular methods followed by analysis of samples from 2 fish processing plants. This research shows the presence of a diverse microbial community in fish processing environment where known spoilage microorganisms are typically in high relative numbers along with various other bacterial species. Total viable counts showed the presence of bacteria in high numbers on processing surfaces during fish processing where few species typically dominated the community. *Photobacterium phosphoreum* was the most apparent species followed by genera such as *Flavobacterium*, *Psychrobacter*, *Chryseobacter*, *Acinetobacter* and *Pseudoalteromonas*. All these species are known fish associated bacteria that live on the skin and in the digestive tract of a living animal. To our knowledge, this is the first study where molecular methods are used to screen microbial communities in fish processing plants. This research has therefore contributed a database on bacterial diversity in fish processing plants that will be used in the future to improve processing and storage methods in the fish industry.

English keywords: *Seafood, fish processing environment, bacteria, diversity, spoilage bacteria, safety, quality*

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1. Introduction

1.1. Marine and fish microbiology

For many years the dominating bacterial species of the ocean were believed to be members of *Vibrio*, *Alteromonas* and *Pseudomonas* to name a few, as a result from studies on cultivating flora from seawater and marine fishes. To date evidence for extremely complicated and diversified community structure in the world's oceans have been published. Ongoing ocean genomic studies are now providing more comprehensive description of the organisms and processes that shape microbial community structure, function and dynamics in the sea [1]. An effort to give insight into the great microbial diversity in different geographic locations in the sea on the viral-, prokaryotic and eukaryotic level has been launched by the Global Ocean Sampling (GOS) Expedition [2]. Data consisting of more than 7.7 million sequencing reads from 41 sampling site on 8000 km route from the North Atlantic, southwards the Panama Canal and onwards towards the South Pacific has revealed more new genes, proteins and diversities than might have been thought [3]. Yet, this is only a fraction of the total biological diversity present in the ocean at greater depths and in other geographical areas.

Remarkably, in most of the genomic oriented studies on bacterial communities in the ocean, the typical bacterial species (or their phylotypes) found in fish and during storage of fish are always present in low relative quantities [1, 2, 4, 5]. These typical teleost fish associated bacteria have been described in the past by cultivation methods and the recent cultivation-independent technologies have actually confirmed their presence in the skin or digestive tract of fishes in the highest relative quantities [6-11]. A good example on how the habitat and major niches of selected microorganisms has been mapped as a travel through different niches within the ocean is that of *Vibrio fischeri*. It involves a heterotrophic travel through the stomachs of marine fish, acting as catalysts for chitin degration and the light organs of squid where they multiply to high concentrations and constantly inoculate the surrounding water [12]. Other similar study is on *Photobacterium* spp. which is closely related to *Vibrio fisheri* and also inhabits the light organ of many fish species. This is a group that has been demonstrated to play a major role during spoilage of many fish species. By using strains isolated from various sources e.g. skin of cod, haddock, salmon and light organs of the deep-sea fish *Chlorophthalmus albatrossis*, it has been demonstrated that strains formerly assigned as *Photobacterium phosphoreum* could be separated into three different species; *P. phosphoreum, P. iliopiscarium* and *P. kishitanii*. They showed that in the light organs of deep sea fishes *P. kishitanii* was the sole symbiont but not *P. phosphoreum* which was the general notation until then. *P. phosphoreum* and *P. iliopiscarium* show a highly

similar phenotypic traits and 16S rRNA sequence similarity which has led to questioning with their species divergence [13]. However, distinct differences in their *gyrB* gene and the absence of the *luxABFE* genes in *P. ilipiscarium* clearly discriminates the species. Using this approach, 3 strains isolated from spoiled cod fillet, previously described as *P. phosphoreum* were reclassified as *P. iliopiscarium* [14]. This information opens a new window for the research on the role of *Photobacterium* in fish spoilage and questions regarding the interactions of these two closely related species during spoilage in fish.

1.2. Undesirable bacteria in fish

Spoilers

Shelf life and food quality are important concepts for food producers and consumers. This is of most importance when handling fresh fish where the shelf life can vary from few days up to few weeks at the most. Fish is one of the most susceptible foods for spoilage where sensory attributes diminish relatively fast. Underlying causative agents for the spoilage is a specific bacterial growth which is governed by the fish species in question, the fishing grounds, season, and the processing and storage conditions. The North-Atlantic cod is a cold-adapted fish species which has been captured for human consumption for centuries. It is a perishable commodity and, for that reason, preservation methods like freezing or salting have traditionally been used to extend its shelf life. There is, however, an increasing demand for chilled fresh fish products as well as products in consumer-friendly packaging. The quality and shelf life of such fish products is greatly affected by the handling and processing as well as the temperature and the time that goes between catching and packaging [15, 16].

The natural flora in the epidermis mucosa of newly caught North-Atlantic cod from the Baltic, Icelandic and North Sea has been characterised using 16S rRNA clone analysis, revealing *Photobacterium*, *Psychrobacter*, *Pseudomonas*, *Acinetobacter*, *Pseudoalteromonas*, and *Flavobacterium* among the commonly found species on cod epidermis [6]. It was reported that *Psychrobacter* spp. was the most abundant species of a 16S rRNA clone library followed by *Photobacterium* spp. Upon catching and slaughtering the fish transforms to a microbial ecosystem of its own where the ecological principles of succession are as valid as in any other ecosystem. This environment consists of a high nutrient content with an oxygen tension favourable to the proliferation of fast-growing heterotrophs also responsible for the spoilage of food. The deterioration of fish freshness starts soon after catch by autolytic activity of endogenous enzymes, followed by oxidation of lipids and microbiological breakdown of tissues leading to fish spoilage [17-19]. Until now, the process of fish spoilage has been investigated intensively with regard to

sensory evaluation, chemical changes of volatile and non-volatile compounds and microbiological growth by cultivation methods [19-24].

Pseudomonas spp. and *Shewanella putrefaciens* were early recognised as putative spoilage inducers in fish muscle and have since then been found in various fish species from fresh- and marine waters as well as in other foods [25-27]. Characterisation of *Pseudomonas* spp. isolated during spoilage of gilt–head sea bream in Greece showed that *P. lundensis* was the predominant species but *P. fluorescens*, *putita* and *fragi* were also found [28]. These species are likely to be among the main fish spoilage organisms in the genus. *Pseudomonas* spp. and *S. putrefaciens* are generally associated with spoilage of fish stored under aerobic conditions. *Photobacterium phosphoreum* has been reported as the main spoilage organism in modified atmosphere (MA) packed fish [29], being CO_2–tolerant and producing trimethylamine (TMA) from trimethylamine oxide [30]. Storage under superchilled conditions has been shown to delay *P. phosphoreum* growth in cod fillets while H_2S-producing bacteria, most likely *S. putrefaciens,* were not affected and reached high levels [15]. The bacterial microflora in these environments are often complex and many other species have been detected besides the ones already mentioned *e.g*. *Brochothrix thermosphacta, Aeromonas* spp., *Vibrio* spp. and members of the Enterobacteriaceae [23].

Pseudomonas spp. is frequently used as bacterial indicator for spoilage and is present in different food types such as fish, meat and chicken [19, 31, 32]. *P. phosphoreum* can also serve as a spoilage indicator but has not been investigated as intensively as *Pseudomonas* spp. Fast and accurate detection and quantification of these microorganisms in combination with appropriate model systems can therefore provide an important tool for quality assessments of fish. In spite of all attempts to delay the spoilage of fresh fish using storage conditions that inhibit bacterial growth, the fish will eventually spoil. Changes of environmental conditions will delay or even stop the typical spoilers from growing but at the same time a new niche is opened for other bacteria. Because of the short shelf life of fish, traceability and information on quality parameters are of great importance during trading of fish. It has been shown before that the correlation between the quality as judged by sensory evaluation and total bacterial counts is not as accurate as compared to the SSO counts [19]. Quantification of the SSOs can be achieved by conventional cultivation strategies but it is too time consuming to benefit the industry and international trade. Therefore, the development of accurate and rapid quantitative assays could assist the industry to improve internal quality controls and processing management.

Biofilms – microbial shelters

In most natural environments, microbes attach to surfaces, multiply and form biofilms which provides enhanced resistance to external disturbances. In this state the biofilm associated cells are

more resistant to many toxic substances such as antibiotics, chlorine and detergents [33, 34]. The formation of biofilms is usually depicted as a series of discrete stages in life cycle which begins when planktonic cells contact surfaces, either randomly or by chemical attractants. The next steps involve irreversible attachment when cells have multiplied and have started to secrete extracellular polymeric substances such as polysaccharides, proteins and DNA. After that the biofilm maturates and disperses [34, 35]. Bacteria living in biofilms are believed to communicate by chemical signalling although orchestrated behaviour of the community is a matter of dispute. A change in gene expression when cells go from planktonic state to biofilms is unquestionable and is the underlying cause of different cell behaviour that characterizes biofilms [36]. Although no single mechanism is responsible, many species use quorum sensing to modulate surface attachment, motility, extracellular polymeric production and dispersal [37]. Secreted polymers are defining feature of biofilms but the functions of them are not yet entirely clear. They promote surface attachment and provide structural support but also offer protection from external threats or help secreting strains to grow toward nutrient rich locations [35, 38]. A mature biofilms is usually composed of channels and cavities to allow the exchange of nutrients and waste [37].

Biofilms are not restricted to natural habitats as food processing facilities are ideal environment for biofilm formation where nutrient rich liquid constantly or periodically covers the surfaces. This can cause problem to the production if proper hygienic preventive measures are not performed. Undesirable bacteria such as spoilers (*Pseudomonas* spp.) and pathogens (*Listeria monocytogenes*) have been shown to form biofilms in food processing plants and if the biofilm grows to mature state the threat of persistent contamination of these bacteria in the food is apparent [39].

Bacterial contamination in fish processing plants

Hygienic design of food processing equipment is becoming recognised as an important parameter for safe and wholesome food production. This is in concordance with the fact that the frequency of food borne diseases has increased globally in recent years [40]. From economical point of view, 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 and safety of the fish e.g. the natural condition when it is captured, the handling on board and in the processing plant. Microbiological breakdown of tissues is one factor that decreases quality. It is unavoidable but can be minimized by incorporating standard hygiene protocols, especially in the early handling and in processing plants [19]. The formation of a microbial biofilm on the surface of fish processing equipment increases the threat of a cross-over contamination of the product [41]. This can have influence on the quality and safety of the final product, especially if specific spoilage organisms or pathogenic bacteria become dominant in the biofilm [42]. 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 [43, 44].

The bacterial species in food processing environment are usually heterotrophs that multiply fast if environmental factors are favourable. The fish processing environment is often humid, with excessive water flow containing nutrients and other important components that promotes bacterial growth. Bacterial species within the processing plant may vary depending on geographical origin of the catch or fish species being processed.

Surface finishing on working surfaces is considered to affect bacterial adhesion [45]. Significantly fewer bacterial cells and less biofilm formation have been observed on electro-polished surface of stainless steel compared to untreated, sandblasted and sanded steel [45]. 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 [46]. Effective hygienic protocols are essential to minimize the formation of biofilms and to prevent contamination of the products [47]. 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 purposes, health issues and governmental regulations [48]. Moreover, some bacteria (e.g. *Pseudomonas* spp.) may have certain resistance mechanisms against antibacterial components commonly used in disinfectants such as quaternary ammonium compounds [49, 50].

Different fish processing establishments have different ways of cleaning and washing their equipment. Water temperature, water hardness, acidity, surface material of equipment, detergent, disinfectant type and 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 *pseudomonads*, 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 [46, 51-53]

1.3 Molecular microbiological ecology.

Since the advent of molecular biology and PCR based strategies, a completely new view on microbial diversity has emerged. The new methodologies have shown the presence of uncultivable populations and genome sequencing has revealed extensive lateral gene transfer between microbial species. This has greatly complicated our conception of microbiological evolution and

speciation. The use of 16S rRNA clone analysis in microbiological ecology has increased enormously for the past years and ever growing sequence databases provide a stronger backbone. The 16S rRNA gene is a suitable gene for ecological studies since it is present in all living bacteria and its evolutionary rate is suitable to distinguish between genera and most species. The transcribed molecule has an important role in the biosynthesis of the living cells where it is a key factor in translation of mRNA to proteins [54]. Its three dimensional structure is highly conserved where the nucleotides of the molecule form hairpins, helices and other tertiary structures important for binding of other molecules. The conserved areas of the 16S rRNA molecule have been used as priming sites for "universal" primers that are able to hybridize to most bacteria present in a sample and in theory, co-amplify the same gene from various bacteria present in one sample at similar efficiency [55].

1.4 Methodologies for the study of microbial populations

The fundamentals of 16S rRNA clone analysis

Today the use of the 16S rRNA gene has become the golden standard of macromolecules used in taxonomic classification and evolutionary relations. The use of several markers in the same run has been used for the same purpose with enhanced discriminatory power. It is the multi locus sequence typing (MLST) approach and is based on sequence information on several housekeeping genes that fulfil the above mentioned criteria [56]. The advantage of the 16S rRNA molecule is that it is present in all bacteria and universal primers have been designed that bind to the majority of known taxa. It is well conserved but with variability in certain areas within the gene which enables both distant and close determination of relations. It is however often too conserved to discriminate between closely related species [57].

The method has been used in two separate applications. First it was used to sequence the gene of pure culture strains and nowadays it is a standardised method in species description and classification of isolated bacterial species. The other application is for the analysis of bacterial community structure and composition in a given environment. In this case, a DNA is isolated from environmental sample containing a mixture of DNA from all organisms present in the sample. The 16S gene is amplified using universal primers and the PCR product is cloned in a vector, typically TOPO cloning or similar applications. Each clone has therefore a 16S rRNA gene from a single bacterium in the community and sequencing sufficient number of clones can give information on species or genus composition in the environment in question. With this approach both cultivable and non-cultivable bacteria can be detected. In order to use the sequence for identification it has to be run against a database containing 16S rRNA sequences and species information [57]. To date

there are 800.159 sequences positioned in the 16S ribosomal database (rdp.cme.msu.edu, accessed in March 2009) and the database is rapidly expanding (Figure 1).

Fingerprinting bacterial communities

In some studies, knowledge of specific bacterial species composition is not required but rather the fingerprint of the community that can be used to evaluate changes in the microbial composition in relation with some intervenic event, depth of a water column, geographic location etc. In these cases the rapid screening of the population structure is preferable over detailed information on species composition which includes more work load and expenses. The most common methods for this purpose are terminal restriction length polymorphism (t-RFLP) and denaturing gradient gel electrophoresis (DGGE) [58, 59]. Both of them depend on an amplification step with universal primers just as in 16S rRNA clone analysis but with a different downstream application. In the t-RFLP method one of the primers (or both) is labelled with a fluorescent dye giving each amplified DNA molecule a label on the corresponding end. Since various bacterial species contain different nucleotide sequences in the 16S rRNA they contain restriction sites at different position. The PCR product is cleaved using restriction enzymes that recognise common sequences (usually four cutters) which results in a labelled DNA fragments of different lengths according to the nearest restriction site to the terminus [60]. The sample is then run on a DNA analyzer in a fragment size mode along with internal size standard (Figure 2). Supporting databases for t-RFLP are available containing a list of organism and their terminal fragment size using specific primers and restriction enzymes (trflp.limnology.wisc.edu/index.jsp). The users can also upload their own datasets based on other primer-restriction enzyme combination for phylogenetic assignment. However, such assignment will never be as accurate as sequencing information since some bacterial species can have differentiated restriction sites in different strains and unrelated bacteria can also share the same terminal restriction site [61].

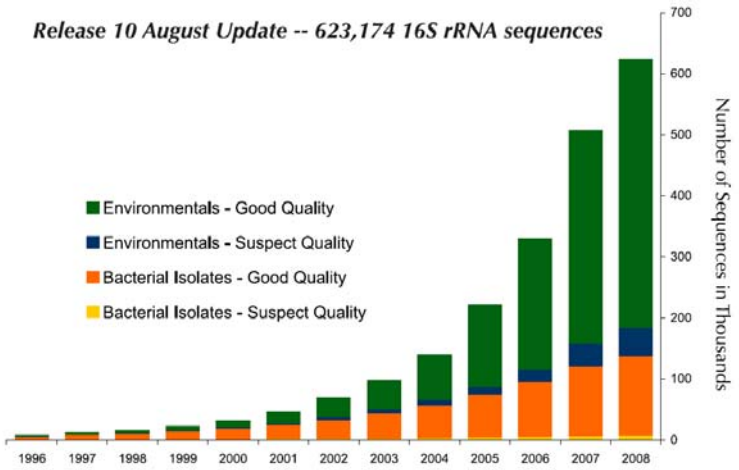

Figure 1. The growth of the 16S rRNA database since 1996 (rdp.cme.msu.edu).

In principal, DGGE is used for the same purpose. Instead of separation by terminal restriction sites, the PCR products originating from different species are separated on an agarose gel containing gradient of chemical denaturant e.g. urea. The GC content and base sequence of the PCR product is the decisive factor of its stability and when it denatures in the increasing concentration of the denaturant in an agarose gel, the migration speed is reduced. Ultimately, this leads to a banding pattern in the gel where each band is theoretically originated from a single species [59]. The main disadvantages of DGGE is that in complex communities, it is possible that one single band is composed of PCR products from more than one species and the method is low throughput compared to t-RFLP which can be analysed in a 96 well format. The advantage of DGGE to t-RFLP is that it is possible to dissect a band from a gel and sequence it for taxonomic classification.

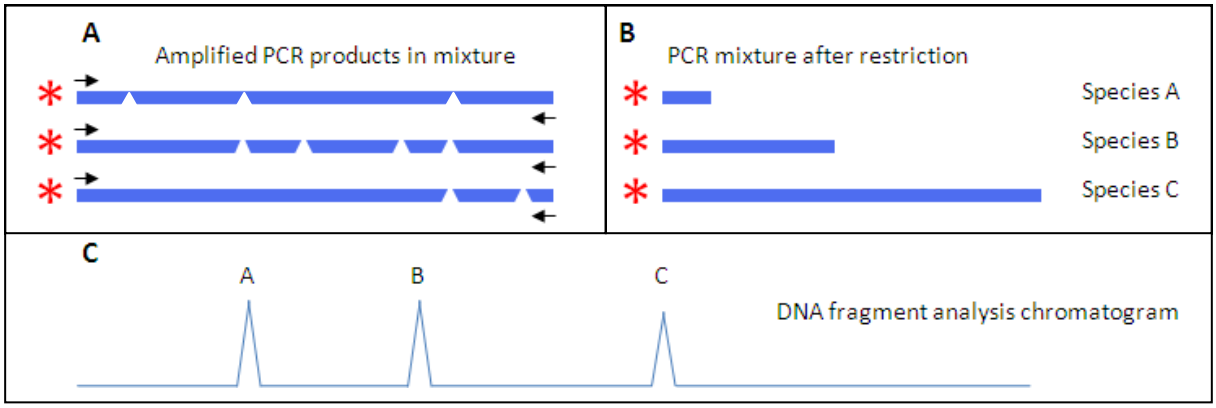

Figure 2. Flowsheet of the fundamentals of t-RFLP when used on mixture of three cultures (Species A, B and C). To start with they are coamplified with PCR resulting in a labelled PCR product (indicated by red asterisk). The three species contain restriction sites on several locations within their 16S gene (A). Upon restriction, only the terminal fragment is detectable in a DNA fragment analyser due to the labelling (B and C).

Projects aims

The objective of this project is to study the ecological importance of known spoilage bacteria, pathogens and other less known- or uncultivable bacteria in sea products and processing environment using molecular based methods for assessing microbial diversity and abundance. Furthermore, monitor the changes of bacterial community composition and changes in the processing chain and during storage of fresh fish in chilled and superchilled environment. The results will be put into perspective with current knowledge obtained by cultivation.

2. Materials and Methods

2.1 Experimental layout

During the project period two processing facilities were visited (A and B). Facility A produces mostly cod and haddock and both these species were on the processing lines at the days of sampling while facility A produces mostly red fish and saithe. Information on sampling tours are summarized in Table 1.

Sampling

Bacteria attached to processing surfaces (biofilm) were sampled by rubbing a cotton swab tightly on the surface using a 50 cm^2 steel frame to standardise the surface size. The swab was dipped in Day and Engley (D/E) neutralizer (Difco, Franklin Lakes, NJ, USA) before capturing the bacteria. The swab was released into 5 mL of maximum recovery diluent (MRD) buffer (Oxoid, Hampshire, UK) and shaken vigorously.

Whole fish (raw material) and final product was collected in the processing plants. Prior to cultivation the samples were minced and 25g sample was diluted ten times in MRD and stomached for 1 min. One side of a whole fish was aseptically skinned; pieces of flesh removed, diluted tenfold in cooled MRD and stomached for 1 min.

Drain samples were collected by entrapping bacteria in sterile gauze. The gauze was placed in the running water of the drains and kept there while the processing plant was being inspected, typically for 30-60 minutes. It was then placed in a sterile container and diluted in 10 mL of D/E neutraliser.

Cultivation

Microbiological counts were evaluated for both the skin and the flesh of haddock. A piece of skin were aseptically cut from one side of each fish, giving a total area of 50 cm^2, and mixed with 60 ml of cooled Maximum Recovery Diluent (MRD, Oxoid, Hampshire, UK) for 1 minute in a stomacher (Stomacher Lab Blender 400, A.-J. Seward Laboratories, London, UK). Successive tenfold dilutions were done as required. The other side of each fish was aseptically skinned, pieces of flesh removed, minced and diluted tenfold in cooled MRD. Cultivation methods used are described by Olafsdottir et al. [15]. Presumptive Pseudomonas were cultivated on modified Cephaloridine Fucidin Cetrimide (CFC, 22°C) agar (Oxoid) based on Stanbridge and Board [62], total viable psychrotrophic counts (TVC) and H2S-producing bacteria on Iron Agar (IA, 17°C).

Results are either represented as Colony forming units (CFU) per gram (flesh or product), cm^2 (skin or processing surfaces) or sample (drains and running conveyor belts). Samples from the drains

were collected by immersion of gauze in the water stream for filtration of the bacteria. In these samples the results are presented as CFU/sample since a volume parameter is not known.

DNA extraction

The MRD buffer added to the cotton swab of the surface samples and the first dilution of fish samples prepared for cultivation were used for DNA extraction. One mL of these samples were used for DNA extraction. Liquid draining from the flesh of a fresh fish was used to extract DNA in some cases to increase bacterial density in the sample. DNA extraction procedure were done as previously described [63].

Fingerprinting bacterial communities

Extracted DNA from duplicate samples was pooled prior to PCR for the Terminal restriction fragment length polymorphism (t-RFLP) analysis. The PCR was performed with 9F forward primer (sequence above) with a 5´ FAM terminal label and HEX labelled reverse primer 805R. The labelled PCR products were digested with *Hae*III, *Alu*I or *Msp*I (Fermentas, Hanover, MD, USA) in a 10 µL reaction volume for 2 h. The digested PCR product was diluted 1:20 and 2 µL 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 (v4.0) using the AFLP analysis method. Peaks below a threshold level of 50 were excluded except where a clear trend of same t-RF was detected in other samples.

Statistical analysis of t-RFLP profiles. The relative abundance of each t-RF in the profile was calculated by dividing the respective peak area of each t-RF with the total peak area generated between 50-600 bp. The profiles from different combinations of labelled primers and restriction enzymes were all combined in one dataset for principal component analysis (PCA) to enhance the analytical power of the model. PCA of t-RFLP profiles from different fish samples was performed using the Unscrambler version 9.5 (Camo ASA, Oslo, Norway). The data was not weighed and full cross validation was used.

16S rRNA analysis

PCR reaction was done by amplifying the 16S rRNA gene with universal primers, 9F and 1544R (5'-GAGTTTGATCCTGGCTCAG-3 and ´5-CCCGGGATCCAAGCTTAGAAAGGA -3´ respectively). The reaction volume was 25 µL which contained Teg polymerase (Prokaria, Reykjavík, Iceland) at 0.05 U/µL, 1.5mM Mg_2Cl, 400 nM primers and 5 µL of template DNA obtained from the fish matrix. Thermal program was as follows: 5 min at 95°C, 35 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. PCR products were analysed by electrophoresis in 1% agarose gel. Cloning and sequencing was performed as described by [64].

3. Results and discussion

3.1. Viable bacteria in processing environment

Samples collected at various locations within each processing plant showed considerable amount of bacteria on the surfaces. Bacterial loads on processing surfaces (steel/plastic) ranged from 0 up to 560.000 CFU/cm^2 in the processing plants. Bacterial loads in the raw material being processed at the time of sampling ranged from 6.000-1.300.000 CFU/cm^2 on the skin and 0-820 CFU/g in the flesh. The final product, rinsing water at heading and clean liquid ice also contained bacteria in relatively high concentrations (Tables 2-6).

In March 2009, a high bacterial contamination was observed throughout processing plant A, generally showing higher counts in the afternoon than in the morning when the same surface are compared (Table 3). In June the same year this was inverted, showing lower bacterial loads on processing surfaces in the afternoon even though a 6 day old material containing 1.300.000 CFU/cm2 was being processed. It is likely that in this case the plant had been cleaned (or rinsed) prior to processing of the old material which explains the low surface counts obtained (Table 4). The age of the saithe being processed in plant B is not known but the results show that soon after processing starts a bacterial load up to 4 log units is present on processing surfaces (Table 5 and 6). Unused processing lines showed that no bacteria were detected on steel surfaces but 14.000 CFU/cm^2 were observed on a clean plastic surface.

In general a large variation of bacterial contamination was observed between samples, sampling visit and processing plants.

Rinsing of the fish in a water bath is usually the first stage of processing. It is of high importance that this water is renewed periodically as this point in the processing line has the highest rate of cross contamination of bacteria between individual fishes. In this study the total viable counts (TVC) of the rinsing water was from 97.000-1.300.000 CFU/mL which imposes this risk of cross contamination.

Table 1. Sampling scheme of the study.

Processing plant	Sampling period	Time	Fish in processing	Age of material	Average surface T	No. of samples
Processing A	March 09	08:00	Cod (gutted)	2 days	5.3°C ± 4.7	14
Processing A	March 09	14:00	haddock	0 days	3.4°C ± 2.9	16
Processing A	June 09	08:00	Cod	2 days	4.9°C ± 1.8	13
Processing A	June 09	14:00	Cod/haddock	6 days	6.9°C ± 4.5	12
Processing B	February 08	10:00	Saithe	-		22
Processing B	January 09	10:00	Saithe	-		16
Total samples						93

Table 2. Overview of total viable bacterial counts (TVC) in processing plants during two separate visits.

Sample type	Plant A March09	Plant A Jun09	Plant B Feb08	Plant B Jan09	Unit
Processing surfaces	2.800-560.000	38-114.000	0-42.000	1-476	CFU/cm^2
Fish skin	6.000-150.000	1.200-1.300.000	81.000-91.000	730.000	CFU/cm^2
Fish flesh	0-30	35-820	18	650	CFU/g
Product	650-7.000	7.400-10.500	128.000	34.000	CFU/g
Rinsing water	200.000-250.000	97.000	1.300.000	173.000	CFU/mL
Clean liquid ice	500-13.000	3.500-5.800	24	3.000	CFU/mL

Table 3. Bacterial counts in samples collected in processing plant A in March 2009.

Time	Processing area	Sample type	Total	H_2S producing	*Pseudo-monas*	Unit	t-RFLP[1]	16S[1]
	Reception	Cod skin	13.000	0	20	CFU/cm^2	x	
	Reception	Cod flesh	0	0	0	CFU/g	x	
	Heading	Plastic surface	160.000	20.000	32.000	CFU/cm^2	x	
	Heading	Steel surface	60.000	2.000	12.000	CFU/cm^2	x	x
	Heading	Rinsing water	250.000	3.000	63.000	CFU/ml	x	
	Filleting	Steel surface	5.000	1.100	1.200	CFU/cm^2	x	x
08:00	Filleting	Rubber conveyor	1.600.000	2.000	60.000	CFU/sample	x	x
	Filleting	Drain	2.800.000	390.000	720.000	CFU/sample	x	x
	Trimming	Operating table	2.800	500	4.800	CFU/cm^2	x	
	Trimming	Plastic conveyor	4.300	200	12.000	CFU/cm^2	x	
	Trimming	Drain	5.000.000	470.000	590.000	CFU/sample	x	
	Packaging	Cod flesh	650	0	60	CFU/g	x	
	-	Unused liquid ice	13.000	40	110	CFU/mL	x	x
	Reception	Haddock skin	6.000	700	90	CFU/cm^2	x	
	Reception	Cod skin	150.000	9.000	1.900	CFU/cm^2	x	
	Reception	Haddock flesh	>100	>100	10	CFU/g	x	
	Reception	Cod flesh	30	10	0	CFU/g	x	
	Heading	Plastic surface	560.000	8.000	30.000	CFU/cm^2	x	x
	Heading	Steel surface	280.000	8.000	23.000	CFU/cm^2	x	
	Heading	Rinsing water	200.000	14.000	13.000	CFU/ml	x	
	Filleting	Steel surface	14.200	130	410	CFU/cm2	x	
14:00	Filleting	Rubber conveyor	760.000	7.000	40.000	CFU/sample	x	x
	Filleting	Drain	800.000	35.000	210.000	CFU/sample	x	x
	Trimming	Operating table	3.200	40	150	CFU/cm^2	x	
	Trimming	Plastic conveyor	62.000	3.300	15.000	CFU/cm^2	x	
	Trimming	Drain	1.600.000	37.000	40.000	CFU/sample	x	x
	Packaging	Haddock flesh	1.500	100	80	CFU/g	x	
	Packaging	Cod flesh	7.000	1.500	1.400	CFU/g	x	
	-	Unused liquid ice	500	-	50	CFU/ml	x	

CFU – colony forming unit

[1] x represents a sample analysed by either t-RFLP or 16S rRNA analysis

Table 4. Bacterial counts in samples collected in processing plant A in June2009

Time	Processing area	Sample type	Total	H_2S producing	Pseudo-monas	Unit	16S[1]
08:00	Reception	Cod skin	1.200	0	350	CFU/cm^2	
	Reception	Cod flesh	35	0	0	CFU/g	
	Heading	Plastic surface	9.200	700	3.700	CFU/cm^2	
	Heading	Steel surface	8.600	600	1.700	CFU/cm^2	
	Heading	Rinsing water	97.000	10.000	36.000	CFU/ml	x
	Filleting	Steel surface	114.000	2.000	1.300	CFU/cm^2	
	Filleting	Plastic surface	-	-	-	-	x
	Filleting	Drain	-	-	-	-	
	Trimming	Operating table	100	0	0	CFU/cm^2	
	Trimming	Plastic conveyor	-	-	-	-	x
	Trimming	Drain	-	-	-	-	
	Packaging	Cod flesh	10.500	500	3.100	CFU/g	x
	-	Unused liquid ice	5.800	280	450	CFU/ml	
14:00	Reception	Haddock skin	1.300.000	1.333	2.100	CFU/cm^2	
	Reception	Haddock flesh	820	0	0	CFU/g	
	Heading	Plastic surface	36	0	2	CFU/cm^2	
	Heading	Steel surface	38	0	3	CFU/cm^2	x
	Filleting	Steel surface	132.000	800	750	CFU/cm^2	
	Filleting	plastic surface	-	-	-	-	x
	Filleting	Drain	-	-	-	-	x
	Trimming	Operating table	9.200	230	660	CFU/cm^2	
	Trimming	Plastic conveyor	-	-	-	-	x
	Trimming	Drain	-	-	-	-	
	Packaging	Haddock flesh	7.400	1.000	270	CFU/g	x
	-	Unused liquid ice	3.500	100	320	CFU/ml	

CFU – colony forming unit

[1] x represents a sample analysed by 16S rRNA analysis

Table 5. Bacterial counts in samples collected in processing plant B in February 2008.

Processing area	Sample type	Total	H_2S producing	*Pseudo-monas*	Unit	t-RFLP	16S
Reception	Saithe skin	92.000	3.000	450	CFU/cm^2	x	x
Reception	Saithe skin	81.000	100	1.640	CFU/cm^2	x	
Reception	Saithe flesh	18	0	0	CFU/g		
Reception	Sorter 2	17.500	0	0	CFU/cm^2	x	x
Heading	Rinsing water	1.300.000	10.000	29.000	CFU/mL	x	x
Heading	Drain	-	-	-	-	x	
Heading	Delivery to filleting	610	0	0	CFU/cm^2	x	
Filleting	Steel surface	1.500	30	0	CFU/cm^2	x	x
Filleting	Plastic surface	600	10	500	CFU/cm^2	x	
Deskinning	Surface	42.000	500	750	CFU/cm^2	x	
Trimming	Conveyor belt	92.000	70	180	CFU/sample	x	x
Trimming	Conveyor belt	15.000	80	180	CFU/sample	x	
Trimming	Drain	-	-	-	-	x	
Trimming	Clean steel surface	0	0	0	CFU/cm^2		
Trimming	Clean plastic surface	14.000	0	2	CFU/cm^2	x	
Trimming	Drain at sorter	82.000			CFU/sample	x	x
Trimming	Liquid ice	82.000	2.000	300	CFU/mL	x	x
Trimming	Cutting machine plast	3.900	0	200	CFU/cm^2	x	
-	Unused liquid ice	24	0	2	CFU/L	x	
Packaging	Steel surface	500	0	0	CFU/cm^2	x	
Packaging	Saithe product	128.000	0	2.500	CFU/g	x	

CFU – colony forming unit

[1] x represents a sample analysed by either t-RFLP or 16S rRNA analysis

Table 6. Bacterial counts in samples collected in processing plant B in January 2009.

Processing area	Sample type	Total	H_2S	*Pseudo-monas*	Unit	16S
Reception	Saithe skin	726.667	6.667	7.333	CFU/cm^2	x
Reception	Saithe flesh	650	0	50	CFU/g	x
Heading	Rinsing water	173.000	18.000	5.500	CFU/mL	x
Heading	Plastic surface	160	58	9	CFU/cm^2	x
Heading	Steel surface	1	0	1	CFU/cm^2	
Heading	Drain gauze	312.000	27.000	1.940	CFU/sample	
Heading	Delivery to filleting	7	0		CFU/cm^2	x
Filleting	Steel surface	41	0	0	CFU/cm^2	x
Filleting	Rubber conveyer belt in	3.000	20	60	CFU/sample	x
Filleting	Rubber conveyer belt out	3.740	30	2.000	CFU/sample	x
Filleting	Drain gauze	320.000	3.000	640	CFU/sample	x
Cutting	Used liquid ice	39.400	1.000	2.960	CFU/mL	x
Cutting	Drain gauze	554.000	60.000	202.000	CFU/sample	x
Packaging	Steel surface	476	10	9	CFU/cm^2	x
Packaging	Saithe product	34.000	50	400	CFU/g	
-	Unused liquid ice	3.000	20	100	CFU/mL	x

CFU – colony forming unit

[1] x represents a sample analysed by 16S rRNA analysis

3.2. Microbial communities in fish processing plants

Bacterial community characterization was done by fingerprinting (t-RFLP) the microbiota and by 16S rRNA analysis of the dominating bacteria. By screening of all samples for their microbial fingerprint enabled a careful selection of samples which showed the highest diversity for 16S clone analysis.

Fingerprinting of microbial communities

Samples obtained from the March 2009 visit to Processing plant A were analyzed by t-RFLP to document differences of the microbial composition in different areas in the plant. Figure 1 shows a PCA plot of the samples were samples containing similar microbiota are clustered together. The microbiota is somewhat alike in morning and afternoon samples collected in the filleting surfaces (stainless steel and plastic), conveyor belt, unused slurry ice, drains in the trimming area and rinsing water in the heading area (circulated in Fig. 1). Furthermore, three distinctive clusters were obtained in the PCA analysis where one of them showed similar microbial composition of the steel surfaces and the skin of both cod and haddock (Fig. 1). Unused slurry ice showed similar composition as samples collected in the trimming area amongst other samples (Fig. 1)

When comparing the microbiota composition of processing plant A in March 2009 to processing plant B in February 2008 we can see higher difference between the plants in general than within each plant (Fig. 2). The samples from processing plant A line up along the first principal component (PC1) while the samples from processing plant B follow the second principal component. Many of the samples deriving both from plant A and B are positioned on the same location of PC1 but are discriminated along PC2. This indicates common variables (bacteria) in these samples but also some distinctive differences defined by PC2

Comparing the PCA plot of all the samples collected to the 16S rRNA clone analysis (next section) of selected samples, the microbial species in the communities of different sampling points and PCA clusters can be identified.

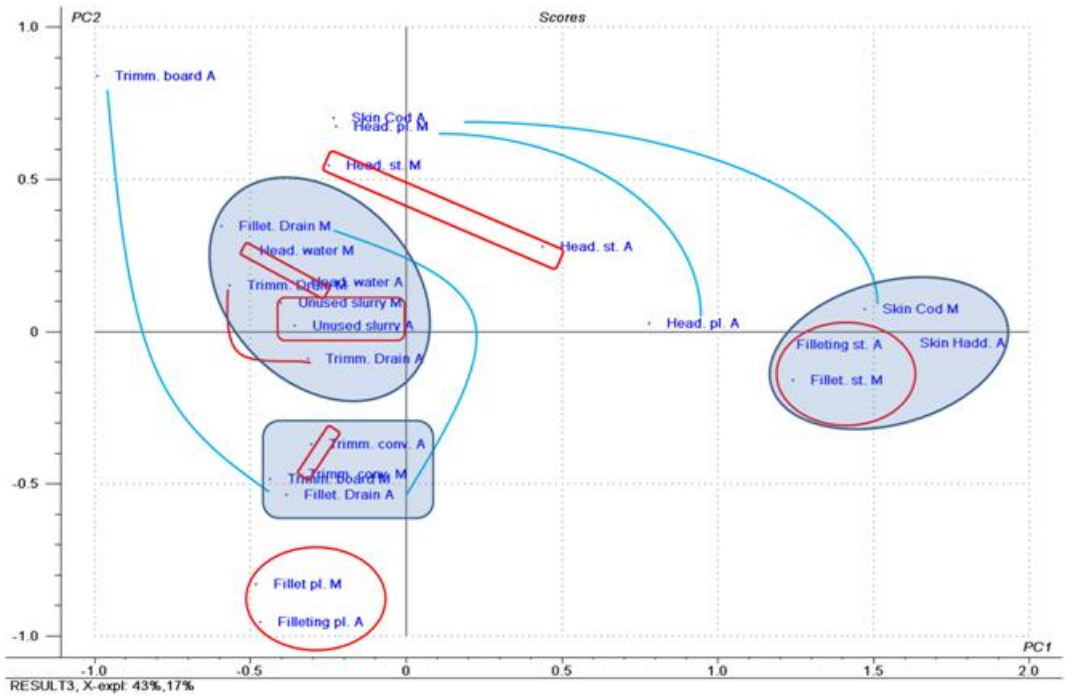

Fig. 1. Principal component analysis of t-RFLP fingerprints obtained in samples collected from processing plant A in March 2009. Samples market with M represent morning samples and A afternoon samples. Surfaces types are abbreviated, pl. for plastic and st. for steel.

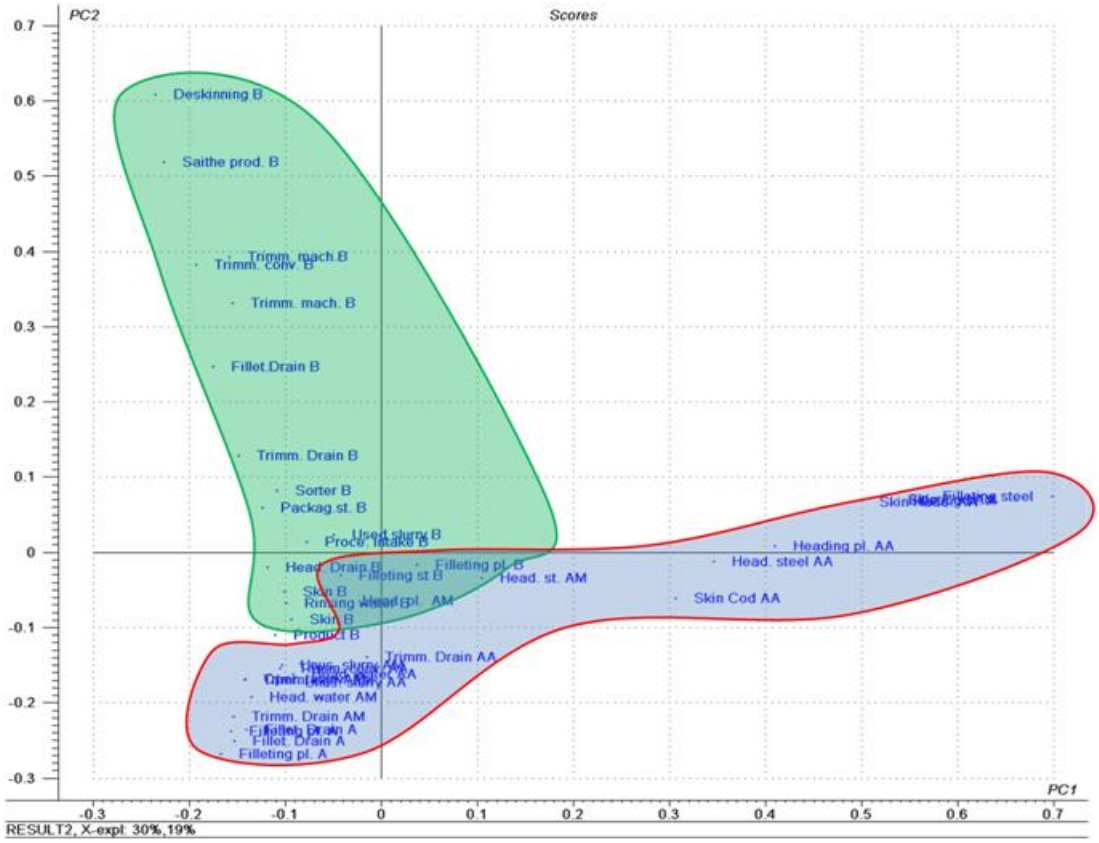

Fig. 2. Principal component analysis of t-RFLP fingerprints obtained in samples collected from processing plant A in March 2009 and plant B in February 2008. Samples market with M represent morning samples and A afternoon samples. Surfaces types are abbreviated, pl. for plastic and st. for steel.

Species identification of microbial communities during fish processing

Bacterial diversity within the processing plants proved to be high. Summarization of the dominating species obtained within each processing plant showed the presence of a typical fish associated microbial flora in different proportions throughout the plants (Fig. 3). The figure illustrates the combined species identification of all samples analysed by 16S rRNA clone analysis in each processing plant. Altogether *Photobacterium phosphoreum* was the most apparent species followed by genera such as *Flavobacterium*, *Psychrobacter*, *Chryseobacter*, *Acinetobacter* and *Pseudoalteromonas*. These groups were present in all the sampling dates except *Pseudoalteromonas* which was not present in processing plant A in June 09. Figures 4-7 show microbial abundance in different sampling points in each processing plant. They demonstrate the presence of the main bacterial groups mentioned above but also other bacteria in lower numbers.

A total of 9 samples were analyzed in processing plant A in March 2009, 5 in the morning and 4 in the afternoon. Overall, *Photobacterium phosphoreum* was the most apparent species but that is mostly due to its high abundance on steel sample in the filleting area (Fig. 4). This bacterium was also present in the heading areas (plastic and steel) but was not detected in other areas in the plant. *Flavobacterium* had the highest distribution and was present in all but two samples while *Acinetobacter* and *Psychrobacter* were present in all but three samples. Other species/genera present showed somewhat random distribution.

When the same processing plant was analyzed in June the same year we could see that *Photobacterium* was still the most abundant species but was more widely distributed this time, being present in all samples except in the drain sample collected in the filleting area (Fig. 5). During this visit *Chryseobacter* had increased in numbers and distribution while *Flavobacterium* and *Acinetobacter* showed similar patterns as in the previous visit, being in moderate amounts and widely distributed. Other species/genera present showed somewhat random distribution but in lower numbers.

The largest microbial diversity was obtained in processing plant B in Feb 08 where 56 different species were identified. This large diversity was mostly due to a single sample collected from the surface of the sorting equipment and showed the presence of 24 different bacterial species (Fig. 6). This time, *Photobacterium* was present in highest relative numbers which is explained by their great dominance on the steel surface in the filleting area and in the slurry ice in the trimming area. *Psychrobacter* had the second highest abundance but with extended distribution among the samples collected, being detected in all samples except the trimming surface (Fig. 6). Again, *Flavobacterium*, *Chryseobacter* and *Acinetobacter* were quite apparent.

When this processing plant was visited again one year later (January 2009), *Photobacterium* was in even higher abundance and wider distribution. In 5 samples out of 13 analysed it showed between 90 and 100% abundance and was detected in 11 samples in total. As before, genera such as *Psychrobacter*, *Flavobacterium* and *Pseudoalteromonas* were among the most apparent genera. Interestingly, *Salinibacter* was found to be present in 82% abundance in an unused slurry ice collected directly from ice machine. Other species/genera present showed somewhat random distribution.

Of all the samples collected, no pathogenic bacteria were detected with the 16S rRNA clone analysis. For detection of pathogenic bacteria a targeted search would have to be done since the 16S rRNA clone analysis will not be able to detect bacteria in low relative numbers.

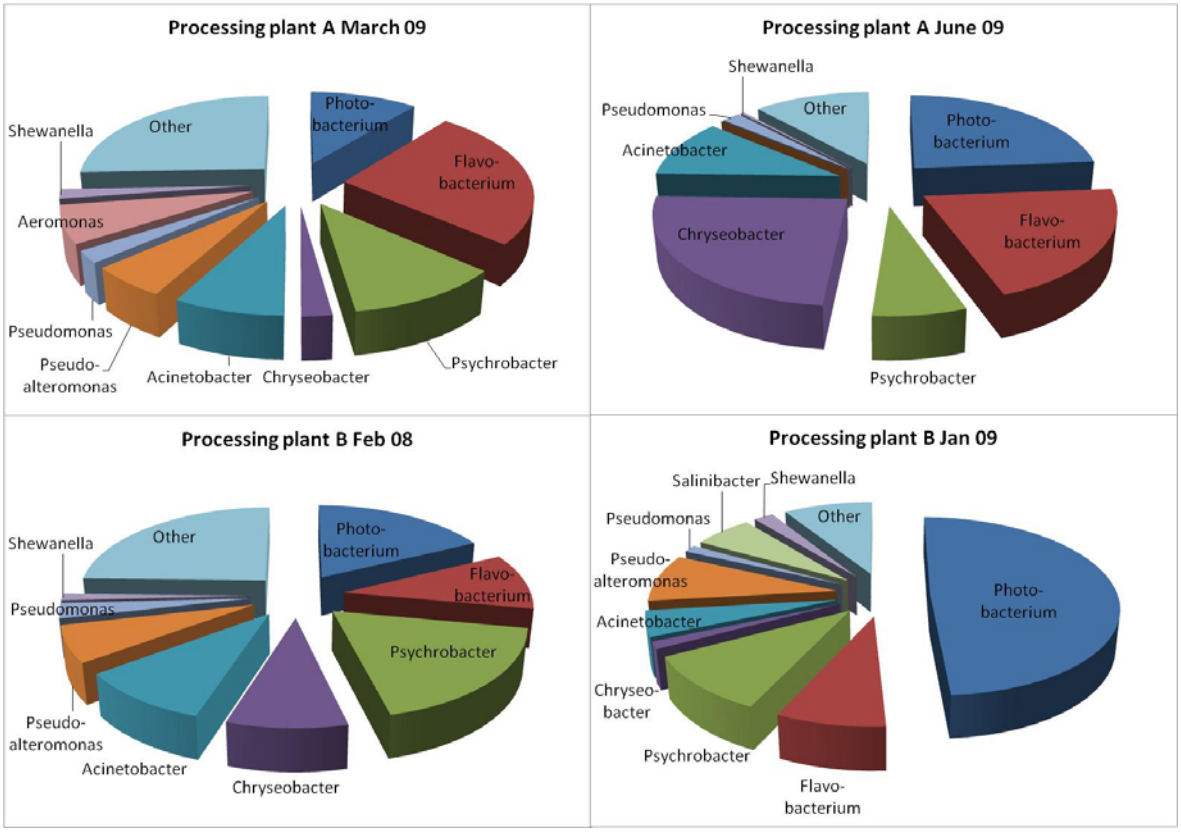

Fig. 3. Relative abundance of dominating species identified in four sampling trips to fish processing plants using 16S rRNA clone analysis.

The results show that the microbial community structure in the processing plants is composed of certain main players which are bacteria commonly associated with wild and caught fish. Many of these bacteria have been identified as active spoilers during storage of fish (*Photobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*) while other are known fish associated bacteria but with less spoilage potential (*Flavobacterium, Psychrobacter, Chryseobacter, Acinetobacter*) [65]. The 16S rRNA clone libraries also demonstrated a large bacterial diversity and up to 100 different bacterial species (taxonomic units) were identified in the study. It is likely that the remaining bacterial species are a mixture of bacteria originating from the fish, processing environment or employees working in the establishment.

Using the 16S rRNA clone analysis it is possible to get a snapshot of the dominating bacteria and the community structure within a sample. Like any other approach this method has also its drawbacks, in this case being only able to detect and identify the dominating members of the community. It is therefore likely that a species with a wide distribution but not detected in a single sample is still there, but at an undetectable level using this method [11, 66].

This is the first study to our knowledge where the use of recent molecular methodology is used to describe the microbial structure and ecology in fish processing environment.

Many studies have been conducted on tracing and identification of specific bacteria such as *Listeria monocytogenes* or *Pseudomonas* spp. in food products and in processing facilities but limited information are available in the literature of microbial communities in processing surfaces [47, 67]. The single report found on microbial ecology in different fish production facilities was carried out in

2003 using conventional cultivation techniques [39]. They investigated the microbiota found in smokehouses of cold smoked salmon, processing surfaces of semi-preserved herring and a caviar processing unit. These products are all highly processed (smoked, sauced and marinated products) compared to the products in the present study. They found bacteria such as Enterobactericeae, *Pseudomonas*, *Corynebacterium*, Neisseriaceae and *Acinetobacter* to dominate smokehouses for salmon while the two first above along with yeasts and lactic acid bacteria were predominately found during processing of herring and caviar. Our results gave quite different results where none of these groups were in any dominance except *Pseudomonas* which still was not in any abundance in our samples. The different microbial structures in these studies are largely explained by the different production units used in the studies. In the present study a recently caught fish was being headed, filleted, trimmed and packed with no additives added. The difference of methodologies (cultivation v.s. cloning) can also influence the results.

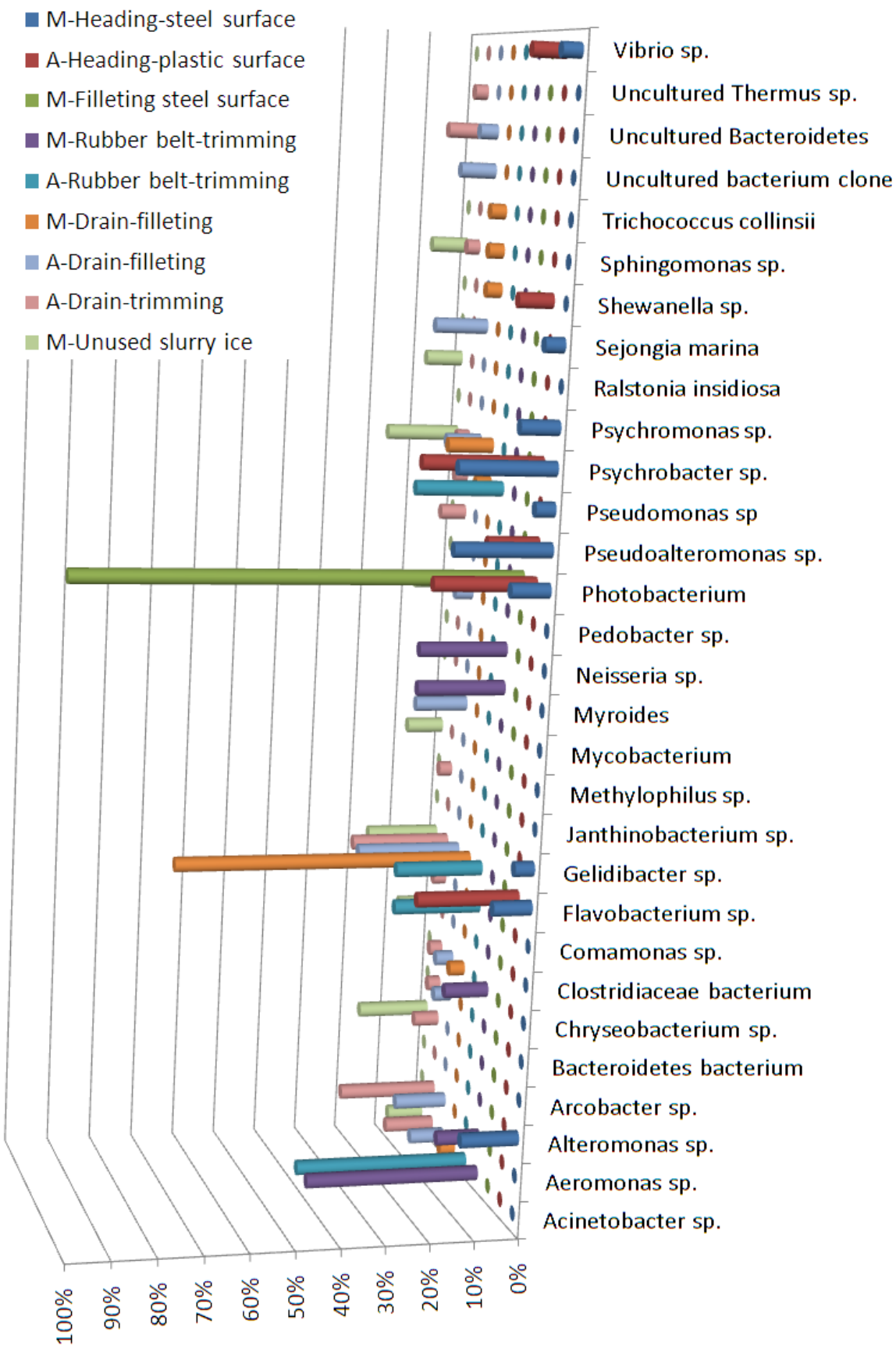

Figure 4. Bacterial composition and abundance in samples collected in processing plant A in March 2009.

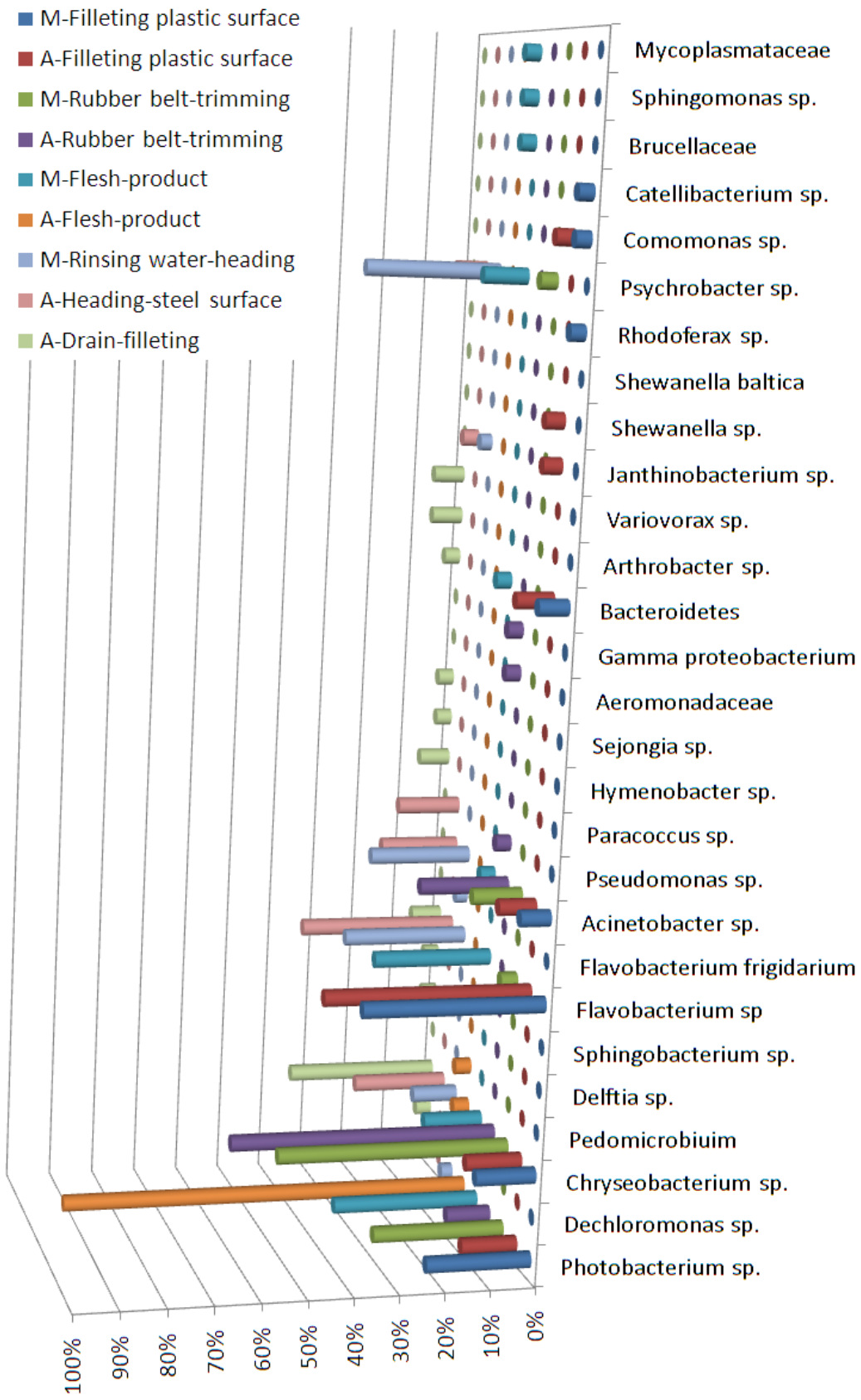

Figure 5. Bacterial composition and abundance in samples collected in processing plant A in June 2009.

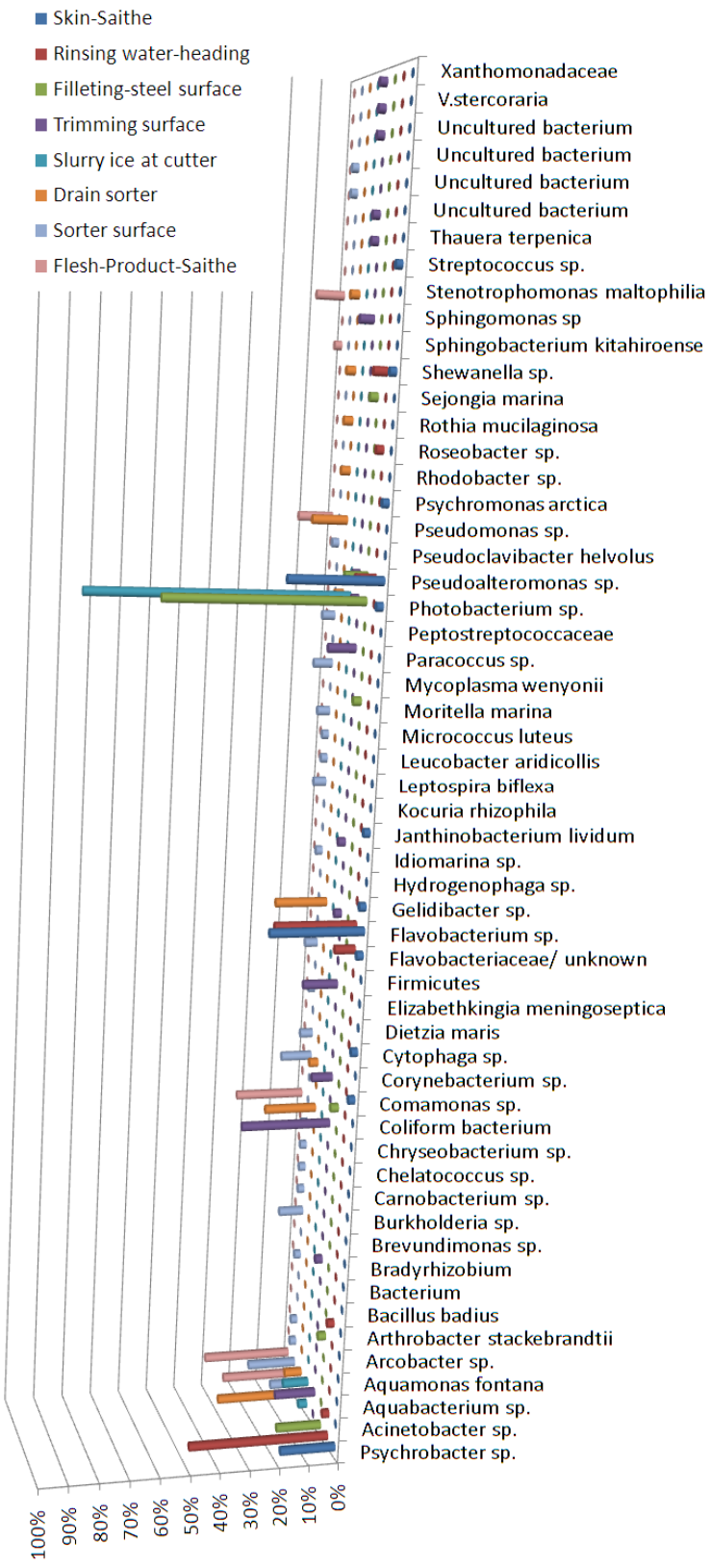

Figure 6. Bacterial composition and abundance in samples collected in processing plant B in February 2008.

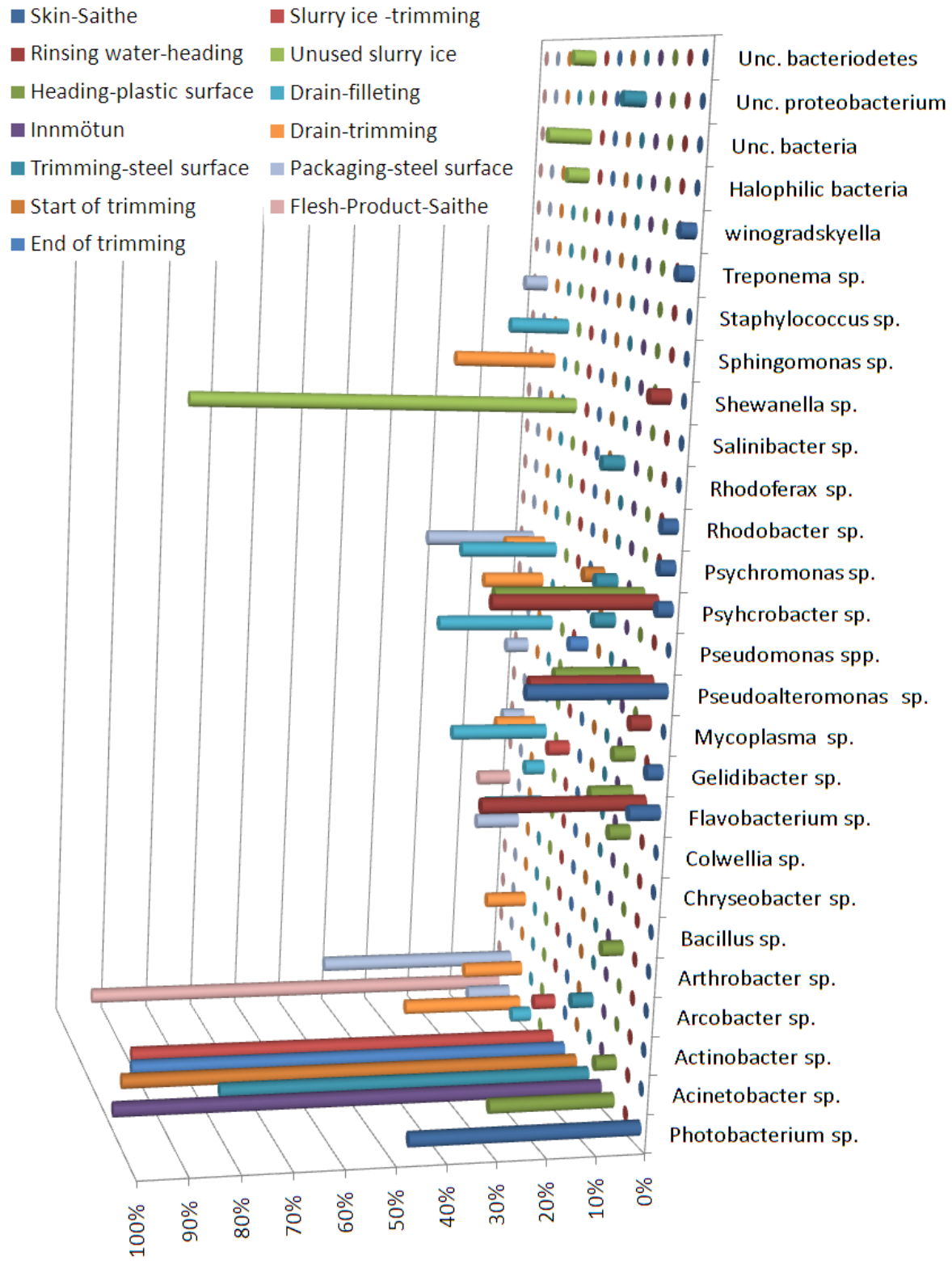

Figure 7. Bacterial composition and abundance in samples collected in processing plant B in January 2009.

4. Conclusion

To our knowledge, this is the first report on microbial community structure in processing environment of fresh fish using molecular analysis. The results indicate a highly diverse microbiota being present in fish processing environment containing various passive microbial visitors in minor quantities. However, the bulk of the microbiota represent groups commonly associated with fish, many of them being specific spoilage organisms. Characterization of microbial communities in processing facilities can contribute to better design of hygienic programs and processing equipment that minimizes the risk of accumulation of undesirable microbes in processing surfaces such as spoilage organisms and pathogens. No pathogenic microbes were detected in the 16S clone analysis although targeted identification of them is needed in order to determine their absence.

This study also demonstrates the usefulness of combining molecular methods to characterise species compositions and conventional methods for enumeration of viable bacterial cells. The most important factor to determine hygienic and quality status of processing environment and products is to have an estimate on bacterial load but information on which bacteria are present and in which proportions is not of less value. Processing plants with high contamination of active spoilers such as *P. phosphoreum* are more likely to produce products with reduced quality and shelf life.

The aim of this study was to explore the microbial systems present during processing of fish. Questions regarding sanitation and cleaning performance of the plants in question are not answered here. This work has provided the first insight of the real microbial world present in the processing environment and further work in this field can provide a better understanding of contamination routes from the environment into the fish products during catching, processing, storage and transportation. Combination of environmental studies such the one presented here to shelf life experiments can furthermore aid new approaches to estimate fish product quality and the development of improved processing and storage method for fish.

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