Effects of storage temperature on bacterial growth rates and community structure in fresh retail sushi

Sunniva Hoel[*1], Anita Nordeng Jakobsen[1], and Olav Vadstein[1]

[1]Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, N-7491 Trondheim, Norway

*Author for correspondence at NTNU - Norwegian University of Science and Technology, Department of Biotechnology and Food Science, N-7491 Trondheim, Norway. Tel: + (47) 73559716, E-mail: sunniva.hoel@ntnu.no.

Running headline: Growth rates and sushi microbiota

Abstract

Aims: This study was conducted to assess the effects of different storage temperatures (4 to 20 °C), on bacterial concentrations, growth rates and community structure in fresh retail sushi, a popular retail product with a claimed shelf life of two to three days.

Methods and Results: The maximum specific growth rate based on aerobic plate count (APC) at 4 °C was 0.06 h^{-1}, and displayed a 6-fold increase (0.37 h^{-1}) at 20 °C. Refrigeration resulted in no growth of hydrogen sulfide (H_2S)-producing bacteria, but this group had the strongest temperature response. The bacterial community structure was determined by PCR/DGGE (Denaturing gradient gel electrophoresis). Multivariate analysis based on Bray-Curtis similarities demonstrated that temperature alone was not the major determinant for the bacterial community structure. The total concentration of aerobic bacteria was the variable that most successfully explained the differences between the communities. The dominating organisms, detected by sequencing of DNA bands excised from the DGGE gel, were *Brochothrix thermosphacta* and genera of lactic acid bacteria (LAB).

Conclusion: The relationship between growth rates and storage temperatures clearly demonstrates that these products are sensitive to deviations from optimal storage temperature, possibly resulting in loss of quality during shelf life. Regardless of the storage temperature, the bacterial communities converged towards a similar structure and density, but the storage temperature determined how fast the community reached its carrying capacity.

Significance and Impact of the Study: Little information is available on the microbial composition of ready-to-eat food that are prepared with raw fish, subjected to contamination during handling, and susceptible to microbial growth during cold storage. Moreover, the data is a good first possibility to simulate growth of APC, H_2S-producing

bacteria and LAB under different temperature scenarios that might occur during production, distribution or storage.

Keywords: Sushi; Ready-to-eat seafood; Spoilage; H_2S-producing bacteria, PCR-DGGE; *Brochothrix thermosphacta*

Introduction

Sushi is a traditional Japanese dish consisting mainly of cooked acidified rice combined with raw fish. Sushi is now available as a ready-to-eat (RTE) product in retail stores, normally with a shelf life of two to three days after production. Fresh retail sushi typically consists of a complete meal, combining a selection of *nigiri* and *maki* sushi with a variety of ingredients. The sushi is offered as a chilled product (≤ 4°C) packed in a plastic tray in normal atmosphere. The diversity of ingredients includes raw or cooked vegetables, and different species of raw fish and raw or cooked seafood combined with rice. The microbiological quality of such combined products is a result of production hygiene (e.g., filleting, slicing and staff hygiene), temperature control and the initial quality of each ingredient. In a previous study, we assessed the microbiological quality of RTE sushi from selected supermarkets in Norway (Hoel *et al.*, 2015). The study revealed large variations in the microbiological quality in the sushi, and we hypothesized that poor temperature control during production, distribution and storage is a main reason for this loss of quality. Refrigeration is an important way of controlling microbial growth in perishable foods, and the time and temperature profile during storage of such food is critical to minimize the risk for development of microbial hazards leading to foodborne illness. Furthermore, proper refrigeration is vital for maintaining the quality of the food by preventing spoilage before the claimed shelf life of the product (Gram *et al.*, 2002; Sivertsvik *et al.*, 2002).

Seafood is more perishable than other high-protein products due to the high *post mortem* pH and high levels of soluble nitrogen compounds in the tissue (Gram and Huss, 1996). In newly processed fresh or lightly preserved fish, the specific spoilage organisms (SSOs) are usually present in low numbers, and constitute only a minor part of the total microbiota. During storage, the SSOs grow faster than the remaining microbiota, and

produce metabolites responsible for off-flavors which eventually lead to sensory rejection (Gram and Dalgaard, 2002; Ryder *et al.*, 2014). The spoilage of unpreserved and chilled fish is mainly a result of the Gram-negative fermentative psychrotolerant bacteria *Pseudomonas* spp. and *Shewanella* spp. *Aeromonas* spp. are associated with the spoilage of seafood from tropical regions (Ryder *et al.*, 2014). The low pH in the sushi rice (pH < 4.6) might increase the selection pressure towards lactic acid bacteria (LAB), including species that contribute to food spoilage (Leroi, 2010). In vegetables, LAB, *Pseudomonas* spp. and *Erwinia* spp. represent the main spoilage microbiota (Lee *et al.*, 2013). The bacteria introduced to the sushi from raw vegetables can contaminate the raw fish, which is an excellent substrate for bacterial growth. In an ingredient-based study of the microbiological quality in sushi, we demonstrated that mesophilic *Aeromonas* spp., Enterobacteriaceae and LAB were introduced to sushi from raw vegetables (Hoel *et al.*, 2015).

Outbreaks of foodborne disease linked to sushi have been caused by bacterial pathogens, such as *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* or viruses (especially norovirus) (NSWFA, 2008; FAO/WHO, 2011; CDC, 2012; Tominaga *et al.*, 2012). *Listeria monocytogenes*, *Aeromonas* spp., and pathogenic *Escherichia coli* must also be considered as risk factors because of their regular presence in raw or lightly preserved seafood and vegetables (Wan Norhana *et al.*, 2010; Xanthopoulos *et al.*, 2010; Di Pinto *et al.*, 2012; Park, 2012; Lunestad *et al.*, 2013; Hoel *et al.*, 2015; Oliveira *et al.*, 2015). During storage, the microbiota of a food product is not static, but affected by several intrinsic and extrinsic factors (den Besten *et al.*, 2017; Liu *et al.*, 2017). For example, temperature may vary throughout the complete production and distribution chain (Koutsoumanis, 2001). Therefore, understanding the dynamics of microbial behavior at

different temperatures is fundamental to determine the critical factors to control microbial spoilage and to assess the shelf life of a product.

The increased consumption of RTE seafood with a shelf life of several days requires more knowledge about the composition and behavior of the microbial community in these products during storage. To our knowledge, this is the first study of retail sushi microbiota as a function of storage temperature. The aim of this study was to assess the effects of storage temperature in retail sushi on the bacterial concentrations, growth rates and community structure. The bacterial counts and growth rates for aerobic plate count (APC), hydrogen sulfide (H_2S)-producing bacteria related to spoilage of fish, and LAB were quantified as a function of five different storage temperatures during a 5-day period. Furthermore, the bacterial community structure during storage was analyzed using a nested PCR/DGGE (Denaturing gradient gel electrophoresis) strategy (Muyzer *et al.*, 1993).

Materials and Methods

Materials, storage regime and sampling

Fresh sushi meals were collected immediately after production at the factory, brought to the laboratory in chilled containers, and put in their respective temperature cabinets (Liebherr, Germany) for storage. The temperatures were monitored using Ecolog TN2 data loggers (Elpro-Buchs AG, Switzerland), and the actual average storage temperatures were 3.5°C ± 0.9, 8.0°C ± 0.4, 12.1°C ± 0.4, 15.9°C ± 0.2 and 20.0°C ± 0.06 (average ± SD). The sushi meals consisted of a selection of 6 pieces of both *nigiri* and *maki* sushi based on farmed Atlantic salmon, halibut and cooked scampi packed in a plastic lidded tray. According to the producer, the raw fish was treated according to the current regulation (EC Regulation

853/2004) which requires freezing to a core temperature of at least -20 °C for not less than 24 hours as preventive treatment for *Anisakis* parasites. The farmed Atlantic salmon is considered to be parasite free and does not need to be frozen (EFSA, 2010) . The pH in the sushi rice was measured after acidification as part of the manufacturer's HACCP (Hazard analysis and Critical Control Point), and was < 4.6. The products had a shelf-life of three days after production, provided storage at 0-4 °C. Three replicate samples were analyzed upon arrival (time t_0) to assess box to box variation. The storage experiment was performed twice. The bacterial growth data obtained in the first experiment were used to decide both the sampling interval during the exponential growth phase, and the duration of storage in the second experiment. The data presented here originate from the second experiment. The samples were stored at 20 and 16 °C for 72 h; at 12 °C for 87 h; and at 8 or 4 °C for 111 h. A new sushi box was analyzed at six to eight different time points for each chosen temperature.

Quantification of microorganisms from sushi

The complete content of a sushi box was mixed in a sterile blender (Invite, Norway) for 30 s, after which a 15-g sample was transferred aseptically to a stomacher bag and diluted 1:10 in physiological saline peptone solution (0.85 % NaCl, 0.1 % peptone). The mixture was homogenized for 60 seconds in a Stomacher 400 lab blender (Seward Medical, UK). Appropriate serial dilutions were made in sterile peptone water and spread on their respective agar plates.

Total aerobic plate count (APC) and H_2S-producing bacteria (representing organisms associated with fish spoilage) were quantified as total and black colonies, respectively, on Lyngby's iron agar (Oxoid, Norway) supplemented with 0.04 % L-cysteine (Sigma-Aldrich,

136 Norway). The plates were incubated at 22 °C for 72 h (NMKL, 2006). LAB were quantified
137 using de Man Rogosa Sharpe agar (MRS, Oxoid) supplemented with 10 mg l^{-1} amphotericin B
138 (Sigma-Aldrich), and incubated under anaerobic conditions at 25 °C for 5 days (NMKL, 2007).
139 Thermotolerant coliform bacteria and *E. coli* were quantified using Violet-red-bile agar
140 (VRBA, Oxoid) with verification tests as described in NMKL method no. 125 (NMKL, 2005),
141 and incubated at 44.5 °C for 24 h. Presumptive *B. cereus* were quantified on Brilliance
142 *Bacillus cereus* agar (Oxoid) and on bovine blood agar, according to NMKL method no. 67
143 (NMKL, 2010), and incubated at 30 °C for 24 and 48 h. *S. aureus* were quantified using Staph
144 Express petri films (3M, Norway) incubated at 37 °C for 24 h. *L. monocytogenes* were
145 quantified using Rapid L mono agar (Bio-Rad Laboratories AB, Norway), as described by the
146 manufacturer, with modifications. Only 15 g were sampled and 1 ml homogenized sample
147 was spread on three parallel agar plates. The plates were incubated at 37 °C for 24 and 48 h.
148 Mesophilic *Aeromonas* spp. were quantified and verified according to NMKL method no. 150
149 (NMKL, 2004) using Starch Ampicillin agar incubated at 37 °C for 24 h. To increase the
150 detection limit of this method, 1 ml homogenized sample was spread on three parallel agar
151 plates at each sampling.
152
153 Analysis of the bacterial community structure by PCR/DGGE
154 A total of 30 samples were analyzed by PCR/DGGE, and each storage temperature was
155 represented by 5 or 6 samples in addition to the time zero samples. Total genomic DNA was
156 extracted from 1 ml homogenized sample using the DNeasy Blood and Tissue Kit (Qiagen,
157 Norway), as described in the protocol for Gram-positive bacteria by the manufacturer. A
158 nested PCR (polymerase chain reaction) strategy was applied to avoid a possible co-
159 amplification of eukaryotic 18S rRNA from the food (Bakke *et al.*, 2011), with modification in

the primers used for the external PCR. For the external PCR, the primers 7f (5'-agagtttgatymtggctcag-3') and 1510r (5'-acggytaccttgttacgactt-3') were used to amplify almost the entire bacterial 16S rRNA (Lane, 1991). A fragment of the variable region 3 (v3) of the 16S rRNA gene was then amplified using primers 338f (5'-actcctacgggaggcagcag-3') with a 40 bp GC clamp attached (5'-cgcccgccgcgcgcggcgggcggggcgggggcacgggggg-3') and 518r (5'-attaccgcggctgctgg-3') (Muyzer *et al.*, 1993). PCR products were analyzed on the INGENYphorU DGGE system (Ingeny, The Netherlands) with a 35-55 % denaturing gradient as described by Bakke *et al*. (2013). The gel was run at 100 V for 20 h. As a marker for the DGGE, pooled 16S rDNA products from nine different pure cultures of bacteria were used (Bakke *et al.*, 2013).

For DNA sequencing of excised bands, the DNA were re-amplified using the linker PCR primer 338F-GC-M13R (5'-caggaaacagctatgaccgcccgccgcgcgcggcgggcggggcgggggacgggggg actcctacgggaggcagcag-3') (O'Sullivan *et al.*, 2008) and primer 518r. The PCR products were purified using MinElute PCR Purification Kit (Qiagen), and DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany). Basic local alignment search tool (BLAST) was used to assign taxonomy to the sequences. The sequences were deposited to the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena) and assigned accession numbers LT605062 to LT605073.

Analysis of the DGGE image was performed with the Gel2K software (developed by Svein Norland at Dept. of Biology, University of Bergen, version 1.2.6) to transform the band intensities into histogram peak areas. The peak areas for each DGGE profile were exported to an Excel spreadsheet, and normalized by converting the areas to a percentage of the sum of all peaks for each DGGE profile. DNA sequencing revealed that some of the bands in the

184 DGGE profiles represented plant chloroplast genes. These bands were most abundant in the
185 t_0-samples, and in samples stored at 4 and 8 °C, presumably originating from plant material
186 in the sushi. These bands were excluded from further analysis.
187
188 Calculations and statistical analysis
189 The ln-transformed bacterial counts were fitted to the primary model of Baranyi and Roberts
190 (1994) (available at www.combase.cc) for estimation of the temperature dependent
191 maximum specific growth rates (μ_{max}) and duration of lag phase. Growth rates were also
192 calculated by a linear regression of ln-transformed bacterial counts versus time for the
193 exponential growth phase for comparison. The linear regression for each temperature was
194 based on 4 to 8 observations, depending on the nature of the growth curve. The significance
195 of the regression coefficients was assessed by a t-test (α=0.05). The software SPSS Statistics
196 (Version 22, IBM) was used for statistical analysis on bacterial growth.
197 The μ_{max} obtained from the primary model of Baranyi and Roberts were further modeled as
198 a function of storage temperature using a square root type model (Ratkowsky *et al.*, 1982)
199 (eq. 1).
200 $\mu = (b\ (T-T_{min}))^2$ *(Equation 1)*

201 where b is the slope of the regression line, T is the storage temperature, and T_{min} is the
202 theoretical minimum temperature for growth. For the secondary model, parameter
203 estimation (b and T_{min}) were done by non-linear regression using Gauss-Newton method and
204 least square estimation in SYSTAT version 13 (Systat Software, Inc.).
205 To describe the microbial community diversity of single samples (α-diversity), the
206 following indices were calculated:
207 Band richness = number of bands *(Equation 2)*

208 Dominance = $\Sigma_i (n_i/n)^2$ *(Equation 3)*

209 Shannon = $-\Sigma_i (n_i/n) \times (\ln(n_i/n))$ *(Equation 4),*

210 where n_i is the area of band i, and n is the sum of peak areas in each sample. Band richness,
211 dominance and Shannon diversity index (Shannon, 1948) were calculated for all the DGGE
212 profiles and one-way and two-way ANOVA, followed by Tukey's HSD test was used to test for
213 differences between the groups of sushi samples. Bray-Curtis similarities (Bray and Curtis,
214 1957) were used to compare the DGGE profiles of the different samples (β-diversity).
215 Ordination based on Bray-Curtis similarities was performed using Principal Coordinate
216 ordination (PCO). One-way PERMANOVA (NPMANOVA) based on Bray-Curtis similarities was
217 used to compare the effect of storage temperature, storage time and APC CFU g^{-1} on
218 bacterial community composition (Anderson, 2001). CABFAC factor analysis was used to
219 reconstruct environmental variables from bacterial community composition (Klovan and
220 Imbrie, 1971). The quality of the reconstruction was assessed based on R^2 and regression
221 analysis of predicted versus measured community structure. Similarity percentage analysis
222 (SIMPER) was used to identify the bands contributing most to the differences in community
223 composition between the groups of samples (Clarke, 1993). The software PAST (version
224 2.17c) (Hammer *et al.*, 2001) and SPSS Statistics were used for the statistical analysis of
225 DGGE profiles.
226
227 **Results**
228 <u>Culture based analysis</u>
229 The average concentration of APC in the sushi immediately after production (t_0) was 3.8 log
230 CFU g^{-1} (n=3, SD = 0.11). The estimated duration of the lag-phase by the primary model of

231 Baranyi and Roberts was 54 ± 8 h for APC in the sushi stored at 4 °C, whereas no lag-phase
232 was observed for the other temperatures (8 to 20 °C) (Figure 1). The exponential growth
233 phase ended with APC counts around 7-8 log CFU g^{-1} for all temperatures, and the carrying
234 capacity (Y_{max}) of the system was 7.7 to 9.7 log CFU g^{-1} (Table 1). The only exception was
235 growth at 4 °C which did not display a distinct exponential phase, and hence there was no
236 flattening of the curve during the experiment.

237 H_2S-producing bacteria were detected in two out of three samples at t_0, with a
238 concentration of 1.0 log CFU g^{-1} in both samples (equal to the detection limit). Refrigerated
239 storage resulted in no detectable growth of H_2S-producing bacteria, whereas no significant
240 lag-phase was observed for the growth at other temperatures (> 4°C). The only exception
241 were growth at 12 °C where a lag-phase of 14 ± 5 h were observed.

242 The average concentration of LAB at t_0 was 2.9 log CFU g^{-1} (n=3, SD = 0.05). A lag-
243 phase of 44 ± 10 h was estimated for LAB at 4 °C, while no lag-phase was observed for the
244 other temperatures. The estimated theoretical minimum temperature of growth (T_{min}) for
245 APC, LAB and H_2S-producing bacteria were -5.9, -7.5 and -1.9 °C, respectively (Table 2).

246 Maximum specific bacterial growth rates (μ_{max}) were calculated for APC, LAB and H_2S-
247 producing bacteria. The square root model described well μ as a function of storage
248 temperature for all three bacterial groups (Figure 2). LAB and APC had a higher μ at low
249 temperatures (4-12 °C) compared to the H_2S-producing bacteria (Figure 2). However, the
250 H_2S-producing bacteria demonstrated the strongest response to increased storage
251 temperature, as indicated by their temperature coefficients (Table 2). At 8 °C, which is not
252 an unlikely scenario through the value chain, we observed almost a 2-fold increase in the
253 specific growth rate of APC compared to an optimal storage temperature ($\mu_{4\,°C}$ = 0.06 h^{-1} and
254 $\mu_{8°C}$ = 0.11 h^{-1}). Storage at room temperature (20 °C) resulted in a 6-fold increase of the rate

($\mu_{20°C}$ = 0.37 h^{-1}). For LAB and H_2S-producing bacteria, the $\mu_{20°C}$ was 0.40 h^{-1} and 0.32 h^{-1}, respectively.

The declared shelf life of the sushi was three days after production. The counts of APC at expiration (72h) were 5.2, 6.6, 7.9, 9.3 and 9.1 log CFU g^{-1} at temperatures of 4, 8, 12, 16 and 20 °C, respectively. As a general indicator of the microbiological quality in RTE foods based on raw fish, values of APC above 6 to 7 log CFU g^{-1} is considered unsatisfactory (UK Health Protection Agency). Storage at 4 °C did not result in APC counts above the recommended limit during shelf life, whereas the other storage regimes resulted in values above the recommendation, and growth of H_2S-producing bacteria. The concentrations of these bacteria at expiration were 3.3, 4.2, 7.0 and 6.9 log CFU g^{-1} at temperatures of 8, 12, 16 and 20 °C, respectively. We did not assess the sensory spoilage of the sushi products. However, a strong unpleasant odor was sensed from sushi with APC above 8 log CFU g^{-1} (at 12, 16 and 20 °C).

Potentially harmful bacteria were quantified at t_0 and at expiration time (72h) at all temperatures. *L. monocytogenes*, *S. aureus* and *E. coli* were not detected in any sample. Presumptive *B. cereus* was detected in two out of three samples at t_0 (average 2.3 log CFU g^{-1}), and after 72 h at 16 and 20 °C (3.4 and 6.3 log CFU g^{-1}, respectively). Mesophilic *Aeromonas* spp. was analyzed at all sampling points for all temperatures, and was detected in sushi stored at 12 °C (48 h) and 20 °C (27 h and 72 h). The number of *Aeromonas* spp. quantified at 12 °C was equal to the detection limit of the method (1 log CFU g^{-1}). For storage at 20 °C, mesophilic *Aeromonas* spp. was detected after 27 h (2.9 log CFU g^{-1}) and increased to 5.5 log CFU g^{-1} at 72 h.

<u>PCR/DGGE analysis of bacterial community structures during storage</u>

279 The microbial communities associated with sushi samples stored at different temperatures
280 were also investigated by the cultivation independent method PCR/DGGE. Diversity indices
281 (band richness (eq. 2), dominance (eq. 3) and Shannon diversity index (eq. 4)) were
282 calculated based on the DGGE. Band richness and Shannon diversity index increased with
283 storage temperature and storage time, whereas dominance decreased. The three indices
284 were significantly different in the t_0 samples compared to the other samples for the variables
285 temperature and time ($P<0.05$). A simple main effect analysis showed that both time and
286 temperature had significant effects on band richness ($P=0.003$ and 0.013, respectively)
287 without a significant interaction effect ($P=0.509$). Moreover, band richness and Shannon
288 diversity index increased significantly with increasing concentrations of APC (Table 3). The
289 APC serves as a variable combining the two experimental variables temperature and time.
290 Principal Coordinate Ordination (PCO) based on Bray-Curtis similarities were done to
291 compare the bacterial community structure based on the DGGE profiles. When grouped
292 according to storage temperature (t_0 and 4, 8, 12, 16 and 20 °C), the samples clustered with
293 a large degree of overlap in the two-dimensional plot (Figure 3A). The ordination of samples
294 grouped according to storage time (days) generated a similar plot, but with less separation
295 of the groups on axis 1 (data not shown). Ordination of samples according to APC (log CFU g^{-1})
296 were well separated (Figure 3B), indicating that dissimilarity can be explained by this
297 variable which is an integration of both temperature and time. In all cases, 44.4 % of the
298 variance was explained in the two-dimensional plot (28.5 and 15.9 % on axis 1 and 2,
299 respectively).
300 A one-way PERMANOVA was used to test for differences in the bacterial community
301 structure between the temperature groups using Bray-Curtis similarities, and demonstrated
302 significant differences between the t_0 samples and the sushi stored at 12 °C ($P=0.013$), 16 °C

303 (P=0.019) and 20 °C (P=0.020). There were also significant differences between samples
304 stored at 4 °C and 12 °C (P=0.030), and at 16 °C (P=0.016) and 20 °C (P=0.008). No significant
305 differences were observed with respect to storage time only. The only exceptions were the
306 expired sushi (4 days storage) stored at 4 and 8 °C, that were significantly different from the
307 t_0 samples (P=0.033). As indicated by the PCO, the community structure in sushi with an APC
308 < 6 log CFU g^{-1} was significantly different from sushi with an APC > 6 log CFU g^{-1} (P<0.05). In
309 fact, there were significant differences between all the groups of bacterial concentrations,
310 with the exception of the 4 to 6 log CFU g^{-1} group which was not significantly different from
311 the <4 log CFU g^{-1} group. The CABFAC factor analysis verified that the bacterial community
312 composition was more strongly correlated to the APC than to the storage time and
313 temperature (R^2 of 0.941 versus 0.803 and 0.891 for storage time and temperature,
314 respectively). Moreover, there was a linear relationship between the reconstructed and
315 measured APC, with the intercept not significantly different from zero (0.39 ± 0.302), and
316 the slope not significantly different from 1 (1.00 ± 0.012).
317
318 Identification of bacterial community members and their importance to differences between
319 samples
320 A total of 36 bands were excised from the DGGE gel, and 27 were successfully sequenced.
321 Out of these, 10 were identified as chloroplast genes, and excluded from further analysis.
322 After removal of the bands representing chloroplasts, the remaining bands were assigned to
323 27 band classes (representing a unique vertical position at the gel). A SIMPER (Similarity
324 Percentage) analysis based on Bray-Curtis similarities was done to evaluate how much each
325 band class contributed to the observed differences between the DGGE profiles (Table 4). The
326 SIMPER scores for profiles grouped according to storage temperature, storage time and APC

(log CFU g^{-1}), demonstrated that four bands accounted for nearly 50 % of the differences between the profiles (data for the storage time and APC not shown in the table). Three out of these bands were identified as *B. thermosphacta.* Sequences from excised bands were used to identify the sushi microbiota related to different storage regimes (Table 4). The dominating organism was *B. thermosphacta,* which was detected in all samples. The identified microbiota was dominated by Gram positive bacteria. The only Gram negative bacterium identified was *Psychrobacter* spp., which appeared after 72h at 4°C, 48h at 8 °C and in earlier samples (before 39 h) stored at higher temperatures. *Weisella* sp. was detected in all samples stored at ≥ 8 °C. Other identified bacteria were lactic acid bacteria (*Enterococcus* sp., *Carnobacterium* sp. and *Lactobacillus* sp.), which were all absent in the t_0 samples.

Discussion

Based on previous findings (Hoel *et al.*, 2015), the present study was conducted to assess the effects of poor temperature control during the chilled food chain on bacterial concentrations, growth rates and community structure in retail sushi. Instead of suggesting possible time-temperature scenarios, the present study was conducted with isothermal storage temperatures over a wide range. An understanding of the bacterial growth rates, in particular those of the spoilage organisms, is important to assess the spoilage of the product. To our knowledge, this is the first study published on the microbiological quality and development of the bacterial community structure in retail sushi during storage.

The spoilage rate of fish and shellfish is highly temperature dependent and can be inhibited by the use of cold storage to reduce bacterial growth by increasing the bacterial lag phase and thus improving the shelf life (Sivertsvik *et al.*, 2002). The declared maximum

storage temperature of the sushi was 0 to 4 °C, and our data demonstrated that refrigeration (≤ 4°C) is an efficient hurdle to suppress bacterial growth during shelf life, particularly for the H_2S-producing organisms associated with spoilage of chilled air stored fish (Gram and Huss, 1996). The observed growth response at storage at 8 °C and above strongly indicated that poor temperature control causes loss of microbiological quality in sushi during its shelf life. A temperature of 8 °C is not unlikely during production, distribution or display of the sushi in stores, especially during the summer time. We were not able to calculate the growth rates of possible pathogens during storage at different temperatures because of the low counts of these bacteria in the products. However, our data demonstrated that a square root model can be used to describe the effect of temperature on the growth of APC, LAB and H_2S-producing bacteria in sushi within a temperature range of 4 to 20 °C. There was no significant lag phase for bacterial growth at temperatures above 4 °C, and our data is a good first possibility to simulate growth for the three bacterial groups under variable temperature scenarios that might occur during production, distribution or storage.

PCR/DGGE analysis of the bacterial community structure was conducted to evaluate how it was affected by the storage temperature. We were not able to sequence all the excised bands, but the SIMPER analysis demonstrated that we did identify three of the four band classes that contributed to nearly 50 % of the observed differences between samples grouped according to storage temperature. A limitation of DGGE is the presence of multiple bands representing one species, which contributes to an overestimation of the bacterial diversity (Ercolini, 2004). We detected four different band classes representing *B. thermosphacta*. One of these bands was absent in t_0, 4 °C and early 8 °C samples, two of these bands were absent in t_0 samples, whereas one band was present at all temperatures.

375 To get a more comprehensive picture of the microbiota, a DGGE approach combined with a
376 culture dependent identification of colonies could be applied. However, the choice of a
377 growth media for the quantification of total aerobic bacteria from complex food matrixes is
378 not straight forward. Broekaert *et al*. (2011) pointed out the limitations of several growth
379 media used in seafood research. Of particular interest, they found that *Brochothrix* were not
380 able to grow on iron agar. Thus, the number of APC reported in our study might be
381 underestimated. Nevertheless, the aim of our study was to assess the effect of different
382 storage regimes on the overall community structure. We have seen that DGGE and 454-
383 pyrosequencing reveal overall the same changes in community structure, despite differences
384 in resolution (unpublished results).
385 Because sushi contains vegetable ingredients, we did experience some
386 contamination with plant organelles, which precludes the use of universal rRNA bacterial
387 primers. Chloroplast and mitochondrial contamination in DNA samples from plant
388 environments can contribute to an overestimation of diversity. Mitochondrial 18S rRNA
389 genes and chloroplast 16S rRNA share a high sequence similarity with bacterial 16S rRNA
390 sequences (Sakai *et al.*, 2004), but the 18S rRNA genes were excluded by the use of a nested
391 PCR strategy. Moreover, it is important to exclude chloroplast bands from community
392 structure analysis.
393 Grouping DGGE samples according to storage time and temperature, we observed
394 large variations within groups and a large degree of overlap between the groups. However,
395 there were significant differences between the start samples (t_0) and the high temperature
396 groups (12-20 °C), and between the 4 °C samples and the high temperature groups ($P<0.05$).
397 When the DGGE profiles were ordinated based on the APC, samples with low (< 6 log CFU g^{-1})
398 and high (> 6 log CFU g^{-1}) APC clustered without overlap, and statistical PREMANOVA

399 analysis confirmed significant differences between the community structures of samples
400 with high and low CFU g^{-1}. The CFU g^{-1} of APC is an explanatory variable that takes into
401 account both temperature and time. Moreover, in the CABFAC factor analysis we
402 reconstructed better the APC (log CFU g^{-1}) than the variables time and temperature from the
403 bacterial community composition. Storage time and temperature are factors that determine
404 how fast the community structure changes, but eventually it converged towards a similar
405 bacterial community structure. Altogether, the analyses of the bacterial communities
406 suggest that APC can serve as an indicator of the storage temperature history of the product.
407 Sushi is a combined product consisting of multiple ingredients with varying degrees
408 of processing. The raw fish is the most perishable ingredient, and the spoilage potential of
409 the sushi is related to bacterial species that are already present in the fish muscle at the time
410 of product assembly. The species present originate from different processing steps, such as
411 fish farming, processing, transportation, and storage conditions (Comi, 2017). Moreover,
412 bacteria are introduced by other ingredients such as raw vegetables (Hoel *et al.*, 2015). The
413 analyzed sushi in our study was made with farmed Atlantic salmon and halibut. The diversity
414 of sushi ingredients is large, and hence also the possible combination of spoilage bacteria. In
415 the present study, the sequence analysis of excised DNA bands from the DGGE gel
416 demonstrated that *B. thermosphacta* was present in all samples, and with multiple bands. *B.*
417 *thermosphacta* is an important spoilage organism in fish, for example in MAP stored Atlantic
418 halibut (Hovda *et al.*, 2007) and meat (Doulgeraki *et al.*, 2012). Several studies have reported
419 its spoilage potential, both in products stored in air and in modified atmosphere (Ercolini *et*
420 *al.*, 2006; Mikš-Krajnik *et al.*, 2016). *B. thermosphacta* is commonly found in fish with low
421 levels of trimethylamine n-oxide (TMAO), such as salmon (Ryder *et al.*, 2014). Furthermore,
422 we found that the microbiota in the sushi was dominated by LAB species. Jaffres *et al*. (2011)

423 reported that *B. thermosphacta* and LAB, mainly represented by the genera *Carnobacterium*,
424 *Vagococcus* and *Enterococcus,* dominated the microbiota of cooked and peeled shrimps in
425 modified atmophere. *B. thermosphacta* and LAB were also identified as the dominating
426 spoilage organisms in cold stored MAP salmon (de la Hoz *et al.*, 2000; Rudi *et al.*, 2004), and
427 Macé *et al*. (2012) demonstrated that MAP raw salmon microbiota were dominated by LAB,
428 *Pseudomonas* and *Photobacterium phosphoreum* at different storage temperatures.
429 Fewer studies have been published on the temperature dependent composition of
430 seafood microbiota stored in normal atmosphere. The effect of storage temperature on the
431 final composition of the spoilage microbiota in shrimps was studied by Dabadé and
432 colleagues (2015). They found that H_2S-producing bacteria, mainly represented by LAB and
433 Enterobacteriaceae, dominated during storage at 28 and 7 °C, whereas *Pseudomonas* spp.
434 were dominant at 0 °C. Shamshad *et al*. (1990) showed that the dominant microorganisms in
435 shrimps were *Moraxella* spp. at low storage temperatures (0-10 °C) and *Vibrio* spp. at high
436 storage temperatures (15-35 °C). Parlapani and Boziaris (2016) described the dominant
437 spoilage organism in whole sea bream stored at low temperatures (0-5 °C) to be different
438 from the dominating organism in fish stored at 15 °C.
439 Our culture dependent analysis revealed a high concentration of LAB in the sushi, but
440 the significance of these results is not clear. While some LAB might contribute to spoilage,
441 others have no impact on the food quality (Leroi, 2010). The different LAB species identified
442 by the PCR/DGGE analysis were not detected in the t_0 samples, whereas they were present
443 in the majority of stored samples. This is typical for spoilage, as the SSOs often consist of a
444 single or a few microbial species, whereas the microbiota found in a product after some time
445 of storage typically include several groups of bacteria (Gram and Dalgaard, 2002). Based on
446 the dominating species identified in our experiment, it is possible that the low pH in the

447 product drives the selective pressure towards a more MAP-like fish microbiota, typically
448 consisting of LAB, Enterobacteriaceae and *Brochothrix* spp. (Lyhs *et al.*, 1998; Joffraud *et al.*,
449 2001; Gram and Dalgaard, 2002).

450 Culture dependent as well as culture independent analysis of potentially harmful
451 bacteria (*S. aureus*, *L. monocytogenes*, *E. coli*, *B. cereus* and mesophilic *Aeromonas* spp.)
452 indicated that they were not a food safety problem in the analyzed sushi. To maintain safe
453 products, the raw materials must be of high quality. We were not able to demonstrate
454 growth of pathogens at temperatures corresponding to refrigeration. *Aeromonas* spp. were
455 able to grow to potentially disease-causing levels only at 20 °C. We have previously reported
456 relatively high numbers of *Aeromonas* spp. in Norwegian retail sushi (more than 4 log CFU g^{-1}
457 in some samples) (Hoel *et al.*, 2015), and it is not unlikely that the growth potential would be
458 different if the pathogen initially was present in higher numbers. The growth potential of
459 the pathogen *Aeromonas* spp., which is also known to grow during refrigeration, should be
460 further explored.

461 In conclusion, the relationship between bacterial growth rates and temperature
462 clearly demonstrates that these products are sensitive to deviations from optimal storage
463 temperature, possibly resulting in the loss of quality during shelf life. Moreover, our data
464 serve as a starting point to simulate the growth of different groups of bacteria under
465 different temperature scenarios in sushi. Regardless of the storage temperature, the
466 bacterial communities converged towards a similar structure and density, but the storage
467 temperature determined how fast the community reached its carrying capacity. Thus, the
468 CFU g^{-1} of APC can be used as a predictor of the previous storage history of sushi. The sushi
469 spoilage microbiota was dominated by the Gram positive bacteria *B. thermosphacta* and
470 genera of LAB.

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Conflict of interest

The authors declare no financial or ethical conflicts of interests regarding the submitted manuscript. All the authors have agreed with the submission.

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Tables

Table 1 Temperature dependent growth kinetic parameters (lag phase duration (h), maximum specific growth rate (μ_{max}, h^{-1}), carrying capacity (Y_{max}, log CFU g^{-1}) (± SE) of total aerobic plate count (APC), lactic acid bacteria (LAB) and H_2S-producing bacteria estimated from the primary model of Baranyi and Roberts in sushi stored at 4 to 20 °C.

Temp (°C)	Lag phase (h)	μ_{max} (h^{-1})	Carrying capacity, Y_{max} (log CFU g^{-1})	R^{2*}	SE (fit)†
APC					
4	54.3 ± 8.5	0.060 ± 0.015‡	ND§	0.703	1.453
8	ND	0.110 ± 0.012	7.74 ± 0.01	0.952	0.720
12	ND	0.163 ± 0.015	8.51 ± 0.01	0.976	0.633
16	ND	0.230 ± 0.023	9.72 ± 0.33	0.964	0.876
20	ND	0.368 ± 0.035	8.86 ± 0.13	0.975	0.637
LAB					
4	44.1 ± 10.6	0.083 ± 0.017	ND	0.786	1.634
8	ND	0.119 ± 0.012	7.19 ± 0.25	0.962	0.670
12	ND	0.189 ± 0.018	7.72 ± 0.18	0.976	0.620
16	ND	0.268 ± 0.030	8.37 ± 0.23	0.965	0.819
20	ND	0.398 ± 0.054	8.49 ± 0.21	0.950	0.994
H_2S-prod.					
4	NA**	NA	NA	NA	NA
8	ND	0.054 ± 0.012	ND	0.788	0.923
12	14.2 ± 5.0	0.131 ± 0.009	ND	0.986	0.420
16	NS††	0.252 ± 0.024	7.05 ± 0.27	0.983	0.637
20	ND	0.318 ± 0.022	7.02 ± 0.16	0.987	0.566

* R2: Coefficient of determination
† SE (fit): Standard error of fit
‡The linear model was the best fit
§ND: Not detected
**NA: Not analyzed due to no growth
††NS: No significant lag phase detected

Table 2 Parameters of the secondary square-root type model for the effect of temperature on the growth rates of total aerobic plate count (APC), lactic acid bacteria (LAB), and H_2S-producing bacteria (H_2S) in sushi, where b (± SE) is the slope of the regression line, T_{min} (±SE) is the theoretical minimum temperature for growth, and R^2 represents the fit of the model.

	b	T_{min} (°C)	R^2
APC	0.023± 0.002	-5.9 ± 1.8	0.985
LAB	0.023± 0.001	-7.5 ± 1.4	0.991

H_2S	0.026 ± 0.004	-1.9 ± 2.6	0.970

Table 3 Average diversity indices (±SD) for the sushi with different concentrations of aerobic plate count (APC). Different superscript letters indicate significant differences (P< 0.05) based on ANOVA and multiple comparison by Tuckey test. n=4 for <4, n=7 for >4<6, n=10 for ≥6<8, n=9 for ≥8 log CFU g^{-1}.

Log APC CFU g^{-1}	Band richness	Dominance	Shannon
< 4	7.8 ± 0.5^{a}	0.36 ± 0.07^{a}	1.3 ± 0.2^{a}
>4<6	10.4 ± 1.7^{b}	0.25 ± 0.07^{b}	1.7 ± 0.2^{b}
≥6<8	14.9 ± 1.1^{c}	0.19 ± 0.04^{bc}	2.0 ± 0.2^{c}
≥8	15.8 ± 1.5^{c}	0.14 ± 0.02^{c}	2.2 ± 0.1^{d}

Table 4 Taxonomic assignments of excised DGGE bands arranged in descending order of contribution to the differences between DGGE profiles grouped according to storage temperature, as represented by the SIMPER (Similarity percent) score. Band classes with SIMPER scores less than 1.0 % were removed from the table as none of these were analyzed (B9, B17, B4, B3, B11, B24, B31, B2, B21).

Band class[*]	Storage temp. (°C)[†]	SIMPER score (%)[‡]	Closest relative in GenBank database (% similarity)	ENA given accession no.
B27 (n=3)	4,8,12	16.34	*Brochothrix thermosphacta* (100 %)	LT605064, LT605071
B26 (n=2)	4, 8	14.28	*Brochothrix thermosphacta* (100 %)	LT605063, LT605070
B25 (NS)[§]		9.71		
B10 (n=1)	8	9.06	*Brochothrix thermosphacta* (100 %)	LT605072
B1 (n=2)	12	8.67	*Weisella ceti* (99 %)	LT605066, LT605067
B16[**]				
B16-A (n=2)	8, 12	8.54	*Enterococcus durans* / *Enterococcus faecium* (98 %)	LT605065
B16-B (n=1)	16		*Carnobacterium* sp. (99 %)	LT605068
B16-C (n=1)	20		*Carnobacterium* sp. (98 %)	
B15 (n=2)	4, 8	5.66	*Psychrobacter* sp. (98 %)	LT605062, LT605069
B22 (NA)[††]		4.09		
B13 (NS)		4.00		
B14 (NS)		3.29		
B29 (n=1)	16	2.34	*Brochothrix thermosphacta* (99 %)	LT605073
B6 (n=1)	20	2.07	*Lactobacillus* sp. (100 %)	LC075530.1[§§]
B28 (NA)		1.91		

B20 (NA)		1.65		
B7 (NA)		1.56		
B12 (n=1)	20	1.45	*Lactobacillus* sp. (99 %)	FR692017.1
B8 (NA)		1.41		
B5 (NA)		1.05		

[*]After removal of bands representing chloroplasts, the remaining bands were assigned to 27 band classes and n = number of sequences successfully obtained from each band class

[†]The storage temperature that the actual band was excised from. The band might also be present at other temperatures

[‡]SIMPER (similarity percentage) score for DGGE profiles grouped after storage temperatures

[§]NS = No sequence obtained from that band class

[**]Four bands were analyzed from B16, which yielded three different sequence matches

[††] NA = Not analyzed (no DNA excised)

[§§]Due to the short nature of two sequences, these were not deposited in a public database. Instead, the Acc. No. of their closest relative from the GenBank database is given in the table.

Figure legends:

Figure 1 Log CFU g^{-1} of A) total aerobic plate count, B) Lactic acid bacteria, and C) H_2S-producing bacteria in sushi during storage at temperatures 4 °C (○), 8 °C (▲), 12 °C (x), 16 °C (■), and 20 °C (●).

Figure 2 Specific growth rate (μ) in sushi as a function of storage temperature for total aerobic plate count (●), lactic acid bacteria (▲), and H_2S-producing bacteria (■). Notice square root transformation of μ-axis. The lines are fit of the square root model to the data (see Table 2 for parameters).

Figure 3 Principal coordinate ordination (PCO) based on Bray-Curtis similarities for bacterial communities in sushi, where each point represents an individual DGGE sample. Coordinates on Axis 1 and 2 represent 28.5 % and 15.9 % of the observed variance, respectively. The solid lines represent the convex hulls of each group. A) Samples grouped according to storage temperatures; t_0 (●), 4 °C (+), 8 °C (□), 12 °C (■), 16 °C (Δ) and 20 °C (○). B) Samples grouped according to level of APC; < 4 log CFU g^{-1} (+), 4-6 log CFU g^{-1} (□), 6-8 log CFU g^{-1} (■), and ≥ 8 log CFU g^{-1} (Δ).

Figure 1

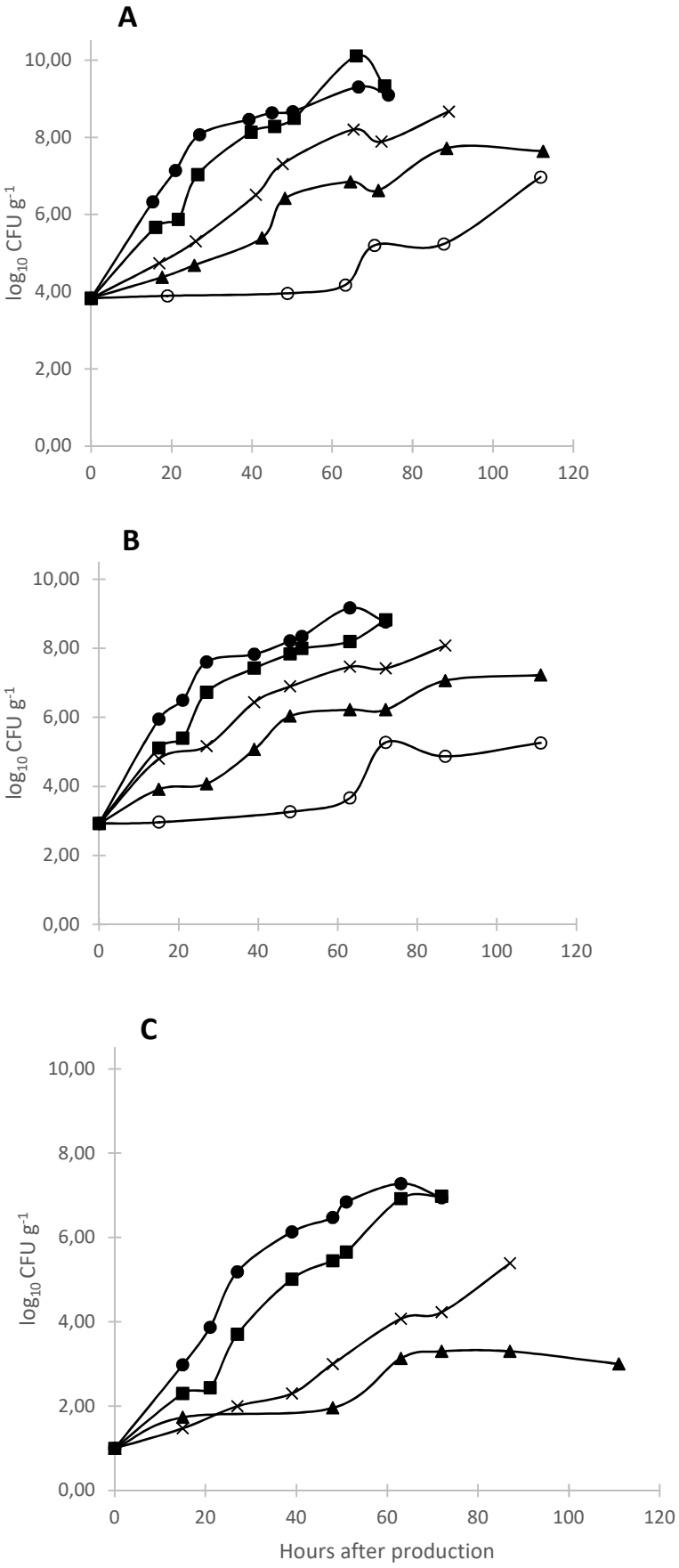
A
B
C
log10 CFU g-1
10,00
8,00
6,00
4,00
2,00
0,00
0
20
40
60
80
100
120
Hours after production

Figure 1 Log CFU g^{-1} of A) total aerobic plate count, B) Lactic acid bacteria, and C) H_2S-producing bacteria in sushi during storage at temperatures 4 °C (○), 8 °C (▲), 12 °C (x), 16 °C (■), and 20 °C (●) .

Figure 2

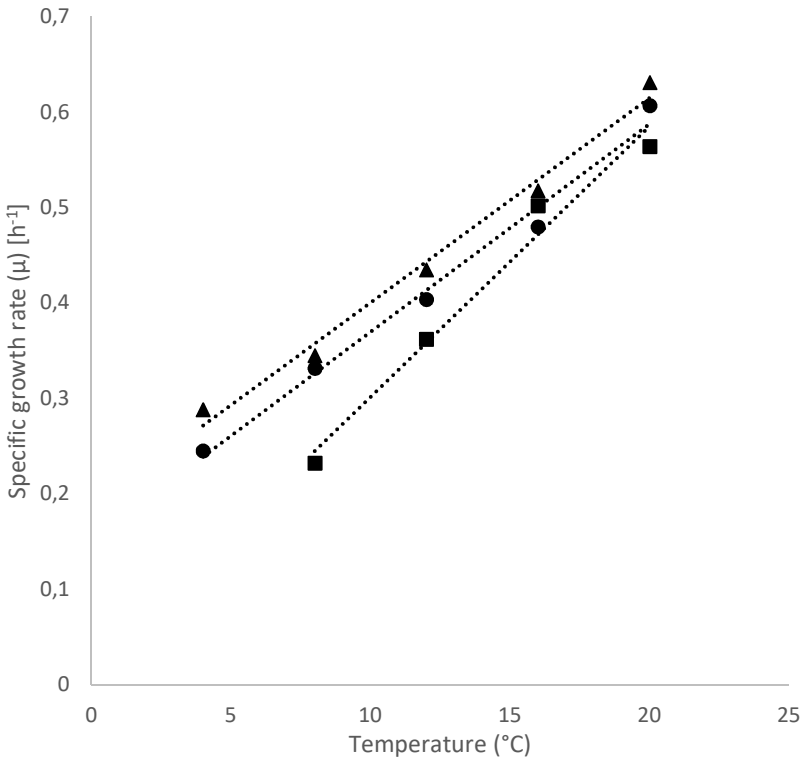

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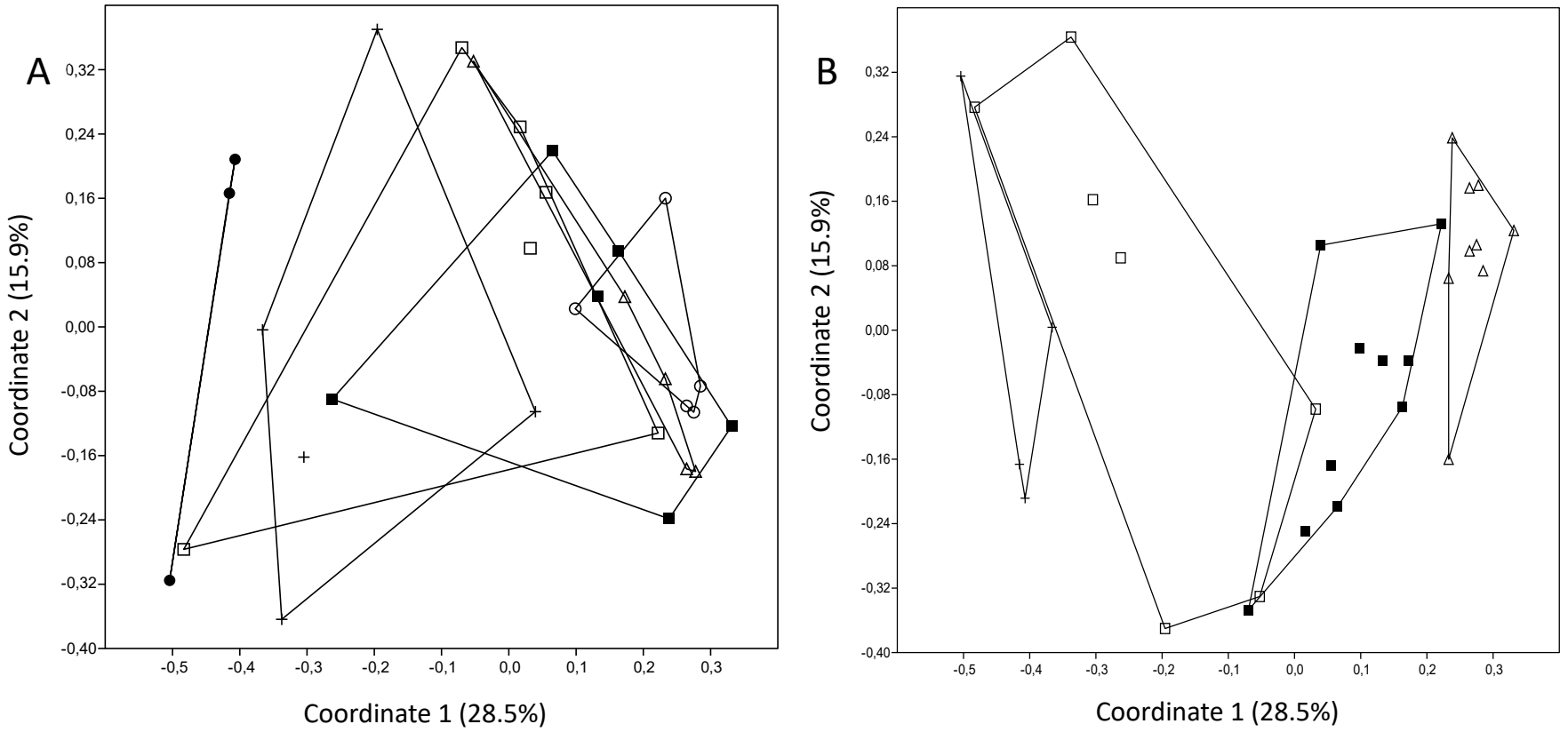
Figure 3



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