**Identification and semi-quantification of Atlantic salmon in processed and mixed seafood products using Droplet Digital PCR (ddPCR)**

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Journal: SEAFOODTOMORROW special issue 2 Food and Chemical Toxicology

**Abstract**

Fishery products are often subject to substitution fraud, which is hard to trace due to a lack of morphologic traits when processed, gutted, or decapitated. Traditional molecular methods (DNA barcoding) fail to identify products containing multiple species and cannot estimate original weight percentages. As a proof of concept, an Atlantic salmon (*Salmo salar*) specific ddPCR assay was designed to authenticate mixed food products. The method proved to be specific and able to accurately quantify *S. salar* whenusing DNA extracts, even in the presence of DNA from closely related salmon species. The ddPCR estimates correlated well with the percentage of *S. salar* in artificially assembled tissue mixtures. The effect of common salmon processing techniques (freezing, smoking, poaching with a Bellevue recipe and marinating with a ‘gravadlax’ recipe) on the ddPCR output was investigated and freezing and marinating appeared to lower the copies detected by the ddPCR. Finally, the assay was applied to 46 retail products containing Atlantic or Pacific salmon, and no indications of substitution fraud were detected. The method allows for a semi-quantitative evaluation of the *S. salar* content in processed food products and can rapidly screen Atlantic salmon products and flag potentially tampered samples for further investigation.

**Running title**

Authentication of mixed salmon food products with ddPCR

**Keywords**

Seafood; mixed and processed food products; rhodopsin; salmonid DNA; food fraud

**Highlights**

1. A species-specific assay for Atlantic salmon with a low limit of detection was designed using ddPCR.
2. Quantification of the method was validated by testing selectivity, limits of quantification and detection, and the effects of processing.
3. The method was successfully applied to retail samples with no evidence of fraud.
4. The method can identify and semi-quantify *S. salar* specific tissue in processed and mixed food products.

**Abbreviations**

ANOVA Analysis of Variance

Cytb Cytochrome b

dMIQE Minimum Information for Publication Quantitative Digital PCR Experiments

ddPCR Droplet Digital Polymerase Chain Reaction

EU European Union

FAO Food and Agriculture Organization of the United Nations

HPLC High-performance liquid chromatography

ICETA Institute of Sciences, Technologies and Agroenvironment of the University of Porto

ILVO Flanders Research Institute for Agriculture, Fisheries and Food

ITS Internal Transcribed Spacer

LOD Limit of Detection

LOQ Limit of Quantification

MAP Modified Atmosphere Packaging of Fish and Seafood

mtDNA Mitochondrial DNA

NTC non-target control

qPCR Real-time PCR (quantitative PCR)

Tukey HSD Tukey honestly significant difference

# Introduction

Seafood sourced from wild stocks and farming plays an important role in the human diet (FAO, 2020). However, fish stocks are under pressure globally and as a consequence, this pressure on supplies has been leading to a rise in different fraudulent practices (Costello et al., 2020; Fox et al., 2018; Long et al., 2020; Worm et al., 2006). Substitution is a widespread problem, where a highly-priced fish is replaced with a cheaper alternative (Bénard-Capelle et al., 2015; Christiansen et al., 2018; Luque and Donlan, 2019), which can occur at every part of the supply chain (Deconinck et al., 2020; Gordoa et al., 2017). Species adulteration occurs when a non-declared or non-specified species is added to a primary processed raw material to lower production costs or hide dubious material (Fox et al., 2018).

Salmonids are an important resource of commercial and game fish worldwide (Herrero et al., 2011; Kitano et al., 1997). Commercially important salmon species are commonly divided into two groups: the *Oncorhynchus* and *Salmo* genus (Rasmussen and Morrissey, 2009; Stearley and Smith, 1993). Since 2013, salmonidshave been the most commonly farmed marine fish (19 % of the total value of internationally traded fish in 2018). *Salmo salar* encompasses 90 % of the farmed salmon market and over 50 % of the total global salmon market (FAO, 2020, 2018, n.d.). Salmons can differ greatly in consumer price, with wild-caught Pacific salmons being more expensive than aquaculture salmon and *S. salar* being the most expensive aquaculture salmon. After processing, morphological identification is no longer possible, making these species susceptible to market substitution (Feng et al., 2017; Rasmussen et al., 2009). Salmonidae species are mislabelled in 7 % of all cases, and among the Salmonidae, wild-caught Pacific salmon belong to the most mislabelled species (17 %), followed by Atlantic salmon (3 %) and rainbow trout (3 %) (Luque and Donlan, 2019).

Currently, the most commonly used technique to identify fish in processed food products is DNA barcoding (Hebert et al., 2003; Luque and Donlan, 2019), which uses Sanger sequencing to determine the DNA sequence of the food product. The DNA sequence is then compared to a DNA reference database. Public databases, like GenBank (NCBI, Bethesda, USA) can be prone to inconsistencies (Li et al., 2018; Mioduchowska et al., 2018), leading to the generation of more specific databases, designed to cope with fraud, such as the SEAFOODTOMORROW database (Deconinck et al., 2020; SeafoodTomorrow, n.d.) and Fishtrace (“FishTrace,” n.d.). Although DNA barcoding is a very valuable technique to identify single species food products, but it cannot identify or quantify food products containing multiple species. To identify species in mixed samples, species-specific assays paired with PCR (endpoint PCR, qPCR or ddPCR) can be used or next-generation amplicon sequencing can be applied, but the latter is less appropriate for quantification (Lundberg et al., 2013). At this point, there is little evidence on the correlation of DNA based quantification of a fish species in a mixed food product with the percentage mentioned on a food label (Hansen et al., 2020; Laube et al., 2007; Mayer et al., 2019).

While species-specific assays for Atlantic salmonhave already been developed (Atkinson et al., 2018; Hernandez et al., 2020; Herrero et al., 2011) and distributed by companies such as bioMérieux (Marcy-l'Étoile, France) and Eurofins (Luxembourg, Luxembourg), no tool currently exists to estimate the percentage of an Atlantic salmon within a mixed food product. Quantitative PCR (qPCR) has been widely used for the quantification of allergens and species within mixed and heavily processed products, including salmonids (Feng et al., 2017; Herrero et al., 2011, 2010). Droplet Digital PCR (ddPCR) is a more recent tool where samples are partitioned in droplets via microfluidics and fluorescence signals are measured via end-point measurement. For the current application, ddPCR provides three major advantages over qPCR (Huggett et al., 2013; Taylor et al., 2017; Zhao et al., 2016)(Huggett et al., 2013; Taylor et al., 2017; Zhao et al., 2016). First, ddPCR uses no Cq values and this no longer efficient PCR reactions, which makes ddPCR a better technology when trying to reduce impact of contaminants (Huggett et al., 2013; Taylor et al., 2017). Second, ddPCR provides absolute quantification and standard curves, which can be a source of error (Taylor et al., 2017; Zhao et al., 2016). Third, the chance of a false-positive detection (by a related species) can be further reduced by setting a high threshold to distinguish positive droplets from negative droplets.

The goal of the present study was to develop a species-specific *S. salar* ddPCR assay that allows identification and quantification of the percentage of Atlantic salmon in processed and mixed food products. For this purpose, five aims were considered: (1) the design of a ddPCR assay specific to *S. salar*; (2) the accuracy of quantification of the assay was tested in single-species samples; (3) the accuracy of quantification was tested in mixed-species samples; (4) the impact of food processing on quantification results was tested; (5) the ddPCR assay was validated by applying it to processed and mixed retail food products containing Atlantic salmon or Pacific salmon.

# Materials and Methods

## Development of an Atlantic salmon specific ddPCR assay

### Sample collection

Authenticated Salmonidae tissues from nine species involved in the creation of the SEAFOODTOMORROW genetic reference database (Deconinck et al., 2020; SeafoodTomorrow, n.d.) were used to develop a specific ddPCR assay for *S. salar* (Table 1). These tissues were stored on ethanol at -20 °C until extraction. Total DNA was isolated from 200 mg of muscle tissue (or food product) using the NucleoSpin® Food kit (Macherey - Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer instructions. Total DNA was quantified using a QuantusTM fluorometer (Promega, Madison, USA), following the manufacturer’s protocol. DNA extractions were stored at -20 °C until further use.

Table 1 Salmonidae used to test the specificity of the ddPCR assay, including the scientific and common name, the number of the specimen and the sample origin.

|  |  |  |  |
| --- | --- | --- | --- |
| Scientific name | Common name | # Specimen | Sample origin |
| *Oncorhynchus gorbuscha* | Pink salmon | 3 | North East Pacific |
| *Oncorhynchus keta* | Chum salmon | 3 | North East Pacific |
| *Oncorhynchus kisutch* | Coho salmon | 3 | North East Pacific |
| *Oncorhynchus mykiss* | Rainbow trout | 3 | Aquaculture – North-East Atlantic \* |
| *Oncorhynchus nerka* | sockeye salmon | 3 | North East Pacific |
| *Oncorhynchus tshawytscha* | Chinook salmon | 3 | North East Pacific |
| *Salmo salar* | Atlantic salmon | 3 | Commercial |
| *Salmo trutta* | brown trout | 3 | Aquaculture – North East Atlantic & Commercial |
| *Salvelinus alpinus* | Arctic char | 1 | Commercial |

\* Pacific salmon, cultured in the Atlantic ocean.

### Primer and probe design

*Rhodopsin* sequences for *S. salar* and related commercial non-target salmonid species (*S. trutta, Oncorhynchus mykiss, O. nerka, O. keta, O. tshawytscha, O. gorbuscha, O. kisutch*) were downloaded from Fishtrace (“FishTrace,” n.d.) and GenBank (NCBI, Bethesda, USA) (Supplementary Table S1). Sequences were aligned and trimmed appropriately using Mega X version 10.0.5 (Tamura et al., 2007). The aligned sequences were used in AlleleID® v7.85 (PREMIER Biosoft, Palo Alto, USA) to generate specific primers (forward and reverse) and a dual labelled probe (5'-FAM, 3'-BHQ1). The following *S. salar* specific primers were designed: SFT\_Salsal\_Rho\_f: 5'-GCCTTTACCTGGGTCATG-3'; SFT\_Salsal\_Rho\_r: 5'-CGTGTGTAGTAGTCGATTC-3'; SFT\_Salsal\_Rho\_p: 5'-FAM-CTTCTGCTTGCTCCGTGCCC-BHQ1-3', resulting in an amplicon size of 110 bp. In silico testing was performed by blasting the assay against the FishTrace and GenBank databases which illustrated that the primer-probe combination was a 100 % match only with *S. salar.* The primers and probe were manufactured and HPLC purified by Eurofins Genomics (Luxembourg).

### ddPCR procedure

Droplets were generated using a QX200 Droplet Generator (Bio-Rad, Hercules, USA) using DG8 cartridges (Bio-Rad, Hercules, USA). After generation, droplets were transferred to ddPCRTM 96 well plates (Bio-Rad, Hercules, USA). The well-plates were sealed with PCR plate pierceable heat foil using a PX1 PCR Plate Sealer (Bio-Rad, Hercules, USA). The well-plates were then transferred to a T100TM Thermal Cycler (Bio-Rad, Hercules, USA) where PCR was performed in every droplet. Afterwards, the reaction mixture was held at 4 °C for at least 1 h. Amplification was measured in each droplet using a QX200 droplet reader (Bio-Rad, Hercules, USA).

The ddPCR reaction conditions were optimized by testing different reaction conditions on both Atlantic salmon and related salmon (Table1). The optimal conditions were selected based on a clear distinction between droplet populations of target and non-target species. The total DNA concentrations were kept at about 25 ng in a total reaction mix of 20µL, following the manufacturer’s (Bio-Rad, Hercules, USA) instructions (less than 66 ng). Optimization experiments included optimizing annealing temperatures (54 – 65 °C) using a Gradient T100 Thermal Cycler (Bio-Rad, Hercules, USA), varied concentrations of primers (50 nM – 900 nM), and varied concentrations of probes (50 nM to 250 nM). Using the optimal settings from these experiments, the number of cycles was increased from 40 to 45, to increase the difference in fluorescence measured for Atlantic salmon and related salmon, as related salmon should amplify at lower efficiencies and thus expected to increase less than the more efficient reaction for Atlantic salmon, allowing for a threshold to be set between related salmon amplification and Atlantic salmon amplification.

The 20 µL ddPCR reaction mixtures consisted of 10 µL 2x ddPCR Supermix for Probes (no dUTP) (Bio-Rad, Hercules, USA), 1.8 µL forward and 1.8 µL reverse primer (900 nM), 0.25 µL probe (250 nM), 2.5 µL of template DNA and 3.65 µL of nuclease-free water. The reaction mixture was divided into a maximum of 20.000 droplets using a QX200 ddPCR droplet generator (Bio-rad, Hercules, USA). The following cycling protocol was used: one initial denaturation step at 95 °C for 10 min, followed by 45 cycles of 30 seconds of denaturing at 94 °C and 60 seconds of annealing and extension at 64 °C, followed by a final inactivation step of 10 min at 98 °C.

### ddPCR data analysis

Fluorescence data per droplet was initially analysed with Quantasoft 1.7.4.0917 (Bio-rad, Hercules, USA), but for further analysis, the raw data (fluorescence values per droplet) were exported to CSV format and further analysed in R version 4.0.3 (R Core Team, 2018). Reactions with less than 10.000 droplets are returned as “no call” by Quantasoft and were rejected for further analysis as the number of analysed partitions affects the theoretical LOD and LOQ (Deprez et al., 2016). Threshold values for each run were determined following the ddpcRquant protocol, which provides an automated analysis of one-dimensional single-colour ddPCR data by calculating a single threshold value based on the negative controls for each assay run in a ddPCR (Trypsteen et al., 2015). The threshold is usually set within the “rain”, which are droplets without pronounced distribution, spread over the space between positives and negatives (Lievens et al., 2016). After determining the positive and negative droplets, the final concentrations for each reaction were calculated using poison statistics, by dividing the average number of targets per droplet (*λ*) by droplet volume (0.88 nL). λ = -ln x (1-k/n), where k is the number of positive droplets and n is the number of accepted droplets (Huggett et al., 2013). The work was performed according to the Minimum Information for Publication of Quantitative Digital PCR Experiments (Digital MIQE or dMIQE) guidelines for which the checklist can be found in Supplementary Table S2.

## ddPCR quantification of Atlantic salmon DNA

The ddPCR measures *S. salar* DNA as copies per microlitre (cp/µl) and is an absolute quantification of the number of copies of the target sequence present in the master mix. For a pure *S. salar* sample, a linear relation is expected between total DNA (ng) added to the PCR mix and the number of targets measured by the ddPCR (cp/µl). To assess the ratio of *S. salar* in mixed samples, the copy number of *S. salar* is converted to ng DNA based on the linear relationship and then divided by the total ng of DNA (belonging to all DNA holding ingredients) as measured with the QuantusTM fluorometer.

### Working range, LOD & LOQ of the ddPCR assay

The working range is the DNA concentration interval over which the method provides results with acceptable reliability. In this concentration range, the relationship between response and concentration is continuous, reproducible and linear after suitable data transformation (Deprez et al., 2016). The working range was investigated using DNA extracts of three *S. salar* individuals and running two serial dilutions in triplicate: one ten-fold dilution series, with total DNA from 150 ng to 0.0015ng; and one five-fold dilution series with total DNA from 75 ng to 0.024 ng.   
The limit of detection (LOD) (sometimes referenced as sensitivity) is the lowest DNA concentration that can be distinguished from zero, with a specified (95 %) level of confidence (Deprez et al., 2016; Magnusson and Örnemark, 2014). To calculate the LOD, the same dilution series as used for the working range estimation was used to determine the lowest value where no false negatives were detected.   
The limit of quantification (LOQ) is the lowest DNA concentration for which the method provides results with an acceptable uncertainty (Deprez et al., 2016) or the lowest level at which the performance is acceptable (Magnusson and Örnemark, 2014). To determine the LOQ, a level of acceptable uncertainty was chosen at a maximum Coefficient of Variance (CV) of 25 % based on the Codex Alimentarius Guidelines CAC/GL 74-2010 (Codex Committee on Methods of Analysis and Sampling, 2010). From the dilution series used to assess the working range, the highest DNA concentration having a CV value higher than 25 % was considered outside of the LOQ.

### The selectivity of the ddPCR assay

Selectivity is the extent to which the method accurately quantifies the target DNA in the presence of interfering substances that could be present in the samples, also defined as the ability of the method to produce results that are not influenced by matrix effects (Deprez et al., 2016; Magnusson and Örnemark, 2014). The selectivity of the method was assessed by performing triplicate reactions of previously extracted *S. salar* DNA mixed with *O. kisutch* DNA and *O. tshawytscha* DNA (paragraph 2.1.1.) in different ratios (Table 3).

## Quantification of Atlantic salmon in mixed tissue samples

To assess quantification of *S. salar* in mixed samples, tissues from *S. salar* and *O. mykiss* were purchased andmixed at different weight percentages (0 % (non-target control), 10 %, 25 %, 50 %, 75 %, 90 %, 100 %) to assess if the initial weight percentage of *S. salar* is reflected in the final measured DNA concentration. Three fresh *S. salar* fillets (taken from whole specimen) were purchased from three different fishmongers and one *O. mykiss* specimen was purchased from a retailer. Extractions were made by using a total of 200 mg tissue. The exact amount of tissue for each percentage was finely cut and put in a reaction tube with lysis buffer, where a pestle was used to mash and homogenise the salmon tissue mixture. Total DNA after extraction was quantified with QuantusTM fluorometer and diluted to fit within the working range of the method. The ddPCR assay was used to measure the *S. salar* copy concentration which was then converted to ng DNA of *S. salar* and divided by the total ng DNA in the sample. This percentage was then compared with the weight percentages.

## Impact of food processing on the ddPCR assay

To test the effect of food processing on copy number estimates, three fresh *S. salar* fillets were purchased, each from a different fishmonger in Ostend (Belgium), and immediately transported to the lab for processing. Each fillet was divided into five pieces. Each piece was subjected to a different processing treatment (resulting in three biological replicates or pieces per treatment): raw, frozen, poached, marinated and hot smoked. The raw treatment pieces were kept on ice until DNA extraction on the same day as they were purchased.

For the poached salmon recipe, also known as ‘Bellevue’, a vegetable broth was prepared by filling a cooking pot with water and adding a leak, a carrot, an onion (all sliced in half), a bundle of thyme, a laurel leaf and a cube of concentrated vegetable stock. This broth was heated and left boiling for 45 min. Then, three salmon pieces were put in the vegetable broth (while hot, no longer boiling) and left there until the broth cooled down to room temperature. Then, DNA was extracted from the three poached pieces, equally on the day of purchase. To test the effect of freezing, three pieces of the freshly purchased Atlantic salmon fillets (one piece of each fillet) were stored in the freezer for one week, after which DNA was extracted. Marinating was done with the following ‘Gravadlax’ recipe: A pickle mix was made using brown sugar, salt and grated zest of lemon and orange. On the day of purchase, three salmon pieces were coated in the pickle mix and topped with finely chopped dill, after which they were wrapped tightly in cling foil and put in the fridge overnight. The day after, the salmon pieces were cleaned of excess salt and placed in the fridge for three more hours. Finally, the pieces were coated in dill and cut into thin slices, from which DNA was extracted. To smoke the salmon, similarly to marinating, a pickle mix was made using brown sugar and salt. On the day of purchase, three salmon pieces were coated in the pickle mix, wrapped tightly in cling foil and put in the fridge overnight. The day after, the pieces were cleaned of excess salt and put back in the fridge for three hours. A smoker was created by coating the inside of a cooking pot with aluminium foil and putting an aluminium roster in the pot. The bottom of the pot was filled with half a centimetre of sugar, dry rice and tea leaves. The pieces of salmon were put on the roster and the stove was put on maximum heat until smoke was perceived. The salmon pieces were left in the smoke for 10 min, after which DNA was extracted.

The resulting 15 DNA extractions (five treatments with 3 biological replicates) were stored at -20 °C. Each extraction was quantified using QuantusTM fluorometer and diluted to 10 ng/µl, resulting in 25 ng in the final master mix. Each of the 15 extractions was quantified using the optimised ddPCR protocol in triplicate. To investigate whether processing methods affected the quantification results, an analysis of variance (ANOVA) with block design was performed testing the effect of processing with biological replicates as blocking factors. The ANOVA was performed in R (R Core Team, 2018), using the package *car* (Fox and Weisberg, 2019)*.* Assumptions of normality and homogeneity were tested using a Shapiro-Wilk (p > 0.05) test and a Levene’s test (p > 0.05), respectively.

## Identification and quantification of Atlantic salmon from retail food products

The ddPCR assay was used to identify and quantify Atlantic salmon in 46 food products purchased at retailers labelled as Atlantic or Pacific salmon. To test the applicability of the assay to real life samples, a wide range of product types (raw, smoked, steamed, frozen, poached, brined, Modified Atmosphere Packaging of Fish and Seafood, canned and tubbed) was selected to account for the different manners in which salmon are sold (Table 5). The Pacific salmon products were included to investigate whether they were substituted with cheaper Atlantic salmon since this is the most common substitution in salmonids (Luque and Donlan, 2019). Food products were acquired from Belgian (31 samples) and Polish retailers (15 samples). The processing method, commercial and scientific name, and the percentage of Atlantic salmon were recorded. The accuracy of the product labels was assessed, according to the European law, stating that these products require both commercial and scientific names to be present according to the national guidelines (“Commercial and scientific name of the species,” n.d.). A 200mg sample of each retail product was taken and extracted (paragraph 2.1.1.). When chunks of salmon were present in the sample, these were used for DNA extraction, while a representative piece was scooped and extracted when the processed product was fully homogenised. The total DNA was measured with the QuantusTM fluorometer. Where possible, the samples were diluted within the working range of the ddPCR assay. For each sample, three technical replicates were run and the mean was used as final concentration*.* The estimated *S. salar* content was then compared with the declared salmon percentage on the labels.

# Results

## Development of an Atlantic salmon specific ddPCR assay

Among all conditions tested, 64 °C was found to be the optimal temperature where the difference between non-target and target DNA droplet populations was sufficiently large to set a proper threshold (Supplementary Figure S1). Primer and probe concentrations were kept at 900 nM and 250 nM, respectively. Lowering the temperature caused non-target amplification while increasing the temperature resulted in no amplification (data not shown). Increasing the number of cycles above 45 did not increase the number of droplets with amplification, as primers and probes within the droplet are likely depleted. Changing the primer and probe concentrations did not affect the ability to distinguish *S. salar* from other salmonids.

## ddPCR quantification of Atlantic salmon DNA

### Working range, LOD & LOQ of the ddPCR assay

Amplification of the target droplets became indistinguishable from the negative droplet cluster at 150 ng, as the positive cloud became far too thick and also appeared to be lower (data not shown). Below 0.015 no amplification was detected (Table 2). The LOD was set at 0.024 ng (or 0.37 cp/µl), the lowest concentration without false negatives. The first concentration where the coefficient of variance surpassed 25 % (39.10 %) was observed at 1.5 ng (or 26.4 cp/µl) (Table 2). Hence, the LOQ was set at 3 ng total DNA or 50.51 cp/µl. Regression analysis showed a linear relationship (R² = 0.96) between the measured total DNA concentration and the cp/µl measured by ddPCR from 0.024 ng (or 0.37 cp/µl) up to 75 ng (or 1191.17 cp/µl) (Figure 1). Based on this linear relationship, the conversion of the copy numbers per µL to percentage *S. salar* in a sample can be calculated as:

|  |  |
| --- | --- |
|  | *Eq. 1* |

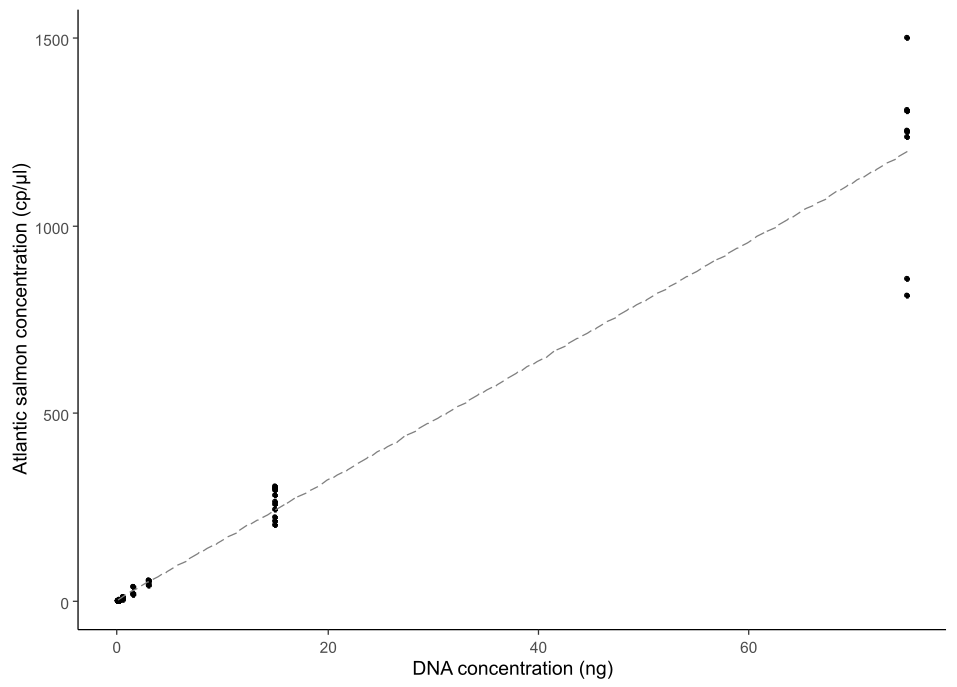


Figure 1 Linear relationship between total DNA (ranging from 0.024 ng to 75 ng, measured by the QuantusTM fluorometer) and the measured cp/µl of target DNA in the ddPCR assay. f(x) = 15.8847 \* x – 4.9527

Table 2 Working range, the limit of detection and limit of quantification of the ddPCR assay. Total DNA is measured by QuantusTM fluorometer. Mean measured copies per microliter (cp/µl) by the ddPCR. SD = Standard deviation. CV = Coefficient of variance. Only the replicates (3 biological, 3 technical) with over 10 000 droplets were included for the analysis. A false negative was recorded when no positive droplets were measured despite DNA being present in the sample.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Total DNA (ng) | Mean measured cp/µl | SD | CV | Replicates | False negatives |
| 150 | 0 | 0 |  | 4 | 4 |
| 75 | 1191.17 | 234.22 | 19.66 | 8 | 0 |
| 15 | 262.38 | 37.44 | 14.27 | 11 | 0 |
| 3 | 50.51 | 6.35 | 12.57 | 5 | 0 |
| 1.5 | 26.43 | 10.34 | 39.10 | 5 | 0 |
| 0.6 | 8.88 | 2.10 | 23.67 | 6 | 0 |
| 0.15 | 1.63 | 0.76 | 46.93 | 8 | 0 |
| 0.12 | 1.37 | 0.59 | 42.73 | 7 | 0 |
| 0.024 | 0.37 | 0.19 | 51.39 | 6 | 0 |
| 0.015 | 0.099 | 0.11 | 109.59 | 9 | 4 |
| 0.0015 | 0 | 0 |  | 6 | 6 |
| 0.00015 | 0 | 0 |  | 4 | 4 |

### The selectivity of the ddPCR assay

*Salmo salar* DNA (Table 1) was mixed with DNA from *O. tshawytscha* and *O. kisutch* (Table 1)at various percentages to investigate whether the presence of other salmon DNA would affect the detection of *S. salar* DNA with the ddPCR assay. The amplitude of the positive droplet population remained the same regardless of the amount of non-target DNA that was added. Both *O. tshawytscha* and *O. kisutch* were amplified by the method, but were visible as a second population of droplets below the target droplet population, allowing for a threshold to be set between the two populations (Figure 2). The estimated DNA percentages were lower than the observed percentages, but still comparable and consistent, indicating that the presence of closely related salmonid DNA in the matrix does not impact the quantification results (Table 3).

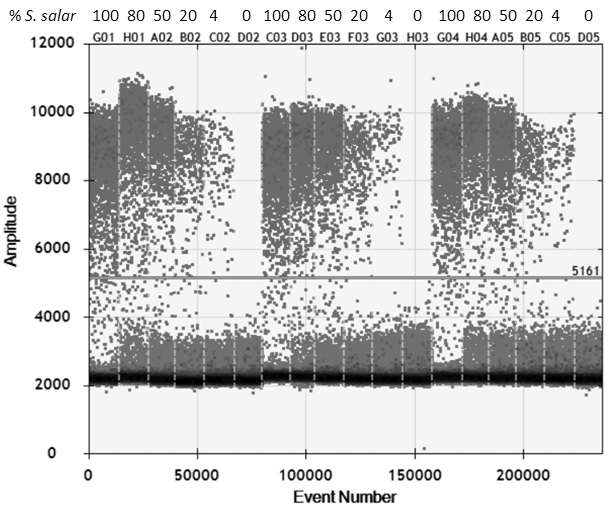


Figure 2 ddPCR amplitude of three times six mixtures of Salmo salar and Oncorhynchus tshawytscha, containing different percentages of S. salar (100, 80, 50, 20, 4 and 0 (NTC)). The x-axis represents the number of droplets measured (event number), the y-axis represents the amplitude measured per droplet. Horizontal line: the threshold at 5161 amplitude. Bottom droplet populations: everything below the threshold value is either rain from S. salar amplifications, background noise, or amplification from related salmonids; everything above the threshold is considered positive droplets and represents amplification from target DNA.

Table 3 Mixtures of Salmo salar DNA with Oncorhynchus kisutch and O. tshawytscha. For every mixture, the Total DNA added, and the amounts of S. salar, O. kisutch and O. tshawytscha are recorded, as well as the final percentage of S. salar in the sample. Results of the ddPCR are recorded as mean measured cp/µl and the standard deviation (SD) and coefficient of variation (CV) are recorded as well. Replicates represent the number of replicates that contained more than 10.000 droplets in total. The estimated S. salar content is calculated using Eq. 1.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **S. salar DNA (%)** | **Total DNA (ng)** | **S. salar DNA (ng)** | **O. kisutch DNA (ng)** | **O. tshawytscha DNA (ng)** | **Mean measured cp/µl** | **SD** | **CV** | **Replicates** | **Estimated S. salar content (%)** |
| 100 | 16.75 | 16.75 | 0 | 0 | 241.27 | 12.79 | 5.3 | 3 | 92.39954 |
| 80 | 16.75 | 13.4 | 0 | 3.35 | 188.86 | 6.33 | 3.35 | 3 | 72.328 |
| 50 | 16.75 | 8.38 | 0 | 8.37 | 114.52 | 1.77 | 1.55 | 3 | 43.8579 |
| 20 | 16.75 | 3.35 | 0 | 13.4 | 33.76 | 2.1 | 6.23 | 3 | 12.92912 |
| 4 | 16.75 | 0.67 | 0 | 16.08 | 9.72 | 1.01 | 10.4 | 3 | 3.722483 |
| 80 | 16.75 | 13.4 | 3.35 | 0 | 193.06 | 5.02 | 2.6 | 3 | 73.93648 |
| 50 | 16.75 | 8.38 | 8.37 | 0 | 113.13 | 3.3 | 2.91 | 3 | 43.32557 |
| 20 | 16.75 | 3.35 | 13.4 | 0 | 42.59 | 4.58 | 10.75 | 2 | 16.31076 |
| 4 | 16.75 | 0.67 | 16.08 | 0 | 10.02 | 0.66 | 6.61 | 3 | 3.837375 |

## Quantification of Atlantic salmon DNA in mixed tissue samples

To accurately quantify *S. salar* in mixed food products, the weight percentage of salmon tissue in mixed tissue samples should be reflected in the DNA percentages estimated with ddPCR. A clear positive linear relationship is observed between the Atlantic salmon DNA in the tissue mix and the measured copies per microliter detected by ddPCR (Figure 3A). Also, the expected Atlantic salmon DNA in the PCR mix, which is extrapolated from the weight percentage and plotted against the concentration measured by the ddPCR (Figure 3B), resulted in a linear relationship (R² = 0.89, p < 0.001). This relationship can be used to more accurately estimate the original Atlantic salmon percentage in a product (Table 4).

|  |  |
| --- | --- |
|  | *Eq. 2* |

Table 4 Validation with mixed tissue samples, where Salmo salar tissue was mixed with Oncorhynchus mykiss tissue at different ratios (200 mg total). Percentage S. salar tissue represents the percentage of S. salar tissue used for extraction, where the rest is O. mykiss. The mean estimated S. salar content (%) is the average estimated S. salar content based on Eq. 2 with its coefficient of variation (CV).

|  |  |  |
| --- | --- | --- |
| Percentage of  *S. salar* tissue (%) | Mean estimated *S. salar* content (%) | CV |
| 10 | 16.05 | 31.74 |
| 25 | 34.04 | 38.34 |
| 50 | 68.21 | 12.63 |
| 75 | 64.65 | 47.99 |
| 90 | 92.3 | 16.20 |
| 100 | 106.3 | 4.40 |

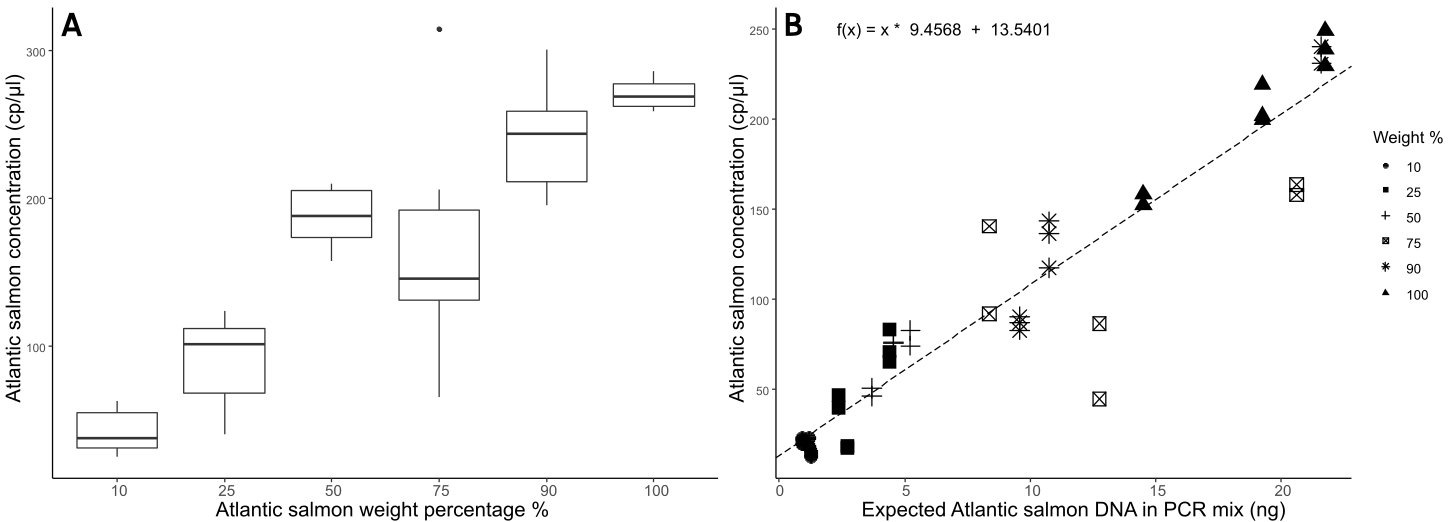


Figure 3 (A) DNA extracts from samples with different weight percentages of S. salar (x-axis), compared to the concentrations measured by the ddPCR assay (y-axis). The top and bottom side of the boxes represent the 75th (Q3) and 25th (Q1) percentile respectively and the horizontal black line in the box is the 50th percentile. The dots are outliers and are considered outliers if their value is less than Q1 – 1.5 \* IQR or greater than Q3 + 1.5 \* IQR (where IQR = Q3-Q1, the length of the box). The upper whiskers are calculated as min(max(x), Q3 + 1.5 \* IQR) and the lower whiskers are calculated as max(min(x), Q1 – 1.5 \* IQR). (B) Linear regression plot of the Salmo salar DNA in the PCR mix (based on initial weight percentages) (x-axis) compared to the concentrations measured by ddCPR (y-axis).

## Impact of processing on the ddPCR assay

ANOVA (Supplementary Table S3) results show that processing significantly impacted the measured concentrations obtained by the ddPCR (p = 1.08e-07) explaining 63 % of the variation, while another 24 % of the variation could be explained by the biological replicates. Tukey’s test (Supplementary Table S4) comparing the means of processing compared to raw fillets indicated significant differences for freezing (p = 8.35e-06) and marinating (p = 0.039), with freezing and marinating resulting in respectively 46 % and 23 % fewer copies than the raw treatment.

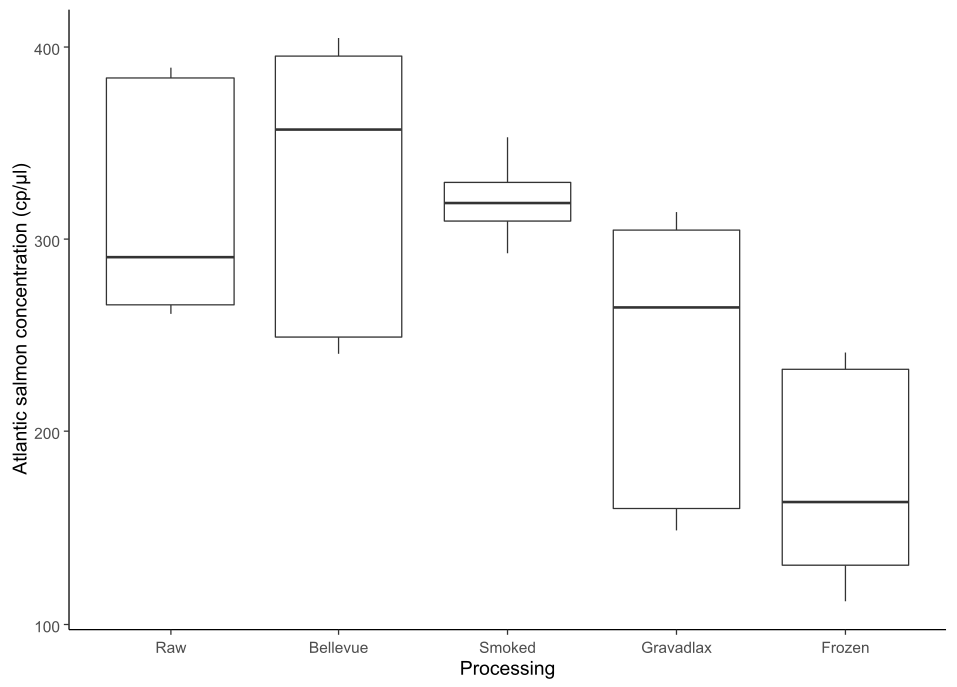


Figure 4 Boxplot graph comparing the effect of various processing procedures of S. salar fillets on the ddPCR measured concentrations. The x-axis contains the processing techniques and the y-axis contains the concentrations measured by the ddPCR for 25 ng total DNA added to the PCR mix. The top and bottom side of the boxes represent the 75th and 25th percentile and the centreline is the 50th. The upper whiskers are calculated as min(max(x), Q3 + 1.5 \* IQR) and the lower whiskers are calculated as max(min(x), Q1 – 1.5 \* IQR) (where IQR = Q3-Q1, the length of the box).

## Identification and quantification of Atlantic salmon in retail food products

A total of 46 retail products were tested (31 Belgian and 15 Polish, Table 5). Out of 138 reactions (three replicates per product), two reactions representing two different products were rejected for having less than 10 000 droplets. For 20 of the 31 Belgian products, the commercial name of the fish was not explicitly stated or an incorrect variant of the species name was used (e.g. C3-C10, where ‘roze zalm’ is used instead of ‘roze Pacific zalm’). Eight Belgian products were ambiguously labelled as generic salmon or trout. Three of the 15 Polish the generic word for salmon, ‘łosoś’, was used. The scientific name was not present on 12 products (10 Belgian, 2 Polish) (“Commercial and scientific name of the species,” n.d., “Gemeenschappelijke Marktordening | Departement Landbouw & Visserij,” n.d., “Handelsbenamingen,” n.d.). Besides the 4 processing techniques tested earlier (Freezing, smoking, poaching and marinating), the gathered retail products also contained additional processing techniques that were not tested in this study (sterilizing, canning and MAP or Modified Atmosphere Packaging of Fish and Seafood).

As anticipated from the label, no Atlantic salmon was detected in any of the 16 food products that contained Pacific salmon (C1-C16). In fifteen out of sixteen Atlantic salmon food products that contained distinct pieces of fish tissue (A1-A16), percentages of *S. salar* above 86 % were recorded, which is relatively close to the 100 % that is expected for single species samples. One frozen sample (A5) was estimated at 52 % instead of 100 %, which is expected as freezing was shown to greatly impact the ddPCR output. Some estimations were above 100 %, which enforces the likelihood that no other DNA is present. From the 14 homogenised products declaring to contain Atlantic salmon (B1-B14), no Atlantic salmon could be detected in three canned products (Polish samples B2, B4 and B5), as their concentrations measured by the ddPCR were below the limit of detection. These three samples were additionally amplified with universal *Cytochrome b* (*cytb*) 364bp primers (van Ginderdeuren et al., 2012) in traditional PCR, but no amplification was detected on agarose gel (Supplementary figure S2). The other results of the homogenised samples were mixed, with some estimations being similar to the percentage mentioned on the label, and others being higher or lower than mentioned on the label.

Table 5 Analysis of 46 commercial retail products. Sample numbers starting with A contained either whole salmon fillets, or easy to discern pieces of a food product. Sample numbers starting with B were completely homogenised where no salmon pieces could be identified visually. C are samples containing Pacific salmon species according to the label. Processing techniques are recorded for every product. The common name on the label was recorded and where the common name on the label was incorrect or missing, the correct commercial designation is given in the column to the right. The scientific name was recorded if present on the label. The percentage of Salmo salar was recorded when declared. Total DNA is the DNA in the master mix, measured by QuantusTM fluorometer. The estimated S. salar concentration was calculated using Eq. 2.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Nr | Processing | Country | Common name on label | Correct common designation | Scientific name | Declared *Salmo salar* % | Other fish | Total DNA added (ng) | Mean measured cp/µl | Estimated salmon content (%) |
| A1 | Smoked | Belgium | Atlantische Zalm |  | *S. salar* | 97 |  | 24.5 | 213.31 | 86.98 |
| A2 | Raw | Belgium | Zalm | Atlantische zalm | *S. salar* |  |  | 11.95 | 110.72 | 87.49 |
| A3 | Raw | Belgium | Zalm | Atlantische zalm |  | 16 |  | 27.5 | 339.82 | 124.2 |
| A4 | Steamed & Smoked | Belgium | Zalm | Atlantische Zalm |  | 28 |  | 30 | 323.77 | 108.92 |
| A5 | Frozen | Belgium | Atlantische zalm |  | *S. salar* | 100 |  | 15 | 81.05 | 52.16 |
| A6 | Smoked | Belgium | Atlantic salmon | Atlantische zalm | *S. salar* | 97 |  | 30 | 316.49 | 106.47 |
| A7 | Belle-vue | Belgium | Atlantische Zalm |  | *S. salar* | 98 |  | 42.5 | 498.87 | 120.08 |
| A8 | Belle-vue | Belgium | Atlantische Zalm |  |  | 98 |  | 26.25 | 293.14 | 111.98 |
| A9 | Smoked | Belgium | Atlantische zalm |  | *S. salar* | 97 |  | 14 | 327.53 | 224.44 |
| A10 | Smoked | Belgium | Atlantische zalm |  | *S. salar* | 97 |  | 14 | 446.91 | 306.24 |
| A11 | Smoked | Belgium | zalm | Atlantische zalm | *S. salar* |  |  | 9.5 | 339.51 | 328.41 |
| A12 | Raw | Belgium | Atlantische zalm |  | *S. salar* |  |  | 3.8 | 106.14 | 214.53 |
| A13 | Smoked | Belgium | Atlantische zalm |  | *S. salar* |  |  | 5.9 | 126.22 | 182.04 |
| A14 | Raw | Belgium | Zalm | Atlantische zalm | *S. salar* |  |  | 4.94 | 124.72 | 206.98 |
| A15 | Sterilised & Brined | Poland | łosoś atlantycki |  | *S. salar* | 40 |  | 0.43 | 18.52 | 105.19 |
| A16 | MAP | Poland | łosoś atlantycki |  | *S. salar* | 23.5 |  | 1.7425 | 38.83 | 129.35 |
| B1 | Canned | Belgium | Zalm en forel | Atlantische zalm & regenboogforel |  | 4 | 4% trout | 26.25 | 1.1 | 0.42 |
| B2 | Canned | Poland | łosoś atlantycki |  | *S. salar* | 25 |  | 3.875 | 0.05 | 0.1 |
| B3 | Canned | Poland | łosoś atlantycki |  | *S. salar* | 25 |  | 5.25 | 0.99 | 1.57 |
| B4 | Canned | Poland | łosoś | łosoś atlantycki | *S. salar* | 26 |  | 0.515 | 0 | 0 |
| B5 | Canned | Poland | łosoś atlantycki |  | *S. salar* | 24 |  | 4.775 | 0 | 0 |
| B6 | MAP | Poland | łosoś atlantycki |  |  | 6.7 |  | 0.6125 | 1.35 | 6.98 |
| B7 | MAP | Poland | łosoś atlantycki |  | *S. salar* | 24 |  | 15.75 | 248.49 | 152.93 |
| B8 | MAP | Poland | łosoś atlantycki |  | *S. salar* | 51 |  | 30 | 312.23 | 105.04 |
| B9 | Tubbed | Poland | łosoś | łosoś atlantycki | *S. salar* | 29 | 7% cod and saithe | 27.5 | 230.62 | 84.29 |
| B10 | MAP | Poland | łosoś atlantycki |  | *S. salar* | 85 |  | 45 | 599.04 | 136.43 |
| B11 | Canned | Poland | łososia atlantyckiego |  | *S. salar* | 30 |  | 4.775 | 6.61 | 11.26 |
| B12 | MAP | Poland | łosoś | łosoś atlantycki |  | 30 |  | 0.595 | 8.76 | 45.7 |
| B13 | MAP | Poland | łososia atlantyckiego |  | *S. salar* | 30 |  | 0.0755 | 2.13 | 14.94 |
| B14 | MAP | Poland | łosoś atlantycki |  | *S. salar* | 30 |  | 1.5325 | 25.07 | 89.43 |
| C1 | Frozen | Belgium | Pacific pink salmon | Roze Pacific zalm | *O. gorbuscha* |  |  | 32.5 | 0.07 | 0.02 |
| C2 | Frozen | Belgium | Rode pacific zalm | Roze Pacific zalm | *O. nerka* |  |  | 27.5 | 0 | 0 |
| C3 | Canned | Belgium | Roze zalm | Roze Pacific zalm |  | 99.20 |  | 35 | 0.07 | 0.02 |
| C4 | Canned | Belgium | Roze zalm | Roze Pacific zalm |  | 99 |  | 15.5 | 0.02 | 0.01 |
| C5 | Canned | Belgium | Roze zalm | Roze Pacific zalm | *O. gorbuscha* |  |  | 27.5 | 0 | 0 |
| C6 | Canned | Belgium | Roze zalm | Roze Pacific zalm | *O. gorbuscha* |  |  | 13.25 | 0 | 0 |
| C7 | Canned | Belgium | Rode zalm | Pacific rode zalm |  |  |  | 37.5 | 0.02 | 0.01 |
| C8 | Canned | Belgium | Roze zalm | Roze Pacific zalm |  |  |  | 32.5 | 0 | 0 |
| C9 | Canned | Belgium | Rode zalm | Pacific rode zalm |  |  |  | 30 | 0 | 0 |
| C10 | Canned | Belgium | Roze zalm | Roze Pacific zalm |  |  |  | 35 | 0 | 0 |
| C11 | Smoked | Belgium | Forel | Regenboogforel | *O. mykiss* | 98 |  | 13 | 0 | 0 |
| C12 | Smoked | Belgium | Regenboogforel |  | *O. mykiss* | 97 |  | 21 | 0 | 0 |
| C13 | Smoked | Belgium | Pacific rode zalm |  | *O. nerka* |  |  | 9.4 | 0 | 0 |
| C14 | Smoked | Belgium | Pacific rode zalm |  | *O. nerka* |  |  | 4.46 | 0 | 0 |
| C15 | Smoked | Belgium | Sockeye zalm | Pacific rode zalm | *O. nerka* | 96 |  | 7.3 | 0.06 | 0.07 |
| C16 | Frozen | Belgium | zalm | Pacific keta zalm | *O. keta* |  |  | 2.2 | 0 | 0 |

# Discussion

Substitution in mixed food products is difficult to investigate using traditional DNA barcoding approaches because resulting sequencing profiles contain confounding signals of the species present in the sample. When applied to experimental samples, the ddPCR assay developed in this paper resulted in a robust, accurate and specific identification of *S. salar*, even in mixed and fully homogenized samples. The percentage estimate as calculated from the analysis appears to be influenced by processing (especially freezing) and the presence of other ingredients, partially limiting the ability to estimate the original *S. salar* content with high accuracy.

## ddPCR identification of Atlantic salmon

Digital PCR systems detect a wide range of copy number concentrations ranging from a single copy to thousands of copies (Deprez et al., 2016). Results showed a clear linear relationship between the amount of DNA and the copy numbers retrieved with the ddPCR assay, allowing predictions in the range from 3 ng DNA (50.51 cp/µl) up to 75 ng DNA (1191.17 cp/µl). Adding too much DNA caused the positive clusters to become indistinguishable from the negative clusters, which was also reported by Mayer et al. (2019). The limit of detection for the designed ddPCR assay for Atlantic salmon was set at 0.024 ng (or 0.37 cp/µl), which is comparable with the theoretical LOD 0.32 cp/µl calculated for 15000 droplets (Deprez et al., 2016). The limit of quantification was set at 3 ng (50.51 cp/µl).

Finally, mixing *S. salar* DNA with other salmonid DNA showed that the assay maintained its linearity in the presence of non-target DNA. Other studies also showed species-specific ddPCR assays to work in complex matrices containing DNA from other species (Deprez et al., 2016; Mayer et al., 2019). Altogether, these results illustrate that the designed ddPCR assay for *S. salar* is highly sensitive, works efficiently over a large range of DNA concentrations and provides repeatable results even when mixed with DNA from other salmonid species.

## Quantification of Atlantic salmon is influenced by processing

Ideally, the quantification of the DNA copy number should not be influenced by the processing procedure of the original tissue. In other words, a given weight of cooked *S. salar* tissue should produce the same number of copy numbers as the equivalent weight of *S. salar* tissue processed in another way. However, the handling of fillets already proved to influence the copy numbers: fresh fillets purchased from fishmongers appeared to result in lower copy number concentrations measured by the ddPCR compared to the tissue that was fixed in ethanol and stored at -20°C from a previous study (Deconinck et al., 2020). The effect of processing was further showcased here, as freezing the samples led to an underestimation of Atlantic salmon copy numbers. To a lesser extent, poaching and marinating (brining and adding dill) also appeared to affect the copy number estimate. Smoking the salmon tissue did not result in significant differences in DNA copy number estimates compared to fresh tissue. It may be possible to create equations for every type of processing, but the duration, effect and differences in recipe per processing type may further influence the results.

Sterilizing, MAP and canning are commonly used processing techniques that were not tested in this study. It has previously been shown that the canning processes (pH change, high temperature and pressure) negatively impacted qPCR results through degradation and fragmentation of the DNA, leading to lower concentrations (Bauer et al., 2003; Hird et al., 2006). In the present study, the extractions from the canned retail products yielded low DNA concentrations (below the LOQ of 3 ng), meaning that such products cannot be accurately quantified. In the case of heavily processed products (canning or freezing), the *S. salar* content estimated through ddPCR will be an underestimation of the original weight percentage. The ddPCR assay returns a value lower than expected, but the label states that the product has been frozen. Moreover, with heavily degraded DNA, the concentration added to the PCR mix may be below the limit of quantification. We conclude that the ddPCR assay can quantify Atlantic salmon within mixed and processed products, but only in an experimental setting.

## Applicability of the assay for retail products

Ambiguous labelling appears to be a widespread problem on the market (Feng et al., 2017; Herrero et al., 2011). The producers are violating the standing EU standards, which states that both the commercial designation and scientific name should be presented, according to EU regulation No 1379/2013 and as such, should be enforced to correctly label products.

Despite the poor labelling of the retail products, no indication for substitution was detected in any of the products, meaning Atlantic salmon was detected in Atlantic salmon products, except for three products, which are discussed below, and no Atlantic salmon was detected in related salmon products. Additionally, all of the Belgian products were also analysed using a novel Salmon HRMA tool designed to identify all of the species in table 1 and all products were found to be correctly labelled. However, none of the Belgian canned products amplified using the HRMA tool (Monteiro et al. In progress), so no conclusive statement can be made for these products. For the products related salmon products C1, C3, C4, C7 and C15, low cp/µl are measured by the assay, but these samples only had between 1 and 3 positive droplets out of 10000 to 15000 droplets in total, which is below the previously determined LOD of 0.37 cp/µl, and the samples that were identified with the HRMA tool were correctly labelled. Additionally, as previously described (Supplementary Figure 1 and Figure 2), amplification could be observed for some of the related salmon products, but these droplet clusters had a fluorescence below the threshold. A previous global meta-analysis showed that Pacific salmon had been substituted in 17 % of the cases, most commonly by Atlantic salmon (Luque and Donlan, 2019). An American study showed that 7 % of Pacific salmon had been substituted with Atlantic salmon (Cline, 2012). In the present study, no Atlantic salmon was detected in any of the Pacific salmon products. The meta-analysis also showed that Atlantic salmon was substituted in 3 % of products (Luque and Donlan, 2019). Another study, which focused on creating an identification assay using qPCR, found 2 out of 13 Atlantic salmon samples in Chilean supermarkets to be substituted (Herrero et al., 2011). In the current study, no Atlantic salmon could be detected in three out of 15 Polish Atlantic salmon labelled products. Although we cannot exclude that these products contained other species than Atlantic salmon, it is most likely that the canning process was responsible for this observation, as all three products were canned and two other canned products also resulted in very low quantification estimates, suggesting that the DNA is extremely degraded (Table 5). These three canned samples also showed no amplification with traditional PCR using short mitochondrial assays (Cytb), so it is likely that the DNA in these samples were to degraded to detect Atlantic salmon. There are only two studies dedicated to the identification of salmon in the European market which indicated a low substitution rate of 5 % and 6 % (Herrero et al., 2011; Muñoz-Colmenero et al., 2017). Together with the results from the present study, this indicates that the substitution of salmon in Europe may not be common.

## Strengths and weaknesses of the assay

One of the goals of this paper was to create a method that allows evaluating the percentage of Atlantic salmon in processed and mixed food products through the use of DNA quantification using ddPCR, comparable to other studies using qPCR (Bojolly et al., 2017; Hansen et al., 2020) or ddPCR (Mayer et al., 2019; Wang et al., 2019).

For food products where salmon pieces were easily picked out of the food product, it would be best to identify the piece of salmon and compare its weight to the rest of the product to assess the weight percentage. For example, A3 (Table 5) was a piece of sushi consisting of salmon on top of rice, where the product was labelled to contain 16% *S. salar*. For these products, it would be better to identify the fish pieces in the product, individually weigh them and compare them with the total weight of the product to estimate the real weight percentage. As such, the salmon pieces were isolated in these products (A3, A4, A15 and A16) and identified using the assay. In all products where salmon pieces were easily picked out from the food matrix, the method returned an estimation close to 100 % or higher. This is to be expected, as whole pieces of one single species would return either 0 or 100 %. For most of the homogenised food products that were not canned, the estimate was higher than the observed percentage mentioned on the label. When inspecting the ingredient list of these food products, several ingredients without DNA, such as lipids, proteins (egg white) and carbohydrates (starch), are listed. These ingredients cannot be traced with DNA analytical methods (Laube et al., 2007). For products that contain ingredients that cannot be traced with DNA analytical methods, the weight percentages on the label cannot reflect the DNA ratios. A DNA extraction of a product with 50 % Atlantic salmon and 50 % ingredients that do not contain DNA will result in 100 % Atlantic salmon DNA. Other factors that could possible influence the ratio of target copy compared to total DNA are variations in mitochondrial DNA and bacterial DNA. Although mitochondrial DNA concentrations are dependent on the tissue type and salmon fillets are always the same muscle (dorsal or ventral muscle) tissue (Alberts et al., 2005; Moraes, 2001). In fresh seafood bacteria only constitute a small fraction of the food, so unless the food is extremely spoiled, this should be negligible (Dulbecco and Ginsberg, 1973; Odeyemi et al., 2020). While overestimation caused be ingredients without DNA interferes with the goal of this study to quantify the Atlantic salmon percentage within mixed food products, as long as food products are not extremely processed, such as the canned samples**,** the ddPCR assay is still useful to quickly identify substitution and dilution fraud, as the overestimation equally indicates that the food product was probably not spiked with a cheaper alternative.

Based on the results, quantification of *S. salar* DNA in food products employing the designed ddPCR assay appears to work relatively well, provided that type of processing, as well as the other ingredients used in the processing of the food product, are known, especially in case these ingredients do not contain DNA. Other studies compared ratios, based on Ct values of qPCR assays for two species, to avoid the impact of processing and added ingredients (Bojolly et al., 2017; Floren et al., 2015; Nagase et al., 2010; Ren et al., 2017; Sánchez et al., 2019). This is interesting since processing would influence the DNA quality of both species, meaning that ratios would remain similar. However, such an approach can only be used when a product contains only those two species, which was never the case in the present study. Currently, no alternatives exist to quantify the DNA of processed seafood that lacks any morphological characteristics (Hansen et al., 2020; Laube et al., 2007). A possible fine-tuning of the current assay, which might allow removing the effects of processing or the need for equations (*Eq. 2*), would be to design an additional PCR assay that allows quantification of universal animal and plant DNA and to incorporate correction factors for different extraction efficiencies (Hansen et al., 2020; Thomas et al., 2014; Vasselon et al., 2018).

# Conclusion

The present ddPCR assay is capable of identifying Atlantic salmon (*S. salar*) reliably and accurately in an experimental setting. However, it appears that in real world examples a lot of factors (variability in DNA per tissue or food type, ingredients without DNA, bacterial DNA, processing) impact the assay. It also provides accurate quantification estimates of Atlantic salmon DNA percentages in mixed fish tissue samples. The method relies on the quantification linearity between the number of fish tissue copies per microliter and the total DNA. When applied to food products, the ddPCR assay is influenced by the type of processing of the food product (mainly freezing and marinating) and by the presence of ingredients that may or may not contain DNA. As such, the ddPCR assay only allows for a semi-quantitative evaluation of the Atlantic salmon percentage present in processed and mixed food products. Despite the its shortcomings, the ddPCR assay may still be useful to detect Atlantic salmon in complex multispecies matrices, as long as they are not too processed, like canned products. Additionally, if the ratio of Atlantic salmon and total DNA are vastly lower than expected, this may serve as an indication of the product contain less Atlantic salmon then mentioned on the label.

# Acknowledgements

We thank Miguel Faria from ICETA for providing salmonid tissues for the assays and Remigiusz Panicz for providing us with the Polish retail samples. We also thank Koen Degelas and Caroline Weydert (Bio-Rad) and Mieke Dhondt (ILVO) for their help with running ddPCR reactions. Additionally, we would also like to thank the reviewers for this study for their extensive input. This project has received funding from the European Union's Horizon 2020 funding programme. Grant Agreement no. 773400 (SEAFOODTOMORROW). This output reflects the views of the author(s) and the European Commission cannot be held responsible for any use that might be made of the information contained therein.

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