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Development of a real-time PCR method coupled with a selective pre-enrichment step for quantification of *Morganella morganii* and *Morganella psychrotolerans* in fish products

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Abstract :

Histamine fish poisoning is common and due to toxic concentrations of histamine often produced by Gram-negative bacteria in fin-fish products with a high content of the free amino acid histidine. The genus *Morganella* includes two species previously reported to cause incidents of histamine fish poisoning. *Morganella morganii* and *Morganella psychrotolerans* are both strong producer of histamine. However, little is known about the occurrence and critical stages for fish contamination with these bacteria. To elucidate contamination routes of Morganella, specific real-time quantitative PCR (RTi qPCR) methods for quantification of *M. morganii* and *M. psychrotolerans* have been developed. Selective primers amplified a 110 bp region of the vasD gene for *M. psychrotolerans* and a 171 bp region of the galactokinase gene for *M. morganii*. These primer-sets showed high specificity as demonstrated by using purified DNA from 23 other histamine producing bacteria and 26 isolates with no or limited histamine production. The efficiency of the qPCR reactions on artificially contaminated fish samples were 100.8% and 96.3% respectively. The limit of quantification (LOQ) without enrichment was 4 log CFU/g. A quantitative enrichment step with a selective medium was included and improved the sensitivity of the methods to a LOQ of below 50 CFU/g in seafood. RTi qPCR with or without enrichment were evaluated for enumeration of *Morganella* species in naturally contaminated fresh fish and lightly preserved seafood from Denmark. These new methods will contribute to a better understanding of the occurrence and histamine production by *Morganella* species in fish products, information that is essential to reduce the unacceptably high frequency of histamine fish poisoning.

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Highlights

► Quantification of *Morganella* species in fish products by RTi qPCR ► Sensitivity of the assay was improved with a quantitative selective enrichment step to 50 CFU/g ► The assays accurately quantified *Morganella* in fresh and lightly preserved fish ► Development of a specific tool to quantify main histamine producing bacteria in fish

Keywords : histamine-producing bacteria, tuna, selective medium, Enterobacteriaceae, galactokinase, typeVI secretion system

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1. Introduction

Histamine fish poisoning (HFP) is common worldwide and due to consumption of a range of dark-fleshed fin-fishes (Dalgaard *et al.*, 2008). Between 2008-2010, HFP was responsible for 51% of all seafood related outbreaks of disease in France and in Europe and for 20% in United States (CDC, 2011; Helwigh *et al.*, 2012; InVS, 2011). HFP is typically caused by consumption of fish muscle containing high concentration of histamine (> 500 mg/kg) which can be observed in fish species with high level of histidine such as Scombridae (tuna, mackerel...), Clupeidae (herring, sardine...) and other species like *Coryphaena hippurus* (mahi-mahi) or *Belone belone* (garfish) (Hungerford, 2010). HFP is a relatively mild illness with allergy-like symptoms that appear some minutes to few hours after consumption of the food. They are mainly characterized by rash, diarrhea, nausea, headache, flushing and sweating (Prester, 2011).

Histamine in fish flesh is produced by decarboxylation of free histidine by bacterial decarboxylase. Gram-negative marine and enteric bacteria have been identified as the main bacterial groups responsible for HFP in fish products (Bjornsdottir *et al.*, 2009; Dalgaard *et al.*, 2008; Veciana-Nogués *et al.*, 2004). Histamine producing bacteria (HPB) can be sub-divided into low and high producers of histamine based on the formation of histamine in a broth culture medium containing histidine. The high histamine producers include mesophilic species such as *M. morganii*, *Enterobacter aerogenes*, *Hafnia alvei*, *Raoultella planticola* and *Photobacterium damselae*, which are able to produce more than 1000 mg/l of histamine in tryptone soy broth supplemented with 2% histidine after 24-48 h incubation at temperatures above 15°C (Bjornsdottir *et al.*, 2009). High histamine-producing and psychrotolerant bacteria have also been described, including *Photobacterium phosphoreum* (Kanki *et al.*, 2004) and *M. psychrotolerans* (Emborg *et al.*, 2006). Both species produce histamine at low temperature until 0°C in fish products (Dalgaard *et al.*, 2006; Emborg *et al.*, 2005).

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Whereas all *Morganella* isolates seem to be strong histamine producers, intra-species variability exists for other species including *P. phosphoreum* (Dalgaard *et al.*, 2006; Emborg et al. 2005; Kim *et al.*, 2002; Klausen *et al.*, 1987). Both *M. morganii* and *M. psychrotolerans* have been isolated from fish products (fresh tuna, tuna in sauce, cold-smoked tuna) responsible for outbreaks (for a review, see Dalgaard *et al.*, 2008). Despites those facts, there is a lack of data on prevalence of *Morganella* spp. in fish products, probably due to the absence of sufficiently sensitive and specific enumeration method. Previous experiments of detection of HPB have been performed on differential media (Mavromatis and Quantick, 2002; Tao *et al.*, 2009) based on color modification of pH indicators once histidine is converted to histamine during bacterial growth. However, these methods are time consuming and unreliable (Bjornsdottir *et al.*, 2009). Nowadays, molecular methods based on RTi qPCR are more reliable for detection, identification and quantification of bacteria but their lack of sensitivity when used with food products remains a problem (Postollec *et al.*, 2011). Bjornsdottir-Butler *et al.* (2011a) developed a RTi qPCR method based on the primers of Takahashi *et al.* (2003) designed on the histidine decarboxylase gene to quantify Gram-negative and high HPB. That method has been set-up for mesophilic species but cannot detect *P. phosphoreum* or *M. psychrotolerans* (experiments in our laboratory, data not shown). More recently, RTi qPCR methods for quantification of *M. morganii* (Ferrario *et al.*, 2012a, 2012b) and viable *P. phoshoreum* (Macé *et al.*, 2013) in fish products have been proposed. However, in those studies the detection limit of RTi qPCR was typically between 3-4 log CFU/g in food products. This limit is too high to study the occurrence of *Morganella* spp. in fish products in which expected contamination level is most likely less than 100 CFU/g. To overcome this problem of the sensitivity of RTi qPCR methods, enrichment steps with selective media have been proposed, allowing quantification of 1-10 CFU/g for e.g. the pathogenic bacteria *Listeria*

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monocytogenes (O' Grady *et al.*, 2008) and *Salmonella* (McGuinness *et al.*, 2009) in different food products.

The objectives of the present study were to develop two specific and sensitive RTi qPCR methods for quantification of *M. morganii* and *M. psychrotolerans* in fish products. Selective primers for both species have been designed and evaluated against isolates of HPB and non-HBP isolates. These RTi qPCR methods used in combination with a new enrichment step reduced the LOQ and increase the methods field of application for fish products.

2. Materials & methods

2.1. *Bacterial strains and pre-culturing*

Bacterial strains used in this study are listed in Table 1. Strains were grown in Brain Heart Infusion (BHI, Biokar Diagnostics, Beauvais, France) at 20°C during 24 h, for *Morganella* strains, and during 24-48 h for other strains, except for *P. phosphoreum* which was cultivated at 15°C in BHI with 2% NaCl. The strains were stored at -80°C in their culture medium with 10% glycerol.

2.2. *DNA extraction*

For bacterial cultures, DNA extraction was performed on 1.5 ml cultures that have reached a concentration of at least 8 log CFU/ml. After centrifugation during 10 min at 8500 x g, the chromosomal DNA of all bacterial isolates was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, S.A., Courtaboeuf, France).

The DNA extraction on fish tissue was adapted from a protocol developed for raw salmon to quantify *P. phosphoreum* by RTi qPCR (Macé *et al.*, 2013). Briefly, 30 g portion of seafood samples (tuna, mackerel or herring) was aseptically weighed in a sterile stomacher bag and 5-fold diluted with sterile peptone-salt water (0.1% peptone, 0.85% salt). Ten milliliters of

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homogenized suspension were filtered on a Nucleospin Filter L (Macherey-Nagel, Hoerdt, France). The following extraction of DNA was performed as described by Macé *et al.* (2013). DNA was purified using the DNeasy Blood & Tissue Kit as described in the Qiagen instruction manual.

2.3.Genomics data and primer design

Shotgun sequencing was performed in Denmark using Roche FLX 454 pyrosequencing on DNA from the *M. morganii* strain U6/1 and on DNA from the type strain of *M. psychrotolerans* U2/3^T = LMG 23374^T = DSM 17886^T (Emborg *et al.*, 2006; Meyer *et al.*, 2008). Sequencing was done using the FLX Titanium sequencing kit and 1 region of an XLR70 pico titre plate per strain. Contigs for each strain were assembled using the Newbler assembler software version 2.0.01.14 provided with the GS FLX instrument and annotated by using the RAST annotation server (Aziz *et al.,* 2008). Genome sequencing and assembly of the *M. morganii* U6/1 and *M. psychrotolerans* U2/3^T resulted in 51 and 28 x coverage on 3,9 Mb and 4,2 Mb size genomes, respectively. Genomes were assembled into 123 (*M. morganii* U6/1) and 292 (*M. psychrotolerans* U2/3^T) large contigs (>500 bp).

Thirty primers pairs were designed for *M. psychrotolerans* and *M. morganii* using the Geneious Software (Geneious version 6.1, Biomatters Ltd.) based on the Primer3 calculation method (Untergasser *et al.*, 2007) and the Primer-Blast software (NCBI, UK). Primers pairs were initially evaluated *in silico* by using the nucleotide Blast program to check their specificity for *M. morganii* or *M. psychrotolerans* against all genomic data of GenBank (NCBI, UK) and tested *in vitro* as described in 2.4 using appropriate hybridization temperatures. The selected primers VasD-F4 (5'-AAATCGCCATCACACTCCTTG-3') and VasD-R4 (5'-TTCAAAACGGGAGTCCTCACTG-3') were designed on the *vasD* gene from the Type VI secretion system of *M. psychrotolerans*. This primer set matched respectively

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position 146-166 and 234-255 of the *M. psychrotolerans* U2/3^T *vasD* gene (Genbank accession number KP069481). For *M. morganii*, the primers GalK-F4 (5'-ACAGTGCTTCGGCGCATCCC-3') and GalK-R4 (5'-GCAGCCACCACGCAGACCTT-3') were obtained on the galactokinase gene (*galK*) and matched respectively position 39-58 and 190-209 of the galactokinase gene of *M. morganii* U6/1 (Genbank accession number KP069480).

2.4. *Real-time PCR amplification*

Inclusivity and exclusivity of primers (TAG Copenhagen, Denmark or Invitrogen, Illkirch, France) designed for *M. morganii* and *M. psychrotolerans* were tested on bacterial DNA extracted from the strains listed in Table 1. Genomic DNA was measured using a Nanovalue (Applied Biosystem, Saint-Aubin, France) and diluted to 4 ng/µl. Specificity of the RTi qPCR assay was tested using 4 ng of DNA.

Real-time qPCR was conducted in a 15 µl reaction volume using the following reaction mixture: 1 U of Iq SYBR® Green Supermix (Biorad, Hercules, US), 300 nM of each VasD or GalK forward and reverse primers, nuclease free H_2O and 1 µl of DNA template. Real-time PCR cycling was performed using a CFX-96 instrument (Biorad, Marnes-la-Coquette, France) or a Mx3000P thermocycler (Stratagene, AH Dianostics, Aarhus, Denmark). The cycling parameters were: 95°C hold for 180 s for initial denaturation and activation of the hot-start polymerase, followed by 40 cycles of amplification of 95°C for 15 s, 60°C for 30 s for *M. morganii*, or 62°C for 30 s for *M. psychrotolerans*. Fluorescence was read at the end of each amplification cycle. At the end of the 40 cycles, a melting curve was conducted between 55°C and 95°C with a 0.5°C/5 s increment read. The cycle threshold (C_T) value was determined using a background limit of 0.02.

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To obtain standard curves relating C_T-values and cell concentrations (log CFU/g), ten-fold serially dilutions of a culture of the type strains *M. psychrotolerans* U2/3^T and *M. morganii* LMG7874^T = CIP A231^T (Table 1) were inoculated on pieces of canned tuna flesh. DNA extraction was performed on each piece of tuna inoculated with decreasing known concentrations of each strain between 2 to 8 log CFU/g. Specific RTi qPCR was performed as described above and enumeration of each strain was made on BHI agar incubated 24 h at 20°C.

For quantification in naturally contaminated products a positive control was made with DNA extracted as described in section 2.2 from 30 g of fish inoculated with *M. morganii* LMG7874^T or *M. psychrotolerans* U2/3^T (9 log CFU/g). Negative controls consisted in DNA extracted from canned tuna and sterile water.

Development of a Morganella *enrichment (MoE) medium*

To improve sensitivity of the RTi qPCR methods, enrichment steps were added. A selective enrichment medium was developed to allow optimal growth of *Morganella* species and to inhibit most of the other HPB. High histamine-producer *M. psychrotolerans* U2/3^T, F39-1, JB-T11, *M. morganii* LMG7874^T, DSM14850^T, 03A11, *R. planticola* DSM3069^T, *E. aerogenes* LMG2094^T, *K. oxytoca* LMG3055^T and *H. alvei* DSM30163^T (Table 1) were selected to test the effect of 15 antibiotics and of the 2-deoxy-D-galactose substrate. This substrate was tested as the hexose analog can be lethal for microorganisms able to metabolize galactose (Alper and Ames, 1975). Tests were performed in honeycomb 2 microplates with 100 wells (Thermo Electron Oy, Vantaa, Finland), each well being filled with 300 µl of Nutrient Broth (NB, CM0001, Oxoid, Basingstoke, UK) with or without different concentrations of antibiotic (Table 2) or with 2-deoxy-D-galactose. NB was supplemented with 0.2 mg/ml of 2-deoxy-D-galactose and incubated with or without an overlay of sterile

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paraffin oil. Wells were inoculated with an overnight pre-culture of each strain at 25°C in NB, at a final concentration of 10^2 CFU/ml. A negative control was performed using non-inoculated NB broth. Growth was followed during 72 h at 25°C by absorbance measurement at 540 nm and with measurements every 20 minutes (Bioscreen C, Labsystem, Helsinki, Finland). Plates were shaked 10 s at medium speed before each measurement. Experiments were done in duplicate and growth data were analyzed by Excel (Microsoft Corporation, Redmond, US) to determine the growth inhibiting effect of the studied antimicrobials.

The final MoE medium was adapted from Emborg and Dalgaard (2008) and consisted of buffered Lucia-Bertani broth added L-histidine and colistin: 10.0 g/l bacto tryptone (211705, Becton and Dickinson Company, Sparks, MD, USA), 5.0 g/l yeast extract (212750, BD), 7.0 g/l KH_2PO_4 (1.04873, Merck, Darmstadt, Germany), 7.0 g/l K_2HPO_4 (1.05104, Merck) and 10.0 g/l L-histidine monohydrochloride monogydrate (Sigma, H8125). The autoclaved (121°C, 15 min) and chilled medium was added a filter sterilized solution of colistin-sulfate salt (Sigma, C4461) to a final concentration of 32 mg/l. pH was adjusted to 6.50.

2.5. Development of a quantitative enrichment procedure

To calculate the concentration of *M. psychrotolerans* or *M. morganii* in fish product from concentrations determined by RTi qPCR after enrichment in MoE, the maximum specific growth rate (μ_{max}) of each *Morganella* species in the MoE medium were determined. Pre-cultures (NB, 25 °C, 12 h) of, respectively, four *M. psychrotolerans* strains (MIX-Mp: U2/3^T, JB-T11, JB-T12, U2/5) or four *M. morganii* strains (MIX-Mm: 25a32, AP28, LMG7874^T, DSM14850^T) were mixed. Appropriate dilutions were inoculated in peptone-salt water, canned tuna and cooked shrimp to obtain a concentration of 250 CFU/ml or 250 CFU/g. Thirty grams of inoculated seafood were five-fold diluted with peptone-salt water and homogenized in a stomacher 400 (Seward Medical, London, UK). 1.00 ml of the homogenate

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was then transferred to 9.00 ml of MoE medium and growth was determined in duplicate by viable counting during storage at 10°C for *M. psychrotolerans* and at 37°C for *M. morganii*. These temperatures for specific incubation were selected for the two species based on the known effect of temperature on the growth rate of *M. psychrotolerans* and *M. morganii* (Emborg and Dalgaard, 2008). In the same way, the peptone-salt water solution with 250 CFU/ml was five-fold diluted and then 1 ml was inoculated in the MoE medium. Samples were removed for bacterial enumeration and DNA extraction after 0, 14, 24, 38, 49, 63 and 86 h for *M. psychrotolerans* and after 0, 1, 3, 4, 6, 7 and 9 h for *M. morganii*. Enumeration of *Morganella* was done on Tryptone Soya Agar (TSA, CMO131, Oxoid) at 25°C after 36 h of incubation. DNA extraction was done with 1 ml of MoE as described above (See 2.2). For each food matrix, the maximum specific growth rate (μ_{max}) of *M. psychrotolerans* and *M. morganii* in MoE was determined by fitting growth data using an exponential model (Eq. 1).

$$\text{Log}(N_t) = \text{Log}(N_0) + (\mu_{max} \text{ x } t)/\text{Ln}(10) \quad (1)$$

where N_0 and N_t are cell concentrations (CFU/ml) at time zero and at time *t*, respectively.

2.6. *Quantification of* Morganella *species in natural contaminated fish products by RTi qPCR*

The RTi qPCR methods were used to quantify *M. psychrotolerans* and *M. morganii* on different fish products from Denmark (Table 3). Tuna loins from the local fish market and herring provided by local fisherman were transported to the laboratory and kept overnight in ice before being processed and analysed. Tuna loins were cut in 8 pieces of 60 g and then vacuum packed. Herrings were eviscerated, tail and head were removed and filets were vacuum packed using a packaging film with low gas permeability. Frozen tuna steaks, hot smoked mackerel, cold-smoked herring and Matjes herring fillets bought in supermarket were already vacuum- or modified atmosphere packed. The fresh products were then stored for one

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week at 2°C whereas previously frozen tuna and lightly preserved products were kept at 10°C. The products were analyzed in triplicate (3 packs) on the day of processing and after one week of storage. Thirty grams of product were 5-fold diluted with chilled peptone-salt water, homogenized with stomacher whereafter RTi qPCR was performed directly as described in 2.4. In addition, for each pack, two times 1.00 ml of the homogenized solution was added to two times 9.00 ml of MoE medium (two tubes) and then incubated respectively during 72 h at 10°C or 7 h at 37°C before DNA extraction and quantification by the RTi qPCR assay (see 2.4). Total viable counts were determined at each time of analysis on spread plates of Long & Hammer agar (L&H) incubated 5 days at 15°C (NMKL, 2006; Van Spreekens, 1974). Plates were also observed in the dark to enumerate luminous *P. phosphoreum* colonies. *Enterobacteriaceae* were enumerated using Tryptone Soya Agar/Violet Red Bile Glucose (TSA/VRBG) agar as previously described (Emborg and Dalgaard, 2008). 24 colonies from the TSA/VRBG agar were isolated and identified by using simple biochemical tests (Dalgaard et al. 2006).

3- Results

3.1 Specificity of the real-time PCR assay

A set of 30 primers pairs has been designed and tested on *M. pyschrotolerans* and *M. morganii* strains. Of these primers, only the VasD-FR4 and the GalK-FR4 primers were specific for *M. psychrotolerans* and *M. morganii*, respectively. Establishment of the specificity of the VasD and GalK primers were tested *in silico* by sequence alignment using the GenBank database and by RTi qPCR on 13 *M. psychrotolerans*, 11 *M. morganii* and 49 HPB or non-HPB isolates (Table 1). The GalK-FR4 primers presented a 100% homology sequence with the *M. morganii* related gene available in the GenBank database (MU9_2965). For the VasD-FR4 primers, partial sequence alignments were obtained with 14 non-

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Enterobacteriaceae vasD sequences. *In vitro* RTi qPCR tests indicated a good specificity of the primers for the 13 *M. psychrotolerans* and the 11 *M. morganii* isolates. The mean C_T values were 16.8 ± 0.3 (n = 13) and 15.2 ± 0.5 (n = 11) with 4 ng of DNA extracted from broth cultures of *M. psychrotolerans* and *M. morganii*, respectively. RTi qPCR exclusivity test on 49 HPB or non-HPB isolates resulted in C_T values higher than 29. That was considered as the C_T threshold for specific detection of *Morganella* species obtained on pure cultures in liquid medium. This threshold was increased to 31 C_T when bacterial DNA was extracted from fish samples (tuna) (data not shown). The melting temperature calculated at the end of each real-time PCR assay was 83.5°C for *M. psychrotolerans* and 86°C for *M. morganii.*

3.2 Efficiency and detection range of the RTi qPCR assay

The standard curve showed a linear relation between cell concentrations (log CFU/g) and C_T values for type strains of both *Morganella* species in canned tuna (Fig. 1). No signal was detected by RTi qPCR for the negative control. The linear relation was determined on a 5 log (CFU/g) range from ca. 3 to ca. 8 log CFU/g for both species (Fig. 1). The linear relations was C_T = -3.30 x viable count (log CFU/g) + 41.60 (R^2=0.99) for *M. psychrotolerans,* with an efficiency of 100.8% on inoculated fish. For *M. morganii* the standard curve was C_T = -3.413 x viable count (log CFU/g) + 44.16 (R^2=0.99) with an efficiency of 96.3% (Fig. 1). Concentrations of *Morganella* lower than 4 log (CFU/g) resulted in C_T values higher than 31 and therefore could not be distinguished from non-specific reactions with other HPB or non-HPB. For that reason, the lower and upper LOQ of each *Morganella* species in fish products using the RTi qPCR method have been set to 4 and 8 log CFU/g respectively.

3.3 Development of an enrichment medium

The substrate 2-deoxy-D-Galactose showed an inhibition of *M. psychrotolerans* growth and was not further studied. Most antibiotics, in the concentrations tested, inhibited growth of

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Morganella strains and had a species specific inhibitory effect on non-*Morganella* HPB (Table 2). In contrast, colistin inhibited the growth of all non-*Morganella* HPB and concentrations up to 32 mg/l did not inhibit growth the *Morganella* isolates (Table 2).

3.4 RTi qPCR quantification using an enrichment step

Three matrixes (peptone-salt water, canned tuna and cooked shrimp) have been used to study the subsequent growth kinetics of *Morganella* strains in MoE. The μ_{max}-values of *M. psychrotolerans* in MoE at 10°C were 0.182 h^{-1} for MIX-Mp inoculated from peptone-salt water, 0.180 h^{-1} from canned tuna and 0.180 h^{-1} from cooked shrimp (Fig. 2). Statistical analysis showed no significant difference between growth rates obtained on the three matrixes ($p > 0.05$), thus growth rate of *M. psychrotolerans* has been set to 0.180 h^{-1}in fish products. The μ_{max}-values of MIX-Mm at 37°C were 1.939 h^{-1} from peptone-salt water, 2.002 h^{-1} from canned tuna, and 2.130 h^{-1} from cooked shrimp. Again, values were not significantly different and growth rate for *M. morganii* has been set at 2.024 h^{-1}.

To complete the enrichment method, standard curves to determine the relation between C_T obtained by RTi qPCR and cell concentrations (log CFU/g) obtained by classical plate count were performed for each species when growing in the MoE medium. The linear relation determined for both species was scaled from 4.0 ± 0.5 to 8.0 ± 0.5 log CFU/ml. For *M. morganii,* the equation was C_T = -3.378 x viable count (log CFU/ml) + 44.95 (R^2=0.99) with an efficiency of 97.71%. For *M. psychrotolerans,* the equation was C_T = -3.464x viable count (log CFU/ml) + 47.69 (R^2=0.99) with an efficiency of 94.54% (Fig. 3).

After enrichment, the initial cell concentration in fish is calculated from the concentration obtained by RTi qPCR in the MoE medium and by taking into account the μ_{max}-value of *M. psychrotolerans* or *M. morganii*, the time of enrichment and the dilution of the fish sample (Eq. 2):

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$$IC = CaE - ((\mu_{max}\, x\, ET) / \ln 10) + \log(DF) \qquad (2)$$

where *IC* is the initial cell concentration in the fish product (log CFU/g), *CaE* the concentration in MoE medium after enrichment as determined by RTi qPCR (log CFU/ml), *ET* the enrichment time (h) and *DF* the total dilution factor resulting from homogenization and transfer of the homogenate to the MoE medium.

3.5 Quantification of Morganella in fish products by RTi qPCR

Just after processing of the studied seafoods, the viable counts on L&H were under 4.2 log CFU/g for fresh fish and under 1.9 log CFU/g for lightly preserved products (Table 3). After storage during one week this concentration increased up to 7 log CFU/g in herring fillets and hot smoked mackerel. Most colonies on L&H agar plates were bioluminescent and the microbiota therefore seemed to be dominated by *P. phosphoreum*. Viable counts of the four others fish product remained under 4.3 log CFU/g. *Enterobacteriaceae* were not detected after processing and remained under 2.6 log CFU/g after one week (Table 3).

RTi qPCR enumeration with or without the enrichment step did not allow to detect the presence of *M. psychrotolerans* or *M. morganii* in the studied fish samples. To confirm these results the MoE medium was plated on TSA/VRBG agar. No *Enterobacteriaceae* was detected in MoE after 7 h enrichment at 37°C, confirming the absence of *M. morganii* in the fish samples. Enumeration on TSA/VRBG plates after 72 h of enrichment at 10°C, for tuna loins, herring fillets and hot smoked mackerel showed bacterial growth up to 8 log CFU/ml.. However, these bacteria on TSA/VRBG agar plates were *Pseudomonas/Shewanella*-like and not *Morganella/Enterobacteriaceae.* These results support those obtained by RTi qPCR on MoE medium with C_T-values higher than 30 and corresponding to concentrations of *M. psychrotolerans* below 5.1 log CFU/ml in the MoE medium and below 1.2 log CFU/g in

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herring fillets and hot smoked mackerel after storage for one week at respectively, 2°C or 10°C.

4 Discussion

After 2009, RTi qPCR methods have been developed for HPB detection and quantification in seafood. These methods have focused on mesophilic HPB species and have not been tested for *M. psychrotolerans* and *P. phosphoreum* (Bjornsdottir-Butler *et al.*, 2011a, 2011b; Ferrario *et al*., 2012a, 2012b). RTi qPCR methods have previously been used in combination with an enrichment step to qualitatively detect pathogenic bacteria including *Listeria monocytogenes* (O'Grady *et al.*, 2008; O'Grady *et al.*, 2009) and *Escherichia coli* (Chern *et al.*, 2011; Taskin *et al.*, 2011) in food products. Spoilage bacteria responsible for sensory defect have also been quantified by RTi qPCR, e.g. *Brochotrix thermosphacta* (Mamlouk *et al.*, 2012) and *P. phosphoreum* (Macé *et al.*, 2013).

The present study focused on quantification of *Morganella* species in fish products as these are strongly histamine producing bacteria and have been responsible for HFP outbreaks. Specific and sensitive quantification methods were missing to study their occurrence and to help management of histamine formation in seafood. Available methods are limited in specificity or in sensitivity to obtain reliable results to improve our knowledge on these HPB. Recently, a RTi qPCR method was developed for quantification of *M. morganii* by Ferrario *et al.*(2012a), but this method was not tested for selectivity in relation to *M. psychrotolerans* although the type strain (U2/3^T) has been available in culture collections since 2007 (Emborg *et al.*, 2006). Primers from Ferrario *et al.*(2012b) were checked *in silico* in the present study and might lead to unspecific reaction with *M. psychorotolerans*. To obtain a specific quantification method for each *Morganella* species primers for *M. psychrotolerans* have been designed on the *vasD* gene as part of the Type VI secretion system, recently discovered in

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Vibrio cholera by Filloux *et al.* (2008). In the *M. psychrotolerans* genome, the type VI secretion system is composed of 17 genes which are missing on the *M. morganii* genome and this allowed the developed RTi qPCR method to specifically detect the *Morganella psychrotolerans* . For *M. morganii*, the choice of primer cites were the galactokinase gene due to absence of this gene in *M. psychrotolerans,* unable to ferment D-galactose (Emborg *et al*., 2006). These two pairs of primers were 100% inclusive for their target species. Without enrichment the developed RTi qPCR methods can be used for specific detection and quantification of *Morganella* species in samples with high concentrations of these bacteria, for example fish products responsible of HFP. This is the first method develop for quantification of *M. psychrotolerans* and it seems useful to increase our understanding of the relative importance of psychrotolerant and mesophilic bacteria responsible for histamine formation e.g. in relation to outbreaks of HFP.

Using a specific enrichment medium, the developed RTi qPCR methods may be sufficiently sensitive to enumerate *Morganella* species in newly processed seafood and within the seafood processing environment. Colistin allowed growth of *Morganella* and reduced growth of some of the other well known and strongly histamine producing *Enterobacteriaceae* during enrichment (Table 2). Furthermore, data for fresh herring and hot smoked mackerel indicated that enrichment in MoE without NaCl limited growth of *P. phosphoreum* and thereby the potential interference from this marine bacterium that often are present in high concentration in fresh and lightly preserved seafood (Dalgaard *et al.,* 1997). Nevertheless, selectivity of the MoE medium in relation to other microorganisms that may influence growth of the *Morganella* species during enrichment deserves further study.

Enrichment at 37°C for *M. morganii* allowed DNA extraction and quantification within one working day. In contrast, for *M. psychrotolerans*, incubation at 10°C was chosen to reduce the growth of *M. morganii* in the MoE medium. Clearly, the suggested enrichment and RTi qPCR

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method for *M. psychrotolerans* is not a rapid method. However, it is the only method available for specific enumeration *M. psychrotolerans* and with a LOQ below 50 CFU/g in fish products. This method seems valuable to obtain information about the occurrence and growth of this strongly histamine producing bacteria that has been responsible for outbreaks of HFP. *M. psychrotolerans* has been isolated sporadically in seafood worldwide (Emborg *et al*., 2006; Macé et al., 2012; Torodo *et al*., 2014). Available information on its occurrence is far from sufficient to quantitatively evaluate its contribution to the risk of histamine formation and HFP. The enrichment and RTi qPCR method suggested in the present study has the potential to overcome this problem.

Lag phases of the *Morganella* species during growth in the MoE have not been studied. For enrichment from fresh or lightly preserved seafood lag phases are likely to be of little practical importance. However, for frozen seafood and more preserved, like salted and/or dried, products significant lag times may be observed during enrichment in MoE. Using eq. 2 to calculate cell concentration in fish products will result in underestimation of cell concentrations when lag times in the MoE medium are observed and this aspect needs further study for the suggest enrichment procedures at 10°C or 37°C. Enrichment steps have previously been proposed to lower the detection limit of RTi qPCR methods (O'Grady *et al.*, 2009, Taskin *et al.*, 2011). However in those studies, the enrichment step did not allow quantification of the initial concentration of the bacteria detected. In the present study, the determination of the growth rate in the enrichment media made it possible to calculate the initial concentration from different enrichment times using Eq. (2). This approach resembles quantitative incubation methods that are known for their ability to enumerate low concentrations of bacteria as shown e.g. for *P. phosphoreum* in fish samples (Dalgaard *et al.*, 1996).

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The LOQ for the developed RTi qPCR methods was limited by the 50-fold dilution during homogenization (x5) and inoculation of the MoE medium (x10). A direct enrichment with 30 g of fish flesh homogenized in 120 ml of MoE medium may lower the LOQ but need further study to evaluate if the growth rate during enrichment will depend on the type of seafood e.g. for fish products with different pH and salt content. Otherwise, a filtration method has already shown good results on pathogenic bacteria and might be tested to increase sensitivity to 1 CFU/g of products (Murakami, 2012).

The methods developed in this study for quantification of *M. psychrotolerans* or *M. morganii* in fish products may be used to survey occurrence of these important HPB in fish products and to improve management of histamine formation.

5 Acknowledgments

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Figure 1: Standard curve showing the relationship between C_T values and log CFU/g for serially diluted culture of *M. morganii* (A) and *M. psychrotolerans* (B) inoculated on canned tuna.

Figure 2: Growth of *M. morganii* (A) and *M. psychrotolerans* (B) respectively at 37°C and 10°C in MoE medium followed by plate count on TSA agar as a function of the time. The line represents a fitted exponential growth model.

Figure 3: Standard curve showing the liner relationship between C_T values and bacterial enumeration (log CFU/ml) of serially diluted *M. morganii* (A) and *M. psychrotolerans* (B) in MoE medium.

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Figure 1

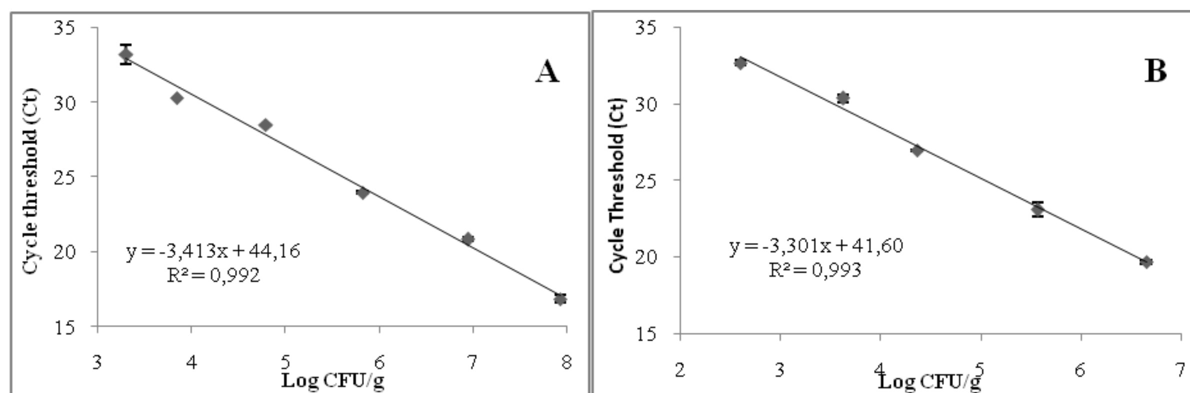

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Figure 2

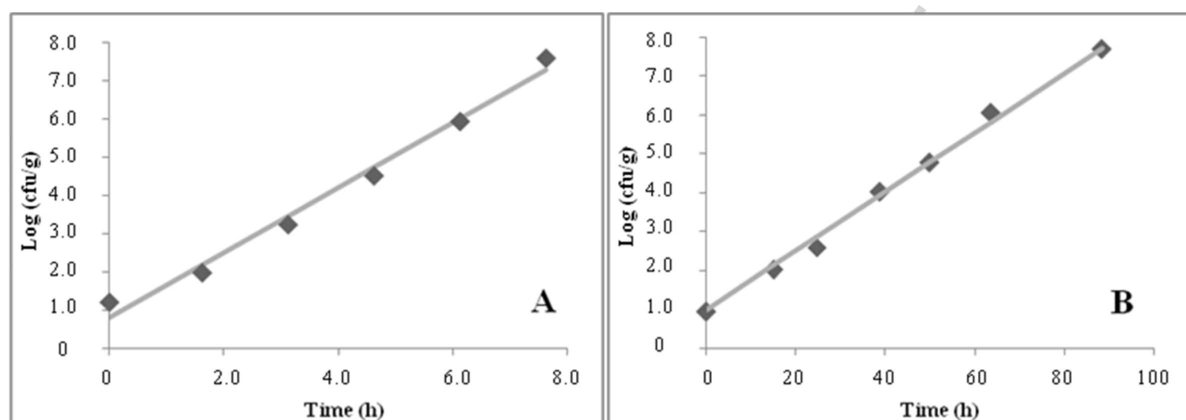

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Figure 3

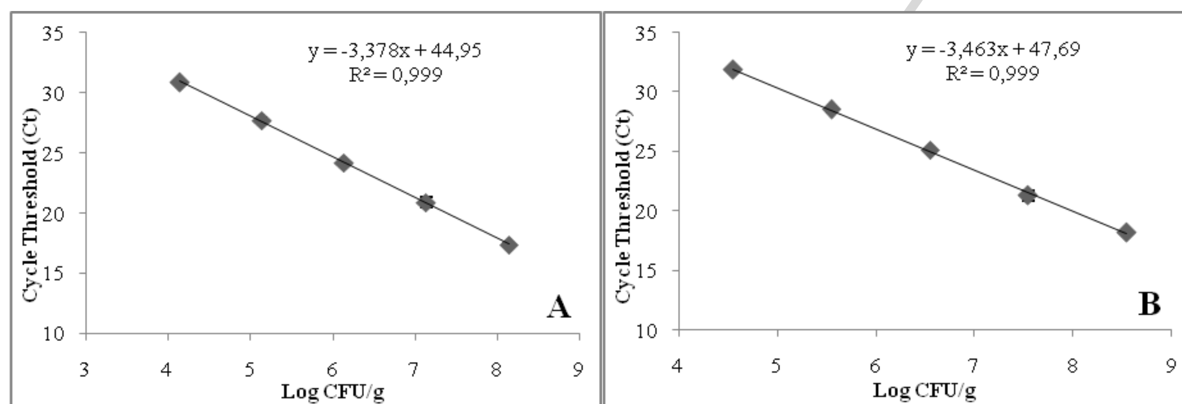

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Table 1

Morganella strains and non-*Morganella* species used in this study.

Bacteria	Strains	Histamine production	Collection	Origin
Morganella psychrotolerans	U2 /3 [T](CIP 109403[T])	High[a]	DTU Food and CIP[1]	Cold-smoked tuna
	U2/4		DTU Food	Cold-smoked tuna
	U2/5		DTU Food	Cold-smoked tuna
	U2/6		DTU Food	Cold-smoked tuna
	U2/7		DTU Food	Cold-smoked tuna
	FD24		DTU Food	Fresh Vacuum-packed tuna
	1F10		DTU Food	Garfish
	2F6		DTU Food	Garfish
	JB-T11		DTU Food	Fresh Vacuum-packed tuna
	JB-T12		DTU Food	Fresh Vacuum-packed tuna
	JB-T16		DTU Food	Fresh Vacuum-packed tuna
	F39-1		DTU Food	Lumpfish roe
	F39-3		DTU Food	Lumpfish roe
Morganella morganii	LMG7874[T]	High[a]	BCCM/LMG[2]	Human stool
	DSM14850[T]		DSMZ[3]	Human sputum
	25a32		DTU Food[b]	Tuna
	M04090		DTU Food[b]	Clinical isolate
	U6/1		DTU Food	Tuna
	NCIMB865		NCIMB[4]	Spoiled fish
	AP28		DTU Food[b]	Yellowtail
	03A11		DTU Food[b]	Smoked fish
	03B10		DTU Food[b]	Smoked fish
	HB2810		Ifremer	Sardine
	HB2811		Ifremer	Sardine
Hafnia alvei	DSM30163[T]	High[a]	DSMZ[3]	NC[b]
	MIP2438		Ifremer/Oniris	Spoiled fresh salmon
	MIP2439		Ifremer/Oniris	Spoiled fresh salmon
	MIP2461		Ifremer/Oniris	Spoiled fresh salmon
	MIP2467		Ifremer/Oniris	Spoiled fresh salmon
	MIP2468		Ifremer/Oniris	Spoiled fresh salmon
	MIP2613		Ifremer/Oniris	Spoiled fresh salmon
	MIP2625		Ifremer/Oniris	Spoiled fresh salmon
Raoultella planticola	DSM3069[T]	High[a]	DSMZ[3]	Radish root
Photobacterium phosphoreum	CCUG16288	High[a]	CCUG[5]	*Etelismarshi*
	1D5		DTU Food	Garfish
	FR7		DTU Food	Fresh Vacuum-packed cod
	EBP3067		Ifremer/Oniris	Salmon
	EBP3079		Ifremer/Oniris	Salmon
	MIP2560		Ifremer/Oniris	Salmon
	MIP2562		Ifremer/Oniris	Spoiled fresh salmon
	MIP2588		Ifremer/Oniris	Spoiled fresh salmon
	MIP2591		Ifremer/Oniris	Spoiled fresh salmon
	MIP2613		Ifremer/Oniris	Spoiled fresh salmon
Photobacterium damselae	CIP102761[T]	High[a]	CIP[1]	Damselfish
Enterobacter aerogenes	LMG2094[T]	High[a]	BCCM/LMG[2]	Sputum
	SF1469		Ifremer	Smoked salmon
	SF1470		Ifremer	Smoked salmon
Klebsiella oxytoca	LMG3055[T]	High[a]	BCCM/LMG[2]	NC[b]
Enterococcus faecalis	CIP105042	Low[a]	CIP[1]	Poultry
***Pseudomonas* spp.**	HB2843	Low[a]	Ifremer	Tuna
Raoultella ornithinolytica	CIP103364[T]	High[a]	CIP[1]	Human urine
Serratia liquefaciens	SF1465	Low[a]	Ifremer	Smoked salmon
Staphylococcus xylosus	DSMZ20029	Low[a]	DSMZ[3]	Fermented meat product

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Listeria monocytogenes	RF190	Low[a]	Ifremer	Shrimp
Shewanella putrefaciens	CIP6929 RF47 RF49	Low[a]	CIP[1] Ifremer Ifremer	NC[b] Vacuum-packed trout Cod fillets
***Psychrobacter* spp.**	CCUG 42949 EBP3029	Low[a]	CCUG[5] Ifremer	Desalted cod Salmon
Psychrobacter aquaticus	MIP2412	Low[a]	Ifremer/Oniris	Spoiled fresh salmon
***Acinetobacter* spp.**	EBP3044	Low[a]	Ifremer/Oniris	Salmon
Vibrio cholerae	RF184	Low[a]	Ifremer	Cooked shrimp
Carnobacterium maltaromaticum	V1	Low[a]	Ifremer/Oniris	Fish
Vibrio parahemolyticus	RF179	Low[a]	Ifremer	Water
Enterococcus faecium	CIP 54.33	Low[a]	CIP[1]	Canned fish
Brochotrix thermosphacta	EBP3084	Low[a]	Ifremer/Oniris	Salmon
Carnobacterium jeotgali	KCTC 13251	Low[a]	KCTC[6]	Shrimp
Leuconostoc gelidum	LHIS2959	Low[a]	Ifremer/Oniris	Mackerel fillets
Carnobacterium divergens	V41	Low[a]	Ifremer/Oniris	Fish viscera
Escherichia coli	CIP 76.24	Low[a]	CIP[1]	NC[b]
Lactobacillus curvatus	LHIS2886	Low[a]	Ifremer/Oniris	Fresh sardine
Lactobacillus fuchuensis	LHIS2997	Low[a]	Ifremer/Oniris	Fresh mackerel
Lactobacillus sakei	LHIS2855	Low[a]	Ifremer/Oniris	Fresh sardine

[a] High: Production of histamine between 200-5000 mg/l in histidine broth. Low: 0 to 200 mg/l of histamine in histidine. broth.

[b] See Emborg *et al*. (2006) for the origin and isolation of these isolates. NC: Not communicated

[1]CIP : Collection de l'Institut Pasteur ; [2]BCCM/LGM : Belgian Coordinated Collections of Microorganisms, [3]DSMZ : Deutsche Sammlung von Mikroorganismen und Zellkulturen; [4]NCIMB : National Collections of Industrial, Marine and Food Bacteria, [5]CCUG : Culture Collection, University of Göteborg, Sweden, [6]KCTC : Korean Collection for Type Cultures.

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Table 2

Antibiotics tested for inhibition of non-*Morganella* species in the enrichment procedure.

Antibiotics	Concentration tested (mg/l)	*M. psychrotolerans*	*M. morganii*			*R. planticola*	*E. aerogenes*	*K. oxytoca*	*H. alvei*
		U2/3^T, JB-T11, F39-1	LMG7874^T	DSM14850^T	03A11	DSM3069^T	LMG2094^T	LMG3055^T	DSM30163^T
Colistin	4 / 8 / 16 / 32	+[a]	+	+	+	- (4)[a]	- (4)	- (4)	- (4)
Ampicillin	1 / 2 / 4 / 8	+	- (8)	+	+	+	+	+	- (2)
Amoxicillin-clavulanat	2;1 / 4;2 / 8;4 / 16;32	+	- (16;8)	+	+	- (2;1)	- (4;2)	+	- (2;1)
Chloramphenicol	1 / 2 / 4 / 8	- (8)	- (4)	+	- (8)	- (4)	- (4)	- (2)	- (2)
Gentamycin	1 / 2 / 4 / 8	- (4)	- (4)	- (8)	- (4)	- (1)	- (4)	- (2)	- (2)
Tobramycin	1 / 2 / 4 / 8	- (4)	- (4)	- (8)	- (4)	- (2)	- (4)	- (4)	- (4)
Florfenicol	1 / 2 / 4 / 8	- (8)	- (1)	- (4)	- (8)	- (4)	- (8)	- (1)	- (1)
Meropenem	2 / 4	- (2)	- (2)	- (2)	- (2)				
Thrimethoprim	2 / 4	- (2)	- (2)	- (2)	- (2)				
Thrimethoprim + Sulfamethoxazol	2 / 4	- (2)	- (2)	- (2)	- (2)				
Oxytetracycline	2 / 4	+ F39-1 / - (2)	- (2)	+	- (2)				
Nalidixicacid	2 / 4	+	+	+	- (2)				
Polymixin B	4 / 8	+	+	+	+				
Ciproflaxin	2 / 4	- (2)	- (2)	- (2)	- (2)				
Norfloxacin	2 / 4	- (2)	- (2)	- (2)	+				

[a]+: Growth observed for all the tested concentrations of antibiotics; - (...): Absence of growth with a known concentration of antibiotics (mg/l).

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Table 3

Microbiological analysis on fish products after purchase or after 1 week of incubation at 2 or 10°C

Fish product	Origin & packaging	Analysis after purchase (log CFU/g)			Analysis after 1 week of incubation (log CFU/g)		
		Total microbiota	*P. phosphoreum* (Bioluminescent)	Enterobacteria	Total microbiota	*P. phosphoreum* (Bioluminescent)	Enterobacteria
Tuna Loin (2°C)	Fish market VP[a]	3.49 (± 0.52)	ND[b]	< 0,69	4.37 (± 0.50)	ND[b]	1.68 (± 0.48)
Frozen and thawed tuna steak (2°C)	Supermarket VP[a]	3.64 (± 0.05)	ND[b]	< 0,69	2.70 (± 0.46)	ND[b]	1.64 (± 0.21)
Herring (2°C)	Fisherman VP[a]	4.20 (± 0.12)	ND[b]	< 0,69	7.88 (± 0.14)	7.88 (± 0.14)	2.27 (± 0.24)
Hot-smoked mackerel (10°C)	Supermarket VP[a]	3.41 (1 sample) ND[b] (2 samples)	ND[b]	< 0,69	7.36 (± 0.26)	7.36 (± 0.26)	< 0,69
Matjes fillets (10°C)	Supermarket MAP[c]	1.86 (± 0.28)	ND[b]	< 0,69	2.12 (± 1.92)	ND[b]	2.61 (± 0.55)
Cold smoked salmon (10°C)	Supermarket MAP[c]	1.23 (± 1.07)	ND[b]	< 0,69	1.70 (± 1.70)	ND[b]	< 0,69

[a]VP: Vacuum packed

[b]ND: Not detected

[c]MAP: Modified atmosphere packaging.

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