Microbiota formed on attached stainless steel coupons correlate with the natural biofilm of the sink surface in domestic kitchens

Running title: ***Microbiota on stainless steel coupons and correlation to the sink surface***

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ABSTRACT

Stainless steel coupons are readily used in biofilm studies in the laboratory as this material is commonly used in the food industry. The coupons are attached to different surfaces to create a "natural" biofilm to be studied further in laboratory trials. Little is however done to investigate how well the microbiota on such coupons represents the surrounding environment. The microbiota of new stainless steel coupons attached to the sink wall for three months and sink wall surfaces in eight domestic kitchen sinks was investigated by next generation sequencing (MiSeq) of the 16S rRNA gene derived from DNA and RNA (cDNA), and by plating/identification of colonies. The average number of colony forming units was about tenfold higher for coupons than sink surfaces and more variation in bacterial counts between kitchens was seen on sink surfaces than coupons. The microbiota in the majority of biofilms was dominated by *Moraxellaceae* (genus *Moraxella*/*Enhydrobacter*) and *Micrococcaceae* (genus *Kocuria*). Results demonstrated that the variation in the microbiota was mainly due to differences between kitchens (38.2%) followed by the different nucleic acid template (DNA vs RNA) (10.8%), and that only 5.1% of the variation was a result of differences between coupons and sink surfaces. The variation between sink surfaces and coupons was smaller for samples derived from RNA than for DNA. Overall, our results suggest that new stainless steel coupons are suited to model the dominating part of the natural microbiota of the surrounding environment, and furthermore suitable for different downstream studies.

Key words: Microbiota, stainless steel coupons, sink surface, domestic kitchens

INTRODUCTION

Studying microorganisms directly *in situ* is challenging for a number of reasons: it is not possible to transport the surfaces to a laboratory for further analysis or perform certain analyses directly, it is not safe to introduce pathogens outside the laboratory and it is difficult to compare different treatments, conditions or surfaces in a systematic and standardized way. Therefore most studies on biofilms are conducted using bacteria collected from environmental biofilms or laboratory strains in laboratory models (Giaouris et al. 2015). Biofilms produced at the laboratory are more or less relevant for the environments they are meant to mimic, with respect to a range of factors such as materials, microbiota, temperatures, nutrients, sanitation regimes and the dynamics for all these factors. To achieve a more realistic biofilm, some studies use the addition of food residues or organic soiling in the biofilm formation (Chaitiemwong et al. 2014; Kuda et al. 2015). Another approach to make the models more realistic is to place coupons at the site to be studied and allow for a natural biofilm to evolve. The biofilm or attached bacteria can then be investigated in different downstream studies, e.g. cleaning and disinfectant studies and/or examination of the survival/establishment of potential pathogens. This will ideally allow studies on biofilms that are more relevant than those produced using laboratory models. The approach has been used to compare hygienic properties of different materials (Guobjornsdottir et al. 2005), identification of microbiota in food production factories (Gunduz and Tuncel 2006; Hood and Zottola 1997; Mettler and Carpentier 1998), detection of biofilm formation (Gibson 1995; Holah et al. 1989) and recently we used this approach to study the effect of kitchen cleaning methods (Rossvoll et al. 2015). Little is however done to evaluate how well the microbiota developed by this approach reflects the microbiota developed *in situ*, where the surfaces can be of a different quality and/or condition than the coupons used.

Next generation sequencing (NGS) technology using the 16S rRNA gene as a taxonomic marker is often used to study complex microbial communities. NGS generates enormous amounts of data helping to reveal a more complete picture of the microbiota compared to traditional plating based analyses, which is dependent on growth conditions such as nutrients, atmosphere and temperature. One drawback of DNA based microbiota analysis is however that it does not discriminate between dead and viable bacteria, and this may limit the applicability when studying matrixes with a high proportion of dead bacteria. In many studies the relative amount of dead bacteria is considered insignificant but when working with biofilms subjected to different environmental stress this may not be the case. DNA will give a good overview of the complete microbiota of the biofilm (dead and active) whilst the use of RNA in principle will estimate the current in situ activity of a community, because cellular rRNA concentration is generally well correlated with growth rate and activity (Bremer 1996; Poulsen et al. 1993). rRNA are also thought to degrade only under certain stress conditions (starvation, stationary phase or following a nutritional downshift) or when an RNA molecule is defective (Deutscher 2003). A biofilm can fulfill several of these criteria, however, a study in *Pseudomonas aeruginosa* biofilms have shown that cells in the bottom portion of the biofilms maintained a high abundance of ribosomal RNAs as well as mRNA for genes associated with ribosome hibernation factors (Williamson et al. 2012).

In this study we aimed to investigate how the microbiota developing on stainless steel coupons placed in domestic kitchen sinks (stainless steel of varying age and condition) for three months compared to the natural microbiota of the kitchen sink surfaces. The samples were collected in a parallel study previously published (Rossvoll et al. 2015). Domestic sinks were chosen as a suitable environment since the material (stainless steel) was comparable to

the coupons, and as these are heavily exposed to and colonized by bacteria (e.g. from raw produce, water and skin microbes). We compared the microbiota between coupons and sink surfaces derived from both DNA (live and dead bacteria) and RNA (potentially active bacteria). In addition we used traditional plating followed by 16S rRNA gene sequencing of isolates to see how well the RNA derived microbiota reflected what could be cultivated.

MATERIALS AND METHODS

Experimental design

The experimental setup of the steel coupons in the domestic kitchen sinks (stainless steel) has previously been described (Rossvoll et al. 2015). Briefly, eight volunteers attached three new stainless steel coupons (AISI 304, 2B; Norsk Stål AS, Nesbru, Norway) in their kitchen sinks in January 2013. The kitchen sinks were all of stainless steel, but of varying age and quality. The surface of the stainless steel coupon was 2 x 6 cm. The volunteers were instructed to use their kitchen sinks as normal, but to avoid the use of disinfectants and direct scrubbing of the coupons. The coupons were left in the kitchen sinks for three months. In April 2013 the volunteers were instructed to sample an area of the size of a coupon (2 x 6 cm) beside each of three different coupons in their kitchen sink. All volunteers were provided with equipment and detailed instructions of how to swab the specific areas in their kitchen sink surface (Hedin et al. 2010).They were also instructed on how to remove the coupons in their sinks with gloved hands to avoid contamination, and place each coupon in a prelabeled 50 ml tube for transportation to the laboratory. The swabs and the coupons were sampled in the morning by the volunteers, brought to the laboratory and analysed within an hour.

Surface sampling and cultivation methods

The swabbing and plating were as described previously (Rossvoll et al. 2015). Briefly, two swabs were used for each coupon/sink area and both swabs were put in the same tube with 3 ml D/E (Dey/Engley) Neutralizing Broth (BD Difco™, New Jersey, USA) and serial 10-fold dilutions were prepared in PBS and spiral plated on Tryptic soy agar (TSA; Oxoid, Basingstoke, UK). The plates were incubated at 25^{o}C for 3 days before determination of cfu and isolating single colonies. A total number of 20 colonies (or less at low cell numbers) were picked at random from plates from each kitchen resulting in up to 60 colonies picked per kitchen. The colonies were restreaked on TSA, incubated at 25^{o}C for three days before preparation for sequencing.

DNA and RNA extraction and cDNA synthesis

The leftover material (swabs in D/E Neutralizing Broth) used for plating (approx. 2 ml per coupon/sink surface) was used to extract DNA and RNA. The Neutralizing broth originating from the swabs from three coupons per kitchen were mixed and then split into two samples; one for DNA extraction and one for RNA extraction. The same were done for the three sink surface areas. For DNA extraction the samples was centrifuged at 13000 *g* for 5 min and then frozen at -20°C for one/two weeks before extraction using the QiaAmp Stool Kit (Qiagen, Valencia, CA). Briefly, the bacterial pellet was resuspended in 500 µl ASL buffer (stool lysis buffer, Qiagen), transferred to Lysis Matrix E (MP Biomedicals, Solon, USA) tubes, and lysed in a FastPrep bead beater (MP Biomedicals) for 40 s at 6 m/s. The samples were centrifuged briefly before adding additional 500 µl ASL buffer and vortexed. The samples were thereafter incubated at 70°C for 5 min, centrifuged at 14 000 *g* for 5 min before

transferring to new tubes, adding 400 µl ASL buffer and following the manufacturer's protocol.

The samples for RNA extraction were added to tubes containing RNA Protect (Qiagen), vortexed for 5 s, incubated 5 min at room temperature, centrifuged at 5000 *g* for 10 min, the supernatant was decanted and the pellets was kept at -20°C/-80°C until extraction using the RNeasy mini kit (Qiagen) and an on-column DNase digestion (Qiagen). Briefly, 700 µl buffer RTL (lysis buffer, Qiagen) (with 40 µl 1M DTT/ml RTL) was added to the pellet, vortexed 5-10 s and then transferred to Lysis Matrix E (MP Biomedicals) tubes, and lysed as described above. The samples were centrifuged at 14000 ×*g* for 5 min before adding ethanol and following the Qiagen protocol from this point. The RNA was measured using nanodrop (NanoDrop Technologies, Inc., Wilmington, USA) and stored at -80°C until cDNA synthesis. The cDNA synthesis was performed using SuperScript™ III reverse transcriptase (Invitrogen, Life Technologies Ltd, Paisley, UK) as recommended by the manufacturer, with and without enzyme (negative control).

PCR and sequencing of colonies

PCR and sequencing was performed as described previously (Rossvoll et al. 2015). Briefly, universal primers (Nadkarni et al. 2002) were used for 16S rRNA gene amplification (V3–V4) and sequencing. DNA was isolated by lysing single colonies using a microwave oven (Sharp Microwave oven R-5000E). The microwave lysis was performed by applying a small amount of the colony on the bottom of the PCR well followed by microwave treatment for 1 min at max power. Amplification was performed using 0.25 μmol l^{-1} of each primer, 10 μl Qiagen multipleks PCR kit (2×) (Qiagen, Oslo, Norway) to a total volume of 20 μl. The cycling conditions, PCR purification and sequencing were performed as described previously

(Omer et al. 2015). The taxonomy was identified using the RDP (Ribosomal Database Project) SeqMatch (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). The thresholds used in the RDP search was: both type and none type strains; both uncultured and isolates; only good sequences >1200nt and KNN=1.

Biofilm microbiota study (NGS)

DNA and RNA (cDNA) from sink surface and coupon samples (described above) were used as template for the NGS (MiSeq, Illumina Inc., San Diego, USA) analysis. A portion of the 16S rRNA gene spanning the variable region 4 (V4) was amplified using the barcoded, universal primer set (515F/806R) (Caporaso et al. 2012). PCR mixture and thermal cycling conditions were the same as described by Caporaso et al. (Caporaso et al. 2012). In addition to the experimental samples, the MiSeq run also contained a control library made from phiX Control v3, which in this run accounted for 50 % of reads. The library quantification and sequencing was performed by the Norwegian Sequencing Centre (http://www.sequencing.uio.no/). The sample pool was quantified using the Invitrogen Qubit, diluted to 2nM, and the MiSeq Protocol provided by Illumina was then followed.

The total number of sequences was 18,162,924. The forward and reverse reads were joined using the QIIME toolkit (Caporaso et al. 2010b) (version 1.7.0) and the barcodes corresponding to the reads that failed to assemble were removed. The total number of sequences that joined were 10,517,341 with an average join length of 49.18. The sequences were then demultiplexed in QIIME allowing zero barcode errors and a quality score of 30 (Q30) resulting in 6,187,913 sequences with a median sequence length of 253 bp. The average number of sequence per sample was 193,372 (min 160,167; max 226,801). Reads were assigned to their respective bacterial id using two-step open-reference operational

taxonomic unit (OTU) picking workflow (Rideout et al. 2014). Briefly, after sequences were demultiplexed and quality filtered, reads were first clustered with a reference database (the Greengenes database (gg_13_5)) pre-clustered at 97% identity. Second, reads that did not group with any sequences in the reference collection were clustered *de novo*. Clustering at 97% identity was carried out using the UCLUST algorithm (Edgar 2010). Reads that did not match a reference sequence were discarded. Representative sequences were chosen for each OTU (cluster centroids) and aligned against the Greengenes core set with PyNAST (Caporaso et al. 2010a). Chimeric sequences were removed in QIIME using ChimeraSlayer. Singeltons were removed resulting in 5,955,225 sequences. In total 5661 OTUs passed the filter. Of these, 48% were 'novel' (i.e. not found in the Greengenes database (gg_13_5).

Statistical analyses

The alpha diversity (observed species) in all kitchens was calculated in QIIME by 100.000 rarefactions, and differences between groups were tested using paired t-tests (Minitab® (Minitab 16.1.1, 2010 (Minitab Ltd., Coventry, UK)). The differences between average bacterial counts were also tested using paired t-tests (Minitab®).

The differences in microbiota were analyzed by principal component analysis (PCA) and 50-50-MANOVA (Langsrud 2002). 50-50-MANOVA is a method for multivariate analysis of variance with a high number of collinear responses and was used to focus on partitioning the variation due to differences between kitchens, sink surface vs coupon and DNA vs RNA, and on identifying the bacterial groups that are significantly different. All analyses were performed at the genus level (level 6 table from QIIME). 50-50-MANOVA was calculated in MATLAB (Release 2013b, The MathWorks, Inc., Natick, Massachusetts, USA) and the taxa were scaled to unit variance in order to remove abundance effects from the analysis.

RESULTS

Total bacterial counts of coupons and sink surface swabs

The average bacterial counts were significantly ($p<0.001$) higher for coupons than corresponding samples taken from the sink surface, with an average cfu of log 6.0 (± 0.4) and log 4.8 (± 0.8), respectively (Figure 1). The bacterial counts on the coupons from all kitchens were similar (not significantly different between kitchens). The bacterial counts on the sink surface on the other hand was significantly different ($p< 0.05$) between the different kitchens, and kitchen no 1 had the highest CFU count. Figure 1 shows the average cfu (log_{10}) for coupons and sink surface (both 12 cm^2) for all kitchens.

Microbiota (NGS)

Overall bacterial composition

The microbiota across all samples (DNA and RNA, coupons and sink surfaces) was dominated by phylum *Proteobacteria* (average 54 %), followed by *Actinobacteria* (34 %), *Firmicutes* (8 %), *Bacteroidetes* (2 %), *Cyanobacteria* (1 %) and *Fusobacteria* (0.2 %). Most *Proteobacteria* belonged to the class *Gammaproteobacteria* (44 %). Figure 2 shows the average relative abundances (percent) of the dominant bacterial taxa (phylum/family level) for DNA (coupon and sink surface) and RNA (coupon and sink surface).

Overall the biofilm samples were dominated by two families; *Moraxellaceae* (genus *Moraxella/Enhydrobacter*) and *Micrococcaceae* (genus *Kocuria*). There were however variation between the kitchens and kitchen no 7 and 7 had a different dominating bacterial population. The sink surface in kitchen no 7 had a more diverse microbiota than the other samples and had high relative values of the families *Staphylococcaceae* and *Streptococcaceae* in addition to *Moraxellaceae* and *Micrococcaceae,* while the sink surface in kitchen no 8

(DNA) was dominated by *Enterobacteriaceae*. Table 1 shows the distribution of taxa down to family level (represented above 5 % in one or more samples) for all samples.

Bacterial diversity within samples

To investigate the bacterial diversity within the different samples an alpha diversity analysis was performed (QIIME). This analysis revealed a tendency (not significant at 5% level) of higher diversity (observed species) in samples derived from DNA than from RNA, and in sink samples compared to coupons.

Bacterial diversity between samples

To investigate the variation in bacterial composition between the samples, a beta diversity analysis (weighted- and unweighted unifrac) was performed (QIIME) (Figure S1). This analysis revealed that it was mainly the low abundant bacteria that were responsible for the difference between the experimental variables (kitchens, RNA/DNA, coupons/sink surface) and that the dominating microbiota was similar for most biofilms. Further statistical analysis was therefore performed on standardized variables (in order to give equal weight to all OTUs regardless of abundance). This analysis revealed some significant differences in the bacterial composition between the experimental variables (kitchen (A); sink surface/coupon (B) and DNA/RNA (C) (see Table 2).

The differences between kitchens accounted for the largest variation in the data, both with regard to main effect (38.2%) and interaction with sink surface/coupon (21.8%). The interaction means that there was a significant difference between sink surface and coupon, but that the bacteria causing the difference were not the same for all kitchens. The variation due to differences between coupons/sink surface (5%) and DNA/RNA (11%) were small in

comparison. Even if these effects were statistically significant, this indicates that the coupon was *in practice* quite representative for the sink surface, and that the main results were similar based on analyses for both RNA and DNA. The differences, however small, are illustrated in principal component (PC) plots in Figure 3. From the scores plot (A) it is clear that there was a separation between samples derived from RNA (green) and DNA (blue) along PC1 (explains 17% of the variance). Note also that the variation in microbiota in sink surfaces (outlined area) was larger than in coupons (filled area), and that this variation was larger in samples derived from DNA than RNA. This indicates that there was a systematic difference between sink surface and coupon for DNA, but not for RNA. The loadings plot (B) shows the significant bacteria (determined from 50-50 MANOVA) as filled circles, and the circle size is proportional to abundance. The taxa of the bacteria significantly different in one or more sample categories are listed in the table in Figure 3. From this we can see that relative proportions of *Acinetobacter*, *Dermacoccus*, *Dermabacteriaceae*, *Chryseobacterium*, *Streptophyta*, *Actinomycetales* and *Comamonadaceae* were significantly different in the microbiota derived from DNA and RNA, where the order *Actinomycetales* (including *Dermacoccus* and *Dermabacteriaceae*) had a higher abundance in RNA derived samples. There were three bacterial taxa significantly different between coupons and sink surfaces (*Streptococcus*, *Chryseobacterium* and *Exiguobacterium*), where *Streptococcus* had a higher abundance in sink surface samples.

Bacterial taxa from isolates plated from coupons

To get a more comprehensive overview of the viable population (bacteria growing aerobically on TSA) of the microbiota on the coupons, the identity of randomly selected isolates were determined (Table 3).

Results from NGS analysis derived from RNA was used for comparisons with the microbiota determined from identification of plated bacteria as the former should in principle reflect the active part of the population. Both methods resulted in the same dominating families/genera; *Micrococcaceae* (genus *Kocuria*) and *Moraxellaceae* (genus *Moraxella/Enhydrobacter*). Bacteria belonging to the genera *Rhodococcus* (f *Nocardiaceae*), *Microbacterium* (f *Micrococcaceae*) and *Brevundimonas* (f *Caulobacteraceae*) were isolated from some coupons, but these genera were not found using NGS. NGS detected *Dermacoccaceae* (0.1-12.4 %) and *Rhodobacteriaceae* 0.1-15.9 %) from most coupons, but these families were not represented among the cultivated isolates.

DISCUSSION

In the present study we investigated how the microbiota developing on stainless steel coupons placed in domestic kitchen sinks (stainless steel) compared to the natural microbiota of the kitchen sink surfaces. In addition we compared the microbiota derived from DNA and RNA to get a picture of the total (live and dead) microbiota and the potentially active microbiota, and last to see how well the RNA derived microbiota reflected what could be plated.

The results showed that the bacterial composition of coupons correlated well with the sink surface, with the best correlation resulting from microbiota derived from RNA samples. The plating results showed higher bacterial counts on coupons than sink swabs. For some coupons, a visible fouling was observed at the lower parts, and that water attached to the fouling. This could produce a more humid environment with higher survival and growth of bacteria compared to the sink surface. The biofilm on the coupons were also younger (three months) compared to the biofilm on the sink walls and one cannot exclude the possibility that in the quantitative analysis a higher proportion of cells were detached from the coupons than the sink surfaces, as it is known that mature biofilms are difficult to remove, and require increased mechanical force e.g. brushing rather than wiping. Further studies are needed to find the optimum attachment time and sampling method. Also, the chemical composition of the biofilm was not assessed and structural and chemical differences between biofilms of the sink surface and coupons cannot be excluded. However, the selective pressure, for example long periods of drying (during the working day and night), was still quite similar for coupons and the sink surface as the dominating microbiota was not systematically different.

Overall, the majority of the biofilms were dominated by *Moraxellaceae* (genus *Moraxella/Enhydrobacter*) and *Micrococcaceae* (genus *Kocuria*). This is in accordance with what have been found by others, although there are variations between studies. The

309 microbiota in domestic kitchen sinks have been studied in some detail by Flores et al., (Flores
310 et al. 2013) where sink samples from four kitchens were investigated together with over 80
311 other kitchen surfaces. Compared to the other surfaces they found the least diverse
312 communities associated with metallic surfaces in and around sinks, which were dominated by
313 biofilm-forming Gram-negative bacteria, including known biofilm-formation organisms like
314 *Sphingomonadaceae*. They found *Moraxellaceae* to be the dominating family in sink basin
315 and sink backsplash. In another study on common household surfaces, *Kocuria* spp. were
316 found to be among the most frequent recovered isolates and the most frequent recovered
317 isolate from kitchen sinks (Saha et al. 2014), and Stellato et al. (Stellato et al. 2015) found
318 *Kocuria* in all sink samples belonging to the pre-processing zones in a cooking center for
319 hospital foodservice.

320 The genus *Enhydrobacter* has been found in widely diverse environments like athletic
321 equipment (Wood et al. 2015), skin (buttocks) (Zeeuwen et al. 2012), toilet samples (Jeon et
322 al. 2013) and a beer bottling plant (Timke et al. 2005). A search in BLAST
323 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed a 100% match to both *Moraxella osloensis*
324 and *Enhydrobacter aerosaccus* for the OTU/isolates representing genus *Enhydrobacter* in our
325 study. Near full length 16S rRNA gene sequences of one random isolate (classified as genus
326 *Enhydrobacter*) confirmed that our isolates was most similar to these two species (99%
327 identity, data not shown). Both *Moraxella osloensis* and *Enhydrobacter aerosaccus* have been
328 found in skin microbiota (Gao et al. 2007; Jeon et al. 2013). *Moraxella osloensis* has also
329 been found in the biofilm of various pipe materials in drinking water distribution systems
330 (Zhu et al. 2014) and is the bacterium responsible for the locker-room smell or shower-curtain
331 odor (Kubota et al. 2012). *Moraxella* spp. was also identified as a part of the microbial
332 population on stainless steel coupons placed in fish and shrimp factories for a three month
333 period (Guobjornsdottir et al. 2005), but not as the dominant genus. *Moraxella* is neither

associated with food borne infections or spoilage. The best sequence match for the OTU/isolates representing genus *Kocuria* was *K. rhizophila* (confirmed by near full length 16S rRNA gene sequencing of a few random isolates). Members of the genus *Kocuria* have been isolated from a wide variety of natural sources, including mammalian skin, soil, the rhizosphere, fermented foods, clinical specimens, freshwater, and marine sediments. The genus has also been isolated from other food production environments (Carpentier and Chassaing 2004; Moretro et al. 2011; Møretrø 2013). Survival in these environments can be explained by resistance to desiccation, biofilm forming abilities and tolerance to chlorine (Leriche et al. 2003; Møretrø 2013). Others have shown that *K. rhizophila* can survive on dry surfaces for several days as well as being tolerant to high salt concentrations in growth medium (Kim et al. 2004; Kovacs et al. 1999). *Kocuria* spp. is not considered to be pathogenic, but in a study on bacteria surviving cleaning and disinfection in food processing plants, a *Kocuria varians* strain increased biofilm production in *Listeria monocytogenes* (Carpentier and Chassaing 2004). Further analyses are however needed if one wants to determine if our isolates represents a threat for safety.

As expected, most of the variation in the microbiota was related to different kitchens and not the sampling site (coupon/sink surface). This variance is likely to be associated with specific selective characteristics such as physical cleaning regimes, food preparation regimes and water availability. In a kitchen sink environment high loads of organic particulate matter such as fats and proteinecous material represent a source of nutrients for attached and/or transient microorganisms. The different kitchens would also have been exposed to different sources of bacteria from raw produce, different microbiota of the residents' skin as well as difference in the faucet water (five of the eight kitchens had different water sources (all public water sources); kitchen no 1 and 4 had the same water source and kitchen no 5, 6 and 8 had the water same source). Flores et al., (Flores et al. 2013) identified three indicator taxa from raw

produce (*Enterobacteriaceae*, *Microbacteriaceae* and *Bacillales*), four from the human skin (*Propionibacteriaceae*, *Corynebacteriaceae*, *Staphylococcaceae* and *Streptococcaceae*) and three from the faucet water samples (*Sphingomonadaceae*, *Methylobacteriaceae* and *Gallionellaceae*). Two of the indicator taxa from the human skin (*Staphylococcaceae* and *Streptococcaceae*) were major taxa in one of the kitchens (no 7) in our study. Further studies are needed to demonstrate the effect of differential usage of the sink.

We found the variation in the microbiota on the coupons to be smaller than the variation between the sink surfaces from the different kitchens. One theory could be that all the coupons were new and of the same steel quality compared to higher variation between the sink surfaces due to different age, produced by different manufacturers and different history of usage. More differences were also found between microbiota on coupons and sink surfaces when using results derived from DNA compared to RNA, indicating differences in the dead population of cells. This was not surprising since the dead cell population will reflect the part of the population not selected for survival and this may be different for a surface exposed to bacteria for years compared to coupons that had been placed in the sink for a three month period. RNA was chosen to illustrate the active taxa since cellular rRNA concentration is generally well correlated with growth rate and activity (Bremer 1996; Poulsen et al. 1993). There are several studies that have used rRNA to characterize the growing or active microbes, and Blazewicz et al. (Blazewicz et al. 2013) found >100 studies that used rRNA for these purposes, including recent studies using rRNA to identify currently active microbes (e.g. (Brettar et al. 2012; DeAngelis et al. 2010; Gaidos et al. 2011; Gentile et al. 2006; Jones and Lennon 2010; Lanzen et al. 2011; Mannisto et al. 2013; Wust et al. 2011)). Blazewicz et al. (2013) however argued that there are conflicting patterns between rRNA content and growth rate indicating that rRNA is not a reliable metric for growth or activity and rather suggested employing rRNA abundance data as an index of potential activity that provides basis for

384 further investigations (Blazewicz et al. 2013). Recognizing that the RNA derived microbiota
385 reflects past, current and future activities in addition to different life strategies we cannot
386 conclude that the RNA observed microbiota reflect the true viable, active bacteria. However,
387 the fact that the microbiota on coupons and sink surfaces correlated better when derived from
388 RNA compared to DNA, and that systematic differences between coupons and sink surfaces
389 were not found in samples derived from RNA, indicate that RNA gave the best picture of the
390 dominating, active microbiota in our study. To investigate this further, we also identified a
391 random selection of isolates plated from the coupons. The plating results showed a high
392 number of cultivable bacteria and, although some differences in the microbiota were
393 observed, the dominating taxa was similar to the NGS result. One must have in mind that only
394 a few isolates were analyzed compared to the high throughput results from the NGS analysis.
395 The plating results are also likely to be influenced by the use of a single culture agar which is
396 unlikely to meet the nutritional requirements necessary to maximize the recovery of all the
397 bacteria present. The NGS results could also have been influenced by the choice of PCR
398 primers and PCR conditions. For example *Microbacterium* (f *Micrococcaceae*) was only
399 detected by plating as previously also reported by Brightwell et al. (Brightwell et al. 2006).
400 Our study clearly shows that both culture-independent and culture-dependent techniques are
401 important to give the best representations of the microbiota in domestic kitchen sinks.

402 The results presented show that stainless steel coupons are suited to model the active
403 and dominating microbiota of the domestic kitchen sink surface, although the coupons in
404 general had a higher microbial load. Such coupons are therefore suited for further studies of,
405 e.g. effects of hygienic procedures (Rossvoll et al. 2015). The methodology could also be
406 developed for use in other environments and could potentially be used to study the ability of
407 pathogens to attach to a biofilm produced *in situ*, an experiment that would not be feasible to
408 perform in e.g. food processing environments or in the domestic environment. Sampling of

409 the surrounding surfaces should, however always be performed as a control. We have also
410 shown that the choice of nucleic acid template will influence the results, and that care should
411 be taken with respect to interpretation of bacterial activity.

412

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567 **TABLES**

568 **Table 1** Relative abundances (percent) of the dominant bacterial taxa (family level) across all samples (all taxa represented above 5 % in one or

569 more samples). The two overall dominating families are highlighted in gray.

570

			Actinobacteria				***Bacteroidetes***	***Cyanobacteria***	***Firmicutes***				***Proteobacteria***								***Other***
			Actinobacteria				*Flavobacteria*	*Chloroplast*	*Bacilli*				*Alphaproteobacteria*					*Gammaproteobacteria*			
			Actinomycetales				*Flavobacteriales*	*Streptophyta*	*Bacillales*	*Exiguobacterales*	*Lactobacillales*		*Rhizobiales*		*Rhodobacterales*	*Rhodospirillales*	*Sphingomonadales*	*Enterobacteriales*	*Other*	*Pseudomonadales*	
		Kitchen no	*Dermabacteraceae*	*Dermacoccaceae*	*Micrococcaceae*	*Promicromonosporaceae*	*Flavobacteriaceae*		*Staphylococcaceae*	*Exiguobacteraceae*	*Leuconostocaceae*	*Streptococcaceae*	*Methylobacteriaceae*	*Rhizobiaceae*	*Rhodobacteraceae*	*Acetobacteraceae*	*Sphingomonadaceae*	*Enterobacteriaceae*	*Other*	*Moraxellaceae*	
DNA	**Sink**	**1**	0.3	3.1	33.6		1.8	0.4		2.8		3.7		0.1	0.1	0.1	0.3	0.3		51.2	2.0
		2	0.1	0.1	3.1		4.2	4.3	0.7	0.4	0.5	3.5	0.3	0.3	0.1	0.4	3.5	12.6	1.0	54.6	10.3
		3	3.5	1.0	9.8	0.1	1.0	0.1			0.3	4.7			0.6	0.3	0.5			73.2	4.9
		4	0.1	11.9	5.8	6.7	6.4	7.2	0.3		0.5	2.9	0.1	2.1	0.4	0.4	1.1	1.1	0.3	33.3	19.5
		5	0.2	0.3	17.3		2.3	5.5	1.3		0.2	4.6	2.1	3.1	5.5	2.7	3.8	0.7		39.0	11.4
		6	1.7	0.5	12.6		0.6	8.0	1.0			12.8	0.1		0.1	0.7	0.1	0.4		50.6	10.8
		7	0.1	0.3	10.1		0.8	1.0	14.3		0.2	24.3	0.5	0.8	0.1	0.1	0.7	4.4		5.8	36.4
		8	0.5	2.9	19.9		0.4	0.7	0.5		1.5	2.4				0.1	0.2	52.4	0.1	5.6	12.7
	Coupon	**1**	1.1	3.4	8.7		4.7	0.2		5.9		3.3		1.1	0.6	0.2	0.9	0.7		62.9	6.4
		2	1.2		10.7		9.3	0.6		3.4		0.4	0.2	1.3	0.5	0.3	2.6	0.3	0.6	60.4	7.8
		3	4.4	3.5	25.7		0.4	0.1	0.1			0.1	0.2		1.3	0.7	0.5	1.0		59.2	2.7
		4	0.5	5.3	15.3		5.7	2.8	0.1		0.1	2.0		1.4	0.2	0.8	1.2	1.0		58.5	4.9
		5	0.1	0.4	23.5		2.2	2.4	0.3		0.1	1.1	0.3	0.5	4.0	1.1	1.1	0.1		58.7	4.0
		6	4.6	1.0	45.0		0.9	1.0	0.3			2.9	0.3	0.4	0.3	2.8	1.2	0.1		34.7	4.4
		7	0.7	0.5	27.6		0.9	0.1	1.0			4.1	0.1	1.6			3.7	0.4	0.2	54.6	4.4
		8	3.2	4.4	39.2		3.2	0.1			0.3	0.5	0.2	0.8	2.4	0.7	1.8	3.8	0.1	34.7	4.7
RNA	**Sink**	**1**	1.3	8.0	56.8		0.2			1.2		0.7		0.1	0.4	0.6	0.2	0.1		28.1	2.2
		2	1.2	0.2	17.2		0.2	0.2	2.3	0.4	0.4	0.6	0.6	0.1	0.6	8.4	10.1	0.4	8.5	44.5	4.2
		3	18.1	2.1	18.2		0.2		0.1			0.1	0.1		3.4	9.4	0.5	0.1	0.1	46.7	0.9

		4	1.1	24.1	15.2		1.9		0.8		1.1	4.4	0.5	5.2	4.5	2.2	1.2		0.4	31.1	6.2
		5	0.1	0.5	33.6		0.1		1.9			1.8	5.3	0.5	17.7	7.8	0.9		0.1	27.7	2.0
		6	2.0	0.5	3		0.3		2.4		0.1	2.9	0.1		0.1	1.6	0.1	0.1		58.3	1.5
		7	0.8	1.0	26.8				41.8		0.2	14.7	0.3		0.1		1.2			4.2	8.7
		8	1.1	6.4	49.5		0.1		1.8		9.2	2.1	0.1		0.5	0.2	0.3	6.9		15.9	5.9
	Coupon	**1**	4.6	12.4	15.2		1.5			5.2		0.8		1.2	3.0	1.3	0.8	0.2		47.6	6.0
		2	7.0	0.1	27.5		2.2		0.1	2.0		0.1	1.0	1.1	5.4	1.4	4.0	0.1	0.9	40.4	6.8
		3	13.9	5.0	31.6										8.4	5.1	0.5	0.1	0.1	34.2	1.1
		4	2.2	11.2	30.4		3.2		0.1		0.1	1.6	0.1	1.5	0.8	3.5	0.4	0.1	0.1	41.9	2.8
		5	1.2	0.7	27.9		0.7		0.4			0.4	0.8	0.3	10.7	3.3	0.5			51.2	1.7
		6	7.4	1.6	51.0		0.3		0.6			0.5	0.3	0.5	1.1	8.5	0.8			24.4	3.0
		7	2.6	1.1	49.4				0.5			1.2	0.6	0.7	0.1	0.2	7.6		0.2	34.9	0.9
		8	10.4	9.4	27.5		1.4				0.2	0.1	0.7	0.7	15.9	2.7	0.9	0.1	0.1	27.2	2.6

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572

573 **TABLE 2.** Explained variance due to the different experimental variables (50-50
574 MANOVA). The analysis is done on the 35 most abundant bacteria on genus level, and
575 variables were standardized to remove abundance effects prior to the analysis.

Source	d.f.	Explained variance (%)	No. of significant bacteria
Kitchen (A)	7	38.2***	9
Sink surface/Coupon (B)	1	5.1***	3
DNA/RNA (C)	1	10.8***	7
A×B	7	21.8***	5
A×C	7	13.5 (ns)	0
B×C	1	2.3 (ns)	0
Error	7	8.2	

576 *** $p<0.001$; ns= not significant at 5% level.

577

578 **Table 3.** Relative abundance (percentage) of the different bacterial taxa (genus level)
579 characterized from isolates cultured from coupons (partial 16S rRNA gene). Total number of
580 sequenced isolated was 113. The two overall dominating families/genera are highlighted in
581 gray. The "n" is the number of isolates/sequences per coupon.

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		Actinobacteria				***Bacteroidetes***		***Proteobacteria***							
		Actinobacteria				*Flavobacteria*	*Sphingobacteria*	*Alphaproteobacteria*			*Gammaproteobacteria*				
		Actinomycetales				*Flavobacteriales*	*Sphingobacteriales*	*Caulobacterales*	*Rhizobiales*	*Sphingomonadales*	*Enterobacteriales*		*Pseudomonadales*		
		Nocardiaceae	*Dermabacteraceae*	*Microbacteriaceae*	*Micrococcaceae*	*Flavobacteriaceae*	*Sphingobacteriaceae*	*Caulobacteraceae*	*Rhizobiaceae*	*Sphingomonadaceae*	*Enterobacteriaceae*		*Moraxellaceae*		*Pseudomonadaceae*
Kitchen no	**Coupon no**	*Rhodococcus*	*Brachybacterium*	*Microbacterium*	*Kocuria*	*Chryseobacterium*	*Sphingobacterium*	*Brevundimonas*	*Rhizobium*	*Sphingomonas*	*Enterobacter*	*unclassified_ Enterobacteriaceae*	*Acinetobacter*	*Moraxella/ Enhydrobacter*	*Pseudomonas*
1	3 (n=6)	17		17	17			50							
	6 (n=6)	17		67	17										
2	2 (n=7)		14		29									57	
	4 (n=4)		50									25		25	
	5 (n=6)			33	50									17	
3	2 (n=7)				43					14				43	
	4 (n=7)				14	14			14	14				43	
	6 (n=4)			25	50	25									
4	2 (n=5)				40			20						40	
	3 (n=5)				20			20						60	
	5 (n=6)	17					17						50		17
5	2 (n=7)				29			14						57	
	3 (n=5)				20									80	
	6 (n=4)				50									50	
6	2 (n=5)		40	20	20			20							
	4 (n=4)				100										
	6 (n=4)		50		25									25	
7	2 (n=3)				67				33						
	4 (n=6)				83									17	
	6 (n=3)									67				33	
8	2 (n=2)		50								50				
	6 (n=7)		29		71										

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591

592 **FIGURE CAPTIONS**

593

594 **Figure 1.** Bar chart showing the average cfu (log_{10}) for coupons and sink surface samples
595 (both 12 cm^2) for eight kitchens (no 1 - no 8). Each bar represents three replicates per kitchen
596 (only two replicates for kitchen no 1 and no 8) and the different shades of grey represent the
597 different kitchens, starting from left with kitchen no 1. The error bars are SEM (standard error
598 of mean).

599

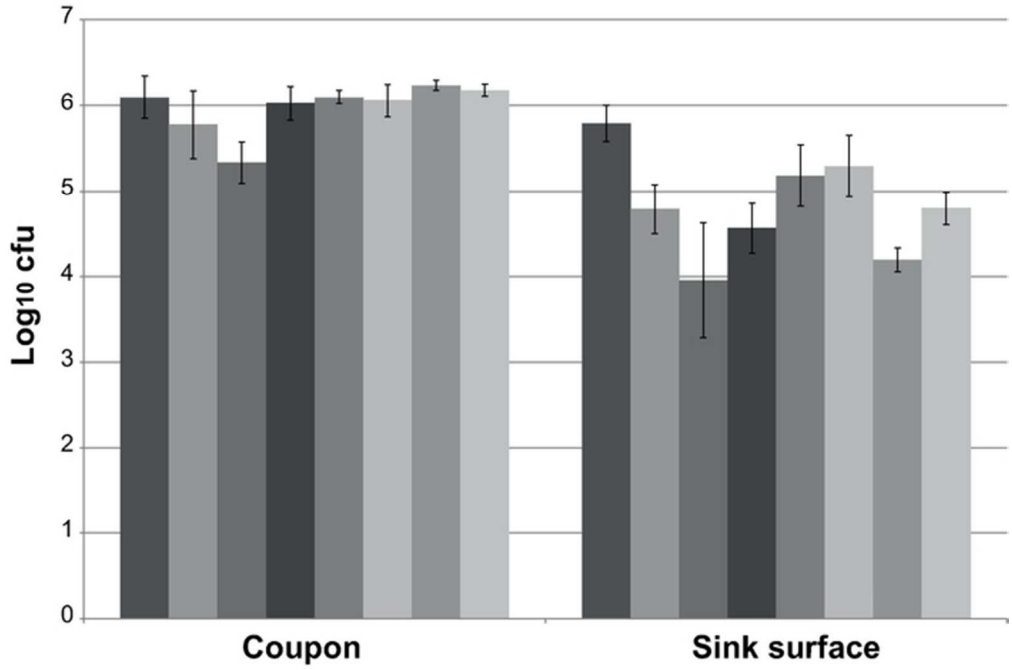
600 **Figure 2.** Average relative abundances (percent) of the dominant bacterial taxa
601 (phylum/family level) for DNA (sink surface and coupon) and RNA (sink surface and
602 coupon). Taxa represented above 5 % in one or more samples is shown. Blue shades:
603 *Proteobacteria*; green shades: *Actinobacteria*; purple shades: *Firmicutes*; apricot shade:
604 *Bacteroidetes*; pink shade: *Cyanobacteria* and grey shade: Other.

605

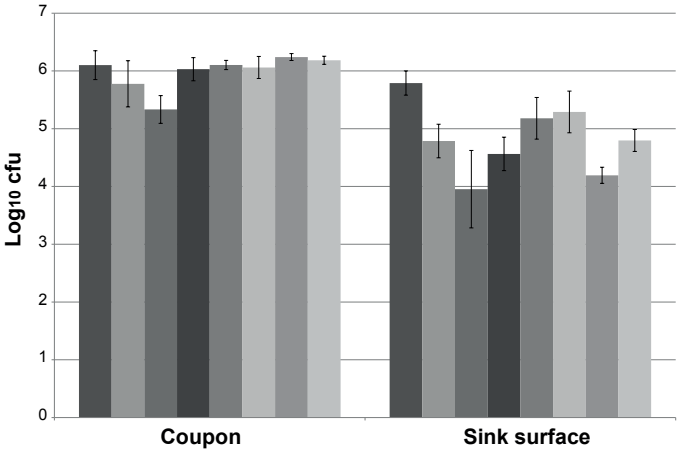
606 **Figure 3.** Overview of results from PCA and 50-50 MANOVA. The scores plot (A) shows
607 the distribution of samples, where labels S/C corresponds to sink surface (outlined area) and
608 coupon (filled area), and colors correspond to DNA (blue) and RNA (green). The loadings
609 plot (B) shows the bacteria significantly different between one or more sample categories
610 (determined from 50-50 MANOVA) as filled circles, and the circle size is proportional to
611 relative abundance. The corresponding table show which bacteria that were significantly
612 different ($p<0.05$) between the sample categories; kitchens (A), sink/coupons (B), DNA/RNA
613 (C) and interaction between A*B. One of the dominating OTU (affiliated with
614 *Enhydrobacter*) had a statistically significantly interaction between sink surface/coupon and
615 kitchen. This means that there was a significant difference in the relative amount of this

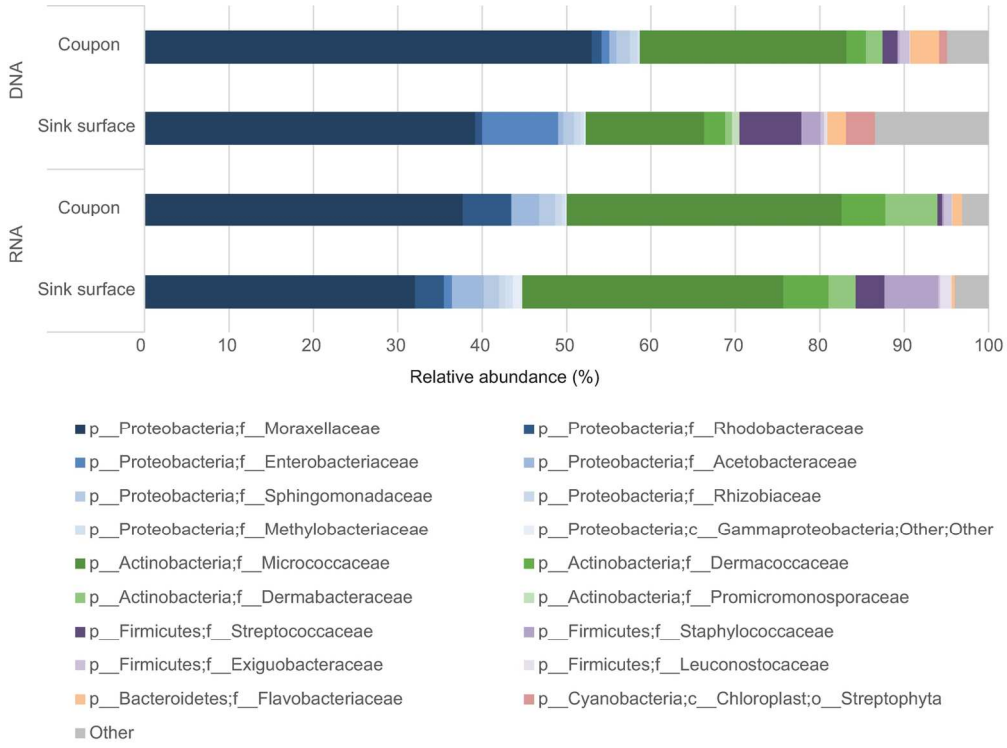
bacteria between sink surfaces and coupons but that this difference was not systematic, that is the relative amount was sometimes higher in sink surface than coupon and vice versa.

Figure S1. Beta diversity analysis. The variation in bacterial composition between the samples shown for unweighted unifrac (top panel) and weighted unifrac (bottom panel). The plots to the left show the samples colored according to nucleic acid template; DNA derived samples (red) versus RNA derived samples (blue). The plots to the right show the samples colored according to the sample type; coupons (red) versus sink surfaces (blue). The beta diversity analysis was performed in QIIME.

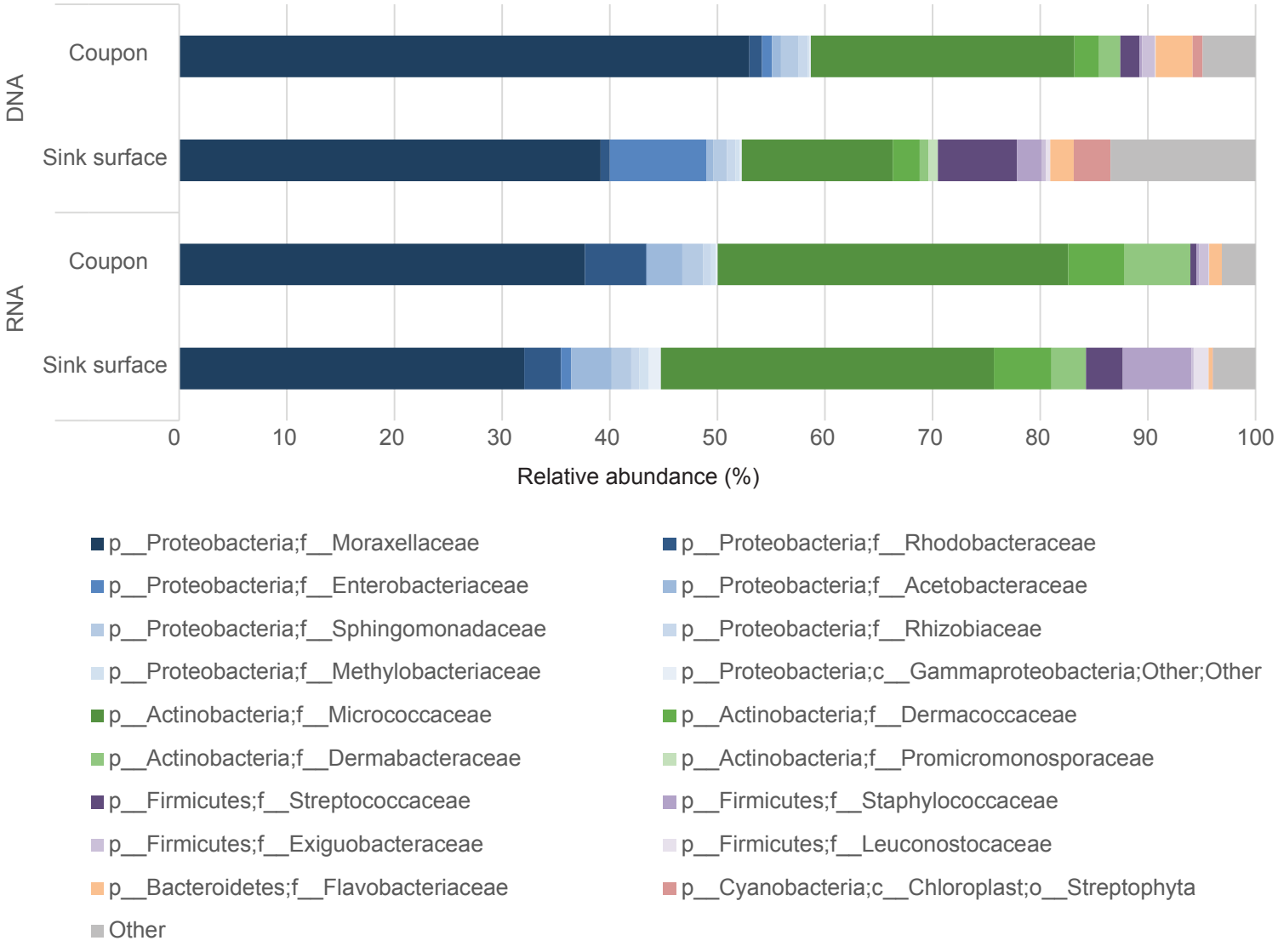


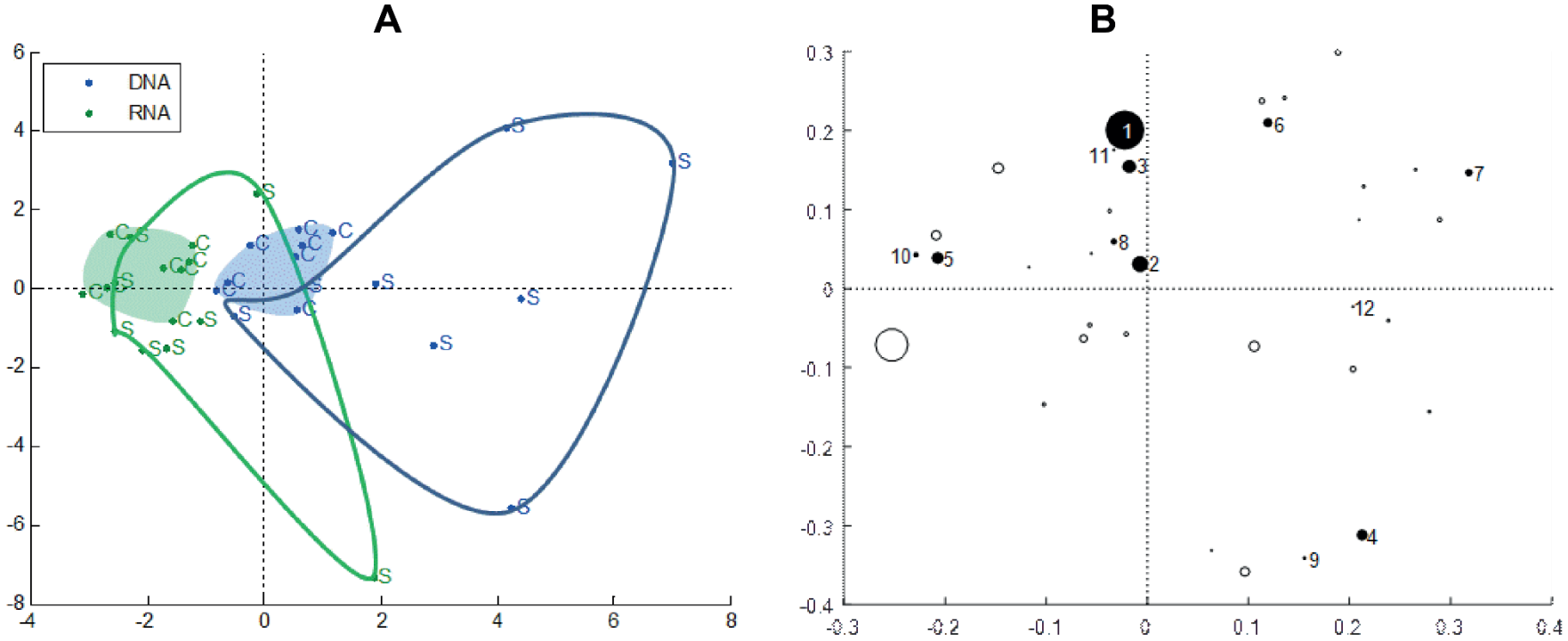
59x39mm (300 x 300 DPI)


7
6
5
4
3
2
1
0
Log10 cfu
Coupon
Sink surface



146x107mm (300 x 300 DPI)


DNA
RNA
Coupon
Sink surface
Coupon
Sink surface
0
10
20
30
40
50
60
70
80
90
100
Relative abundance (%)
p__Proteobacteria;f__Moraxellaceae
p__Proteobacteria;f__Rhodobacteraceae
p__Proteobacteria;f__Enterobacteriaceae
p__Proteobacteria;f__Acetobacteraceae
p__Proteobacteria;f__Sphingomonadaceae
p__Proteobacteria;f__Rhizobiaceae
p__Proteobacteria;f__Methylobacteriaceae
p__Proteobacteria;c__Gammaproteobacteria;Other;Other
p__Actinobacteria;f__Micrococcaceae
p__Actinobacteria;f__Dermacoccaceae
p__Actinobacteria;f__Dermabacteraceae
p__Actinobacteria;f__Promicromonosporaceae
p__Firmicutes;f__Streptococcaceae
p__Firmicutes;f__Staphylococcaceae
p__Firmicutes;f__Exiguobacteraceae
p__Firmicutes;f__Leuconostocaceae
p__Bacteroidetes;f__Flavobacteriaceae
p__Cyanobacteria;c__Chloroplast;o__Streptophyta
Other



	Bacterial taxa	Kitchen (A)	Sink/Coupon (B)	DNA/RNA (C)	A×B
1	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Enhydrobacter	*	ns	ns	*
2	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter	***	ns	*	ns
3	Actinobacteria;Actinobacteria;Actinomycetales;Dermacoccaceae;Dermacoccus	*	ns	*	ns
4	Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus	**	**	ns	**
5	Actinobacteria;Actinobacteria;Actinomycetales;Dermabacteraceae;Other	*	ns	*	ns
6	Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Chryseobacterium	**	*	**	ns
7	Cyanobacteria;Chloroplast;Streptophyta;;	ns	ns	*	ns
8	Firmicutes;Bacilli;Exiguobacterales;Exiguobacteraceae;Exiguobacterium	***	*	ns	*
9	Firmicutes;Bacilli;Lactobacillales;Enterococcaceae;Enterococcus	**	ns	ns	*
10	Actinobacteria;Actinobacteria;Actinomycetales;Other;Other	*	ns	***	ns
11	Proteobacteria;Alphaproteobacteria;Rhizobiales;Other;Other	ns	ns	ns	**
12	Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Other	ns	ns	**	ns