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The significance of *Shewanella* sp. strain HSO12, *Photobacterium phosphoreum* strain HS254 and packaging gas composition in quality deterioration of fresh saithe fillets

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A R T I C L E I N F O

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A B S T R A C T

This study presents the significance of *Shewanella* sp. strain HS012 and *Photobacterium phosphoreum* strain HS254 and the packaging gas composition (67% CO_2 balanced with either O_2 or N_2) related to fillet freshness, microbiological spoilage, and physiochemical parameters of portioned inoculated saithe fillets (*Pollachius virens*). Saithe fillets packaged in CO_2/N_2 gave, independent of inoculum, faster conversion of inosine (HxR) to hypoxanthine (Hx), and thereby increased fillet spoilage (increased H-value), faster increase in dimethyl amine (DMA) and trimethyl amine (TMA) content, the highest level of the tyrosine (Tyr), and the lowest level of lactate. Moreover, it was found that independent of initial microbiota and packaging gas composition, the main bacterial genera after 14 days of cold storage was *P. phosphoreum*. Among samples packaged in CO_2/N_2, the highest Hx and TMA content and the highest H-value were observed in samples inoculated with *P. phosphoreum*. Moreover, among fillets packaged in CO_2/O_2, samples inoculated with *P. phosphoreum* or a mix of both *Shewanella* sp. and *P. phosphoreum* (50:50) tended to have the highest H-value and the highest Hx and TMA levels. No clear evidence was found related to spoilage microbiota's impact on physiochemical parameters such as fillet drip loss or firmness.

1. Introduction

Saithe caught along the Norwegian coast-line is traditionally traded on ice as a fresh product for the Norwegian marked or exported as unprocessed, frozen or dried/salted/smoked products (Hjellnes, Rustad, & Falch, 2020). The share of supermarkets in Norway having possibilities to offer fresh fish is decreasing. Consequently, stakeholders need to implement new concepts for packaging that meet structural changes in the grocery business and fit consumers' demands (Asioli et al., 2017; Carlucci et al., 2015). To meet market demands, stakeholders within the seafood sector focus on value-added product developments (VAPD) involving actions such as, e.g., filleting, portioning, novel cuts, and active packaging concepts (Morrissey & DeWitt, 2014). Among concepts for functional packaging, modified atmosphere (MA) packaging has been widely studied (DeWitt & Oliveira, 2016; Sivertsvik, Jeksrud, & Rosnes, 2002) and has increased in popularity by producers and consumers (Carlucci et al., 2015).

The spoilage microbiota of fresh chilled and aerobic stored cold-water fish species are dominated by Gram-negative, psychrotrophic, aerobic, or facultative anaerobic bacteria such as *Pseudomonas* spp. or *Shewanella* spp. (Gram & Huss, 1996). *Shewanella putrefaciens* has been considered the most efficient H_2S-producing *Shewanella* specie; however, Vogel, Venkateswaran, Satomi, and Gram (2020) identified *S. baltica* as the dominant H_2S-producing specie in ice-stored marine fish. Furthermore, recent work identified inconsistencies in the existing *Shewanella* species classification using 16S rRNA sequences (Torell, Meier-Kolthoff, Sjöling, & Martín-Rodríguez, 2019), and taxonomic and physiological updates are necessary. The specific microbiota and production of spoilage metabolites in seafood depends on several factors, including packaging and storage concepts used (Dalgaard, Gram, & Huss, 1993; Debevere & Boskou, 1996; Gram & Dalgaard, 2002; Jakobsen, Shumilina, & Hoel, 2020; Lerfall, Bjørge Thomassen, & Jakobsen, 2018; Macé et al., 2013). *Photobacterium phosphoreum* often is notified as the specific spoilage organism (SSO) of MA-packaged seafood, especially at high CO_2 concentrations (>60%) (Dalgaard, Mejlholm, & Huss, 1997; Gram & Dalgaard, 2002). *S. putrefaciens* is more sensitive to CO_2 (Boskou & Debevere, 1997) and acts as the dominant spoilage microorganism in vacuum packages or air (Gram & Huss, 1996;

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Karl & Meyer, 2007). The growth of *P. phosphoreum* and *Shewanella* spp. in vacuum and MA packages is related to their ability of anaerobic respiration. In marine fishes, TMAO is a characteristic part of the non-protein nitrogen (NPN) fraction of marine fishes that biologically use TMAO as an osmolyte (Samerotte, Drazen, & Yancey, 2007). However, post-mortem, TMAO serves as a terminal electron acceptor for anaerobic respiration of spoilage microbiota such as, e.g., *P. phosphoreum* and *Shewanella* spp. (Dalgaard, 1995; Vogel et al., 2020). Other potential electron acceptors for anaerobic respiration that could be found are sulphurous amino acids, leading to hydrogen sulphide, methylmercaptan, and dimethyl-disulphide production (Leroi & Joffraud, 2011).

Spoilage of seafood is mainly due to the spoilage microbiota activity (Gram & Huss, 1996; Kuuliala et al., 2018). However, mechanisms driven by autolysis, such as catabolism of nucleotides, denaturation of proteins, and subsequently, changes in textural and water holding properties, are highly relevant regarding seafood freshness and product quality (Delbarre-Ladrat, Chéret, & Verrez-Bagnis, 2006; DeWitt & Oliveira, 2016; Hong, Regenstein, & Luo, 2017; Roth, Slinde, & Arildsen, 2006). The autolytical breakdown of adenosine triphosphate (ATP) to Inosine (HxR) followed by a bacterial conversion from HxR into hypoxanthine (Hx) allows moreover the assessment of seafood freshness and spoilage. Several quality indicators have been suggested whereof the K-value and the H-value are often used to measure seafood freshness and spoilage, respectively (Hong et al., 2017).

The ability of a fish muscle to retain water throughout the value chain is essential to improve consumers' perception, the overall product quality, and the profitability for the producer (Huff-Lonergan & Lonergan, 2007; Rotabakk, Jørpeland, & Lerfall, 2018; Schafer, Rosenvold, & Henckel, 2002). The water holding capacity (WHC) of a muscle and subsequently the drip loss throughout the value chain is an important quality attribute that must be considered to monitor seafood quality. The WHC of seafood is known to be affected by several pre- and post-mortem factors, e.g., stress before slaughter (Lerfall et al., 2015), starvation (Mørkøre, Mazo, & Einen, 2008), state of rigor mortis (Ofstad, 1996; Rotabakk, Jørpeland, & J. L, 2018) and packaging (Lerfall et al., 2018). Lerfall et al. (2018) reported moreover the drip loss of saithe fillets to be affected by the packaging concept. The lowest drip loss was reported of fillets stored in a MA consisting of CO_2 and O_2, followed by an atmosphere of CO_2 and N_2, of which those in vacuum packages showed the highest. Due to differences in spoilage microbiota present among saithe packaged in CO_2/O_2 and CO_2/N_2, respectively, Lerfall et al. (2018) suggested the fillet drip loss to be affected by the present microbiota in addition to other known mechanisms such as, e.g., post-mortem structural changes (Ofstad, 1996) and autolysis (Rotabakk et al., 2018).

Therefore, the present study aimed to study the significance of *Shewanella* sp. and *P. phosphoreum* related to fillet freshness, microbiological spoilage, and physiochemical parameters of MA-packed saithe stored at 4 °C for 14 days.

2. Material and methods

2.1. Fish material and experimental design

The saithe used in the present study was caught on April 23, 2018 by fishermen's using gillnets at the west coast of Norway (approximately 63.5°N, 9°S). All fish were instantly killed by a blow to the head before the fish were gill cut and transferred to a bleeding tank. After that, all fish were decapitated, gutted, and transported in an ice slurry to a local processing plant. Before transported to the Norwegian University of Science and Technology (NTNU, Trondheim, Norway), all fish were packaged in polystyrene boxes and transported on wet ice. Approximately 48 h post-mortem, a selection of 28 saithe ranging from 0.57 to 2.77 kg with a median weight of 1.63 kg was filleted and thereafter divided into portions of 75.1 ± 1.3 g. The fillet portions consisting of the dorsal muscle (n = 198) were randomly distributed into eight groups (24 samples in each group) whereas six samples were used for raw material charactersitics.

Of those groups, four were packaged in a MA consisting of CO_2/N_2 (67/33%), and four in CO_2/O_2 (67/33%), respectively. For each MA (CO_2/N_2 and CO_2/O_2, respectively), one group was inoculated with *Shewanella* sp. strain HS012, one with *P. phosphoreum* strain HS254, and one with a mix of both (50/50). As a control, natural non-inoculated samples were used. The experimental setup is presented in Table 1.

2.2. Bacterial strains and preparation of inoculum

Inoculums of the strains *Shewanella* sp. strain HS012 and *P. phosphoreum* strain HS254, previously isolated from saithe (Lerfall et al., 2018), were prepared by adding 10 mL/L thawed stock cultures (stored at - 80 °C) in separate 250 mL baffeled conical flasks containing 100 mL tryptone soy broth (TSB) (Oxoid CM0129, Oxoid Ltd., Basingstoke, UK). The isolated bacteria were originaly characterized by Gram staining, oxidase and catalase test. Colony appearance and bioluminescence ability on LH agar were tested for *P. phosphoreum* strain HS254, and colony appearance and ability to produce black colonies on iron agar were tested for *Shewanella* sp. strain HS012. The strains were identified by Sanger sequencing of the V3–V9 region of the 16S gene; however, a species separation was not defined for *Shewanella* sp. strain HS012 (*Shewanella putrefaciens* and *Shewanella baltica* were found among the closest relatives/top hits from the Blast search). The growth conditions of *P. phosphoreum* (HS254) was improved by adding 10 g/L sodium chloride (NaCl) to the broth. The cultures were incubated (24 h, 100 rpm) at 22 °C for *Shewanella* sp. strain HS012 and 15 °C *P. phosphoreum* strain HS254. After 24 h, 1 mL of both cultures were transferred to separate conical flasks containing 100 mL broth and incubated (7 °C, 100 rpm) for 24 h to ensure cold-adapted cultures in the early stationary growth phase. Thereafter, the cultures were diluted to an appropriate optical density (OD) (600 nm, Shimadzu UV spectrophotometer, Shimadzu Corporation, Kyoto, Japan) corresponding to a bacterial concentration of 10^6 colony forming units (CFU)/mL. The mixed inoculum was prepared by mixing diluted *Shewanella* sp. strain HS012 and *P. phosphoreum* strain HS254 in a ratio 1:1.

2.3. Inoculation of samples and packaging

The saithe samples were inoculated with the cold-adapted cultures (Table 1). Inocula (750 μL) were dispersed onto the surface in droplets and spread evenly on the surface using a sterile spreader, ending up with a concentration of approximately 10^4 CFU/g salmon. The relatively high concentration of inoculum was applied to ensure detection of the produced metabolites in a naturally contaminated system, as previously described in Jakobsen, Shumilina, Lied, and Hoel (2020). The samples were allowed to dry for 30 min before packaging. After inoculation, samples were packaged in 230 mL semi-rigid crystalline polyethylene

Table 1
Experimental setup showing packaging gas and inoculum for all experimental groups.

Group name	Packaging gas[a]	Inoculum
Control N_2	CO_2/N_2	-
Shew N_2	CO_2/N_2	*Shewanella* sp. (HS012)
Photo N_2	CO_2/N_2	*Photobacterium phosphoreum* (HS254)
Mix N_2	CO_2/N_2	*Shewanella* sp. (HS012) and *P. phosphoreum* (HS254)
Control O_2	CO_2/O_2	-
Shew O_2	CO_2/O_2	*Shewanella* sp. (HS012)
Photo O_2	CO_2/O_2	*P. phosphoreum* (HS254)
Mix O_2	CO_2/O_2	*Shewanella* sp. (HS012) and *P. phosphoreum* (HS254)

[a] The gas composition was 67% CO_2 and 33% O_2 or N_2.

terephthalate (CPET) trays (C2125-1A, Færch Plast, Holstebro, Denmark) using a semi-automatic tray sealing packaging machine (TL250, Webomatic, Bochum, Germany) giving a degree of filling (DF) of approximately 33%. The atmosphere was evacuated to an end vacuum pressure of 0.25 mPa and subsequently flushed with the gas mixture before sealing the top film. The top film used was a 40 μm thick combination of polyethylene (PE), ethylene vinyl alcohol (EVOH), polyamide (PA), and polyethylene terephthalate (Topaz B-440 AF, Plastopil, Almere, The Netherlands). Food-grade CO_2 and N_2 or O_2 were mixed using a gas mixer (MAP Mix 9000, Dansensor, Ringsted, Denmark) to obtain preset packaging gas mixtures of 67% CO_2 balanced with either N_2 or O_2 according to Table 1. Oxygen transmission rate (OTR) was 66–78 $cm^3 \times 25$ μm x m^2 x 24 h x Pa at 23 °C for the tray and 2.5 $cm^3 \times 40$ μm x m^2 x 24 h x Pa at 23 °C for the cover film. After packaging, the trays were stored at 4 °C until analyses (maximum 14 days).

2.4. Headspace gas analyses

The headspace gas composition (O_2 and CO_2) was measured using an oxygen and carbon dioxide analyzer (Checkmate 9900 analyzer, PBI-Dansensor, Ringsted, Denmark). Moreover, the amount of N_2 was assumed to balance O_2 and CO_2 up to 100%. Twenty mL of the headspace gas was collected with a syringe through the top film. Before measurement of the composition, a rubber septum (Nordic Supply, Skodje, Norway) was placed onto the top foil to avoid rupture and introduction of a false atmosphere. In addition, the gas compositions were measured in empty trays immediately after packaging and in sample trays after 2, 5, 7, 9, 11- and 14-days storage.

2.5. Microbiological analyses

A 10-g sample of fish muscle was aseptically transferred to a sterile stomacher bag and diluted 1/10 with sterile peptone water (1.0 g bacteriological peptone and 8.5 g/L NaCl) and homogenized vigorously for 60 s in a Stomacher 400 Lab Blender (Seward Medical Ltd., Gwent, UK). Appropriate serial dilutions were made in sterile peptone water and spread at their respective agar plates. Psychrotrophic aerobic plate count (PC) was quantified by spreading on Long and Hammer agar (L&H) with 10 g/L NaCl to support the growth of the salt requiring *P. phosphoreum* (NMKL, 2006). Plates were incubated at 15 °C for six days. Aerobic plate count (APC) and H_2S-producing bacteria were enumerated by pour plating on Lyngby's iron agar (IA) (Oxoid, Oslo, Norway) supplemented with 0.4 g/L L-cysteine (Sigma-Aldrich, Oslo, Norway) as total and black colonies, respectively. Plates were incubated at 22 °C for 72 h. *Pseudomonas* spp. was quantified on Pseudomonas agar base (CM0559, Oxoid, Oslo, Norway) supplemented with Pseudomonas CFC selective supplement SR0103 (Oxoid, Oslo, Norway) by spread plating and incubated aerobically at 25 °C for 48 h.

2.5.1. Identification of microbiota

Identification of microbiota in the different samples by partial 16S sequencing was performed as described by Lerfall et al. (2018). Single colonies from L&H agar plates with less than 100 colonies were picked, representing each treatment (Table 1). A total of 335 colonies from 22 samples (10 samples at day 0, and 12 at day 14, n = 12 to 27 colonies per sample) representing all experimental groups (n = 8) were included to be identified by partial 16S sequencing. The selected colonies were resuspended in 50 μL of Tris-EDTA buffer in a microtiter plate well, followed by heat treatment at 99 °C for 10 min. The plates were centrifuged at 4500×*g* for 3 min, and 30 μL supernatant was transferred to a new plate, which was stored at − 20 °C until further analysis.

A pair of universal primers (Nadkarni, Martin, & Hunter, 2002) 5′-TCCTACGGGAGGCAGCAGT-3, and 5′-GGACTACCAGGGTATCTAAT CCTGTT-3′ was used for the amplification V3–V4 region of 16S rRNA gene in a Doppio Gradient 732–2551 Thermal cycler, with 2 × 48-well universal blocks (VWR, Oslo, Norway). The reaction mixture, of a total volum of 24 μL, contained 1 μmol/L of each primer, HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) used according to the producer's recommendations, and 1 μL thawed supernatant as a template. The cycling conditions, were as follows: 95 °C in 15 min, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR products were purified with ExoSap-IT (ThermoFisher Scientific, appliedbiosystems™, Oslo, Norway). Sequencing was carried out at Eurofins Genomics, and genus was determined by a search of the approximately 400 bp fragment in the nucleotide database in NCBI/Blast.

2.6. Physiochemical parameters

The pH was measured in the raw material 24 and 48 h post-mortem (initial pH), and in all fish samples at day 2, 5, 7, 9, 11- and 14-post processing with a pH Selective Electrode Intellical™ PHC725 (Hach, Loveland, United States) connected to a Hach Multi-meter (Hach HQ40d multi Portable Meter, Hach, Loveland, United States).

The drip loss throughout storage was calculated gravimetrically according to Lerfall et al. (2018) as the sample weight difference between the raw sample and sample weight at day x (where x = 2, 5, 7, 9, 11, and 14, respectively).

The muscle's textural properties were analyzed in duplicates on each sample at each sampling day using a Texture Analyzer TA-XT2 (SMS Ltd., London, United Kingdom) equipped with a 25 kg load cell and a flat-ended cylinder probe (20 mm diameter, type P/1SP). The force-time graph was recorded and analyzed by the Texture Exponent light software for windows (version 4.12 SMS). The resistance force (N) was recorded with a constant speed of 2 mm/s, and the force (N) required to press the cylinder down to 60% of fillet thickness (F60%) was applied to describe fillet firmness.

2.7. Quantitative NMR analysis of metabolites

The metabolites, alanine (Ala), tyramine (Tyr), valine (Val), inosine monophosphate (IMP), HxR, Hx, lactate, dimethyl amine (DMA), and TMA) were analyzed using a modified method described by Shumilina, Ciampa, and Dikiy (2015). Muscles' sampling was carried out 48 h post-mortem before packaging (day 0), and at day 5, and 11 post-processing. Samples were immediately frozen and stored at −80 °C until further analyses. Metabolite analyses were performed on trichloroacetic acid (TCA) extracts (165.2 g/L) prepared from muscle homogenates as previously described by Shumilina, Ciampa, Capozzi, Rustad, and Dikiy (2015).

1D ^{1}H NMR spectra of all TCA extracts were acquired at 300 K on a Bruker Avance 600-MHz spectrometer (Billerica, United States) equipped with a 5-mm z-gradient TXI (H/C/N) cryoprobe. All NMR analysis were done at the NMR centre of the faculty of Natural Sciences at NTNU, Trondheim. The NMR experiments were acquired with the Bruker pulse sequences noesygppr1d, NS = 48 and RG = 144. The spectra were processed using the TopSpin 3.5pl7 software (Billerica, Massachusetts, United States). The signal of the external standard Trimethylsilylpropanoic acid (TSP) was used for spectral calibration (0 ppm). All peaks were assigned using our previous data (Shumilina et al., 2015, 2016). An integral value used for the metabolite's quantification was an average value of three integrations of the same resonance signal.

2.8. Statistics

The main effects of the experimental design were analyzed by a general linear model (GLM), where the "gas composition" (CO_2/O_2 or CO_2/N_2) and the "inoculum" (not inoculated, inoculated with *Shewanella* sp. strain HS012, *P. phosphoreum* strain HS254, or a mix of both (50/50)) was used as fixed factors and "storage" (days) as a co-factor. Statistical

analysis on microbial counts were done at log-transformed data. Significant differences between experimental groups were moreover analyzed by One-way ANOVA combined with Tukey's pairwise comparison test. Pearson's correlation coefficient (r) was used to calculate the linearity dependence between variables X and Y.

Statistical analyses were performed using an IBM Statistical Package for the Social Sciences statistics software (release 27, IBM Corporation, Armonk, New York, United States). The alpha level was set to 5% ($P < 0.05$). All results are given as an average ± standard deviation (SD) unless otherwise stated.

3. Results and discussion

The fish used in the present study was randomly selected from a commercial catch giving a distribution of experimental fish that varied in size (range 2.5–0.8 kg) and muscle pH (on average 6.6 ± 0.2 24 h post-mortem). The observed variation in muscle pH indicated differences in onset of rigor mortis among individuals (Kristoffersen, Tobiassen, & Olsen, 2006), potentially affecting muscle quality (Borderías & Sánchez-Alonso, 2011; Poli, Parisi, & Zampacavallo, 2005). An utterly randomized distribution of fillet portions allocated to each experimental group (Table 1) was chosen to avoid the effects of differences in quality between experimental samples.

3.1. Packaging gas composition

An average drop in headspace CO_2 concentration (on average 21%) was observed between day zero and day two, followed by a significant increase throughout 14 days storage ($P < 0.001$, on average 11%). The initial drop in headspace CO_2 after packaging is related to the absorption of CO_2 in the aqueous phase of the fillets (Abel, Rotabakk, & Lerfall, 2020). The slight increase in headspace CO_2 observed as a function of storage time did not correlate with bacterial growth, indicating environmental factors (e.g., changes in temperature, atmospheric pressure, etc.) to disturb the system equilibrium, and be the discriminant. The headspace concentrations of CO_2 and O_2 as a function of storage time are presented in Table 2. The results indicated similar storage patterns between groups except for the group ***Photo N₂***, which had on average, a slightly higher headspace CO_2 concentration compared to other groups ($P < 0.001$).

3.2. Microbiological activity

The mean initial PC enumerated on L&H and APC enumerated on IA of the raw material was 3.5 ± 0.3 and 2.5 ± 0.1 log CFU/g, respectively. The microbiological load was within expected levels of commercial caught saithe (Lerfall et al., 2018), at the same level as reported for Atlantic halibut (Rotabakk, Birkeland, & Sivertsvik, 2008), but considerably lower than reported for Atlantic cod fillets (Kuuliala et al., 2018). The mean initial PC and APC of the inoculated samples were Shew: 4.6 ± 0.1 and 4.7 ± 0.0 log CFU/g; Photo: 3.9 ± 0.1 and 3.0 ± 0.2 log CFU/g; and Mix: 4.4 ± 0.2 and 3.4 ± 0.9 log CFU/g, respectively.

The proliferation of PC was significantly affected by the MA, showing increased growth in samples packaged in CO_2/N_2 compared to those packaged in CO_2/O_2 (GLM: $P_{gas\ composition}<0.001$, Fig. 1A). Moreover, independent of the packaging atmosphere, higher counts were seen among samples inoculated with *P. phosphoreum*, or a mix of *P. phosphoreum* and *Shewanella* sp. compared to those inoculated with *Shewanella* sp. only ($P = 0.022$). These results indicate *P. phosphoreum* strain HS254 to be more tolerant to CO_2 than *Shewanella* sp. strain HS012 independent of the balancing gas used (O_2 versus N_2). Opposite to PC, no effect was observed regarding the MA used on APC enumerated on IA ($P > 0.775$, Fig. 1B). In the present study, higher counts were generally observed on L&H compared to IA medium for all samples analyzed (Fig. 1), which supports Kuuliala et al. (2018), who reported higher counts on L&H than IA medium for cod filets during storage due to the dominance of *Photobacterium* spp. H_2S-producing bacteria, defined as black colonies on IA, were initially not detected in neither control groups (***Control N₂*** and ***Control O₂***) nor experimental groups inoculated with *P. phosphoreum* (Fig. 1C). Low initial levels of H_2S-producing bacteria are in accordance with other studies (Gram & Dalgaard, 2002; Olafsdottir, Lauzon, & Kristbergsson, 2006) as H_2S-producing bacteria normally are constituted as a minor fraction of the microbiota on freshly caught fish. In the present study, H_2S-producing bacteria were detected in all samples inoculated with either *Shewanella* sp. or a mix of *P. phosphoreum* and *Shewanella* sp. (Fig. 1C), but limited growth as a function storage time was observed. This observation supports *Shewanella* spp. to be inhibited by the high level of CO_2 used in the packaging system (Debevere & Boskou, 1996; Gram & Dalgaard, 2002; Sivertsvik et al., 2002). Moreover, evaluated counts of H_2S-producing bacteria were observed among samples from the group ***Control N₂*** from day 11 and throughout storage compared to ***Control***

Table 2
Headspace concentration (%) of carbon dioxide (CO_2) and oxygen (O_2) measured in samples (n = 3) of the experimental groups as affected by storage time (stored at 4 °C).

Gruppe	Day	Control N_2	Shew N_2	Photo N_2	Mix N_2	Control O_2	Shew O_2	Photo O_2	Mix O_2	P-Value[b]
CO_2	Initial[a]	67	67	67	67	67	67	67	67	-
	2	$46.5 \pm 0.5^{abc, y}$	$43\pm2^{c, z}$	$46.5 \pm 0.8^{abc, z}$	$44\pm2^{bc, z}$	46 ± 1^{abc}	$48.2 \pm 0.3^{ab, yz}$	$48.5 \pm 0.4^{a, xy}$	$47\pm1^{abc, y}$	= 0.010
	5	$45.3 \pm 0.7^{ab, y}$	$46\pm2^{ab, yz}$	$52\pm2^{a, yz}$	$49\pm2^{ab, xy}$	47.6 ± 0.9^{ab}	$43\pm2^{b. z}$	$47\pm1^{ab, z}$	$47\pm1^{ab, y}$	= 0.042
	7	$50\pm1^{ab, y}$	$48\pm2^{b, xyz}$	$55\pm1^{b, xy}$	$49\pm1^{a, xy}$	49 ± 3^{ab}	$48\pm1^{b, yz}$	$45.2 \pm 0.5^{b, z}$	$47\pm1^{b, y}$	= 0.002
	9	$50\pm2^{ab, y}$	$52\pm2^{ab, xy}$	$55\pm2^{a, xy}$	$56\pm2^{a, x}$	48 ± 1^{b}	$50\pm2^{ab, yz}$	$47\pm1^{b, z}$	$50\pm1^{ab, y}$	= 0.002
	11	57.4 ± 0.8^{x}	54 ± 1^{x}	57 ± 2^{xy}	51 ± 1^{xy}	50 ± 1	51 ± 3^{xy}	52.5 ± 0.8^{xy}	51 ± 4^{xy}	>0.055
	14	$59\pm2^{ab, x}$	$52\pm1^{b, xy}$	$61\pm2^{a, x}$	$59.3 \pm 0.7^{ab, x}$	52 ± 1^{b}	$59\pm2^{ab, x}$	$57\pm2^{ab, x}$	$61\pm3^{a, x}$	= 0.003
	P-value[b]	<0.001	= 0.001	<0.001	<0.001	>0.106	<0.001	<0.001	= 0.002	
O_2	Initial[a]	0	0	0	0	33	33	33	33	-
	2	$0.2 \pm 0.1^{b, xy}$	$0.4 \pm 0.1^{b, xy}$	$0.4 \pm 0.1^{b, x}$	$0.6 \pm 0.2^{b, x}$	48.6 ± 0.6^{a}	$48.1 \pm 0.3^{a, x}$	$48.5 \pm 0.4^{a, x}$	$49\pm1^{a, x}$	<0.001
	5	$0.4 \pm 0.1^{b, x}$	$1.0 \pm 0.3^{b, x}$	$0.1 \pm 0.1^{b, xy}$	$0.3 \pm 0.2^{b, xy}$	48.4 ± 0.6^{a}	$48\pm1^{a, x}$	$50\pm1^{a, x}$	$49.2 \pm 0.8^{a, x}$	<0.001
	7	$0.2 \pm 0.1^{b, xy}$	$0.5 \pm 0.3^{b, xy}$	$0.0 \pm 0.0^{c, y}$	$0.0 \pm 0.0^{c, y}$	47 ± 3^{a}	$48.0 \pm 0.7^{a, x}$	$49\pm1^{a, x}$	$48.1 \pm 0.3^{a, x}$	<0.001
	9	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	47 ± 1^{a}	$47\pm2^{a, x}$	$46.3 \pm 0.8^{a, xy}$	$47\pm1^{a, x}$	<0.001
	11	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	45 ± 1^{a}	$46\pm2^{a, x}$	$42.8 \pm 0.9^{a, yz}$	$45\pm3^{a, x}$	<0.001
	14	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	$0.0 \pm 0.0^{b, y}$	45 ± 1^{a}	$38\pm1^{a, y}$	$40\pm2^{a, z}$	$35\pm4^{a, z}$	<0.001
	P-value[b]	= 0.004	= 0.002	= 0.003	= 0.005	>0.266	<0.001	<0.001	= 0.002	

[a] Initial concentration of CO_2 in the gas mixture was adjusted to 67%, whereas the balancing gas (either O_2 or N_2) was preset to a start concentration of 33% (data of N_2 is not present).
[b] Different lowercase superscripts within each row (abc) and within each parameter and column (xyz) indicate significant differences ($P < 0.05$) between groups by one-way ANOVA and Tukey's pairwise comparison test.

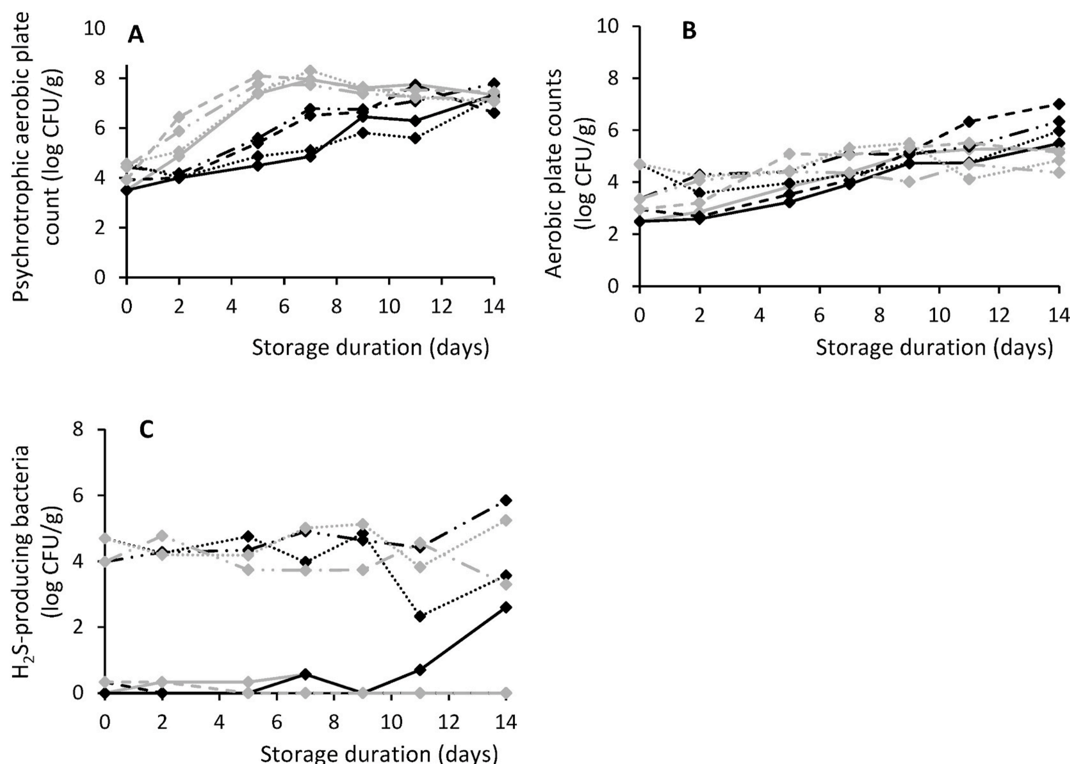

Fig. 1. (**A**) Psychrotrophic aerobic plate count (PC) in saithe portions as affecting by storage time (log CFU/g, $GLM_{gas\ composition}$: $P < 0.001$); $GLM_{inoculum}$: $P = 0.022$; $GLM_{storage}$: $P < 0.001$), (**B**) aerobic plate counts (APC) in saithe portions as affecting by storage time (log CFU/g, $GLM_{gas\ composition}$: $P > 0.775$); $GLM_{inoculum}$: $P = 0.003$; $GLM_{storage}$: $P < 0.001$), and (**C**) H_2S-producing bacteria (black colonies on iron agar) in saithe portions as affecting by storage time (log CFU/g, $GLM_{gas\ composition}$: $P > 0.797$); $GLM_{inoculum}$: $P < 0.001$; $GLM_{storage}$: $P > 0.910$). Each sampling point represents the average value (n = 3). Legends: ——— Control N_2;——— Control O_2;············ Shew O_2;-------- Photo O2; —·—·— Mix O_2;············ Shew N_2;-------- Photo N_2;—·—·— Mix N_2.

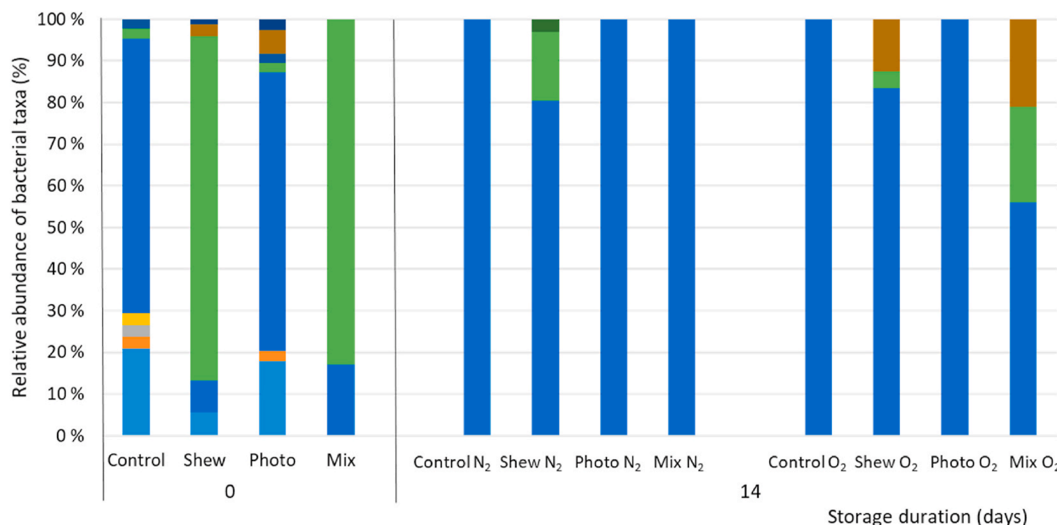

Fig. 2. The relative abundance of the dominating bacterial taxa (genus level) in saithe filets of the raw material (Control) and after inoculation of *P. phosphoreum,* HS254 (Photo), *Shewanella* sp., HS012 (Shew), and a mix of both *P. phosphoreum* and *Shewanella* sp. (Mix). The right side of the figure shows the relative abundance of the dominating bacterial taxa of experimental groups at the end of storage (14 days, 4 °C). Legends: Flavobacterium*; Pseudoalteromonas; Aliivibrio; Janthinobacterium; Photobacterium; Shewanella; Psychrobacter; Pseudomonas; Chryseobacterium; Lactococcus; Streptococcus; Vibrio*.

O_2. This observation indicated an inhibitory effect of O_2 against H_2S producers that are in accordance with Lerfall et al. (2018), and Sivertsvik (2007). On the other side, Kuuliala et al. (2018) found that the growth rate of H_2S- producers was lower at high O_2-concentrations, but the growth was not completely inhibited.

APC can be used as a general indicator for remaining shelf-life (Gram & Dalgaard, 2002), but there are no absolute criteria available stating a maximum threshold level indicating spoilage. However, most guidelines state seafood with APC levels of more than 7 log CFU/g should be considered unsatisfactory for consumption (Mikš-Krajnik, Yoon, & Yuk, 2016; The Centre for Food Safety, 2014). Sensory rejection of MA packaged seafood can although occur before the APC indicate spoilage due to non-microbiological degradation such as, e.g., endogenic denaturation of TMAO and oxidation of lipids and proteins (Dalgaard et al., 1997; Mikš-Krajnik, Yoon, Ukuku, & Yuk, 2016; Parlapani, Mallouchos, & Boziaris, 2014).

Gram and Huss (1996) stated *Pseudomonas* spp. to be a part of the spoilage microbiota in aerobic stored cold-water fish species. However, in the present study, *Pseudomonas* was only detected sporadically, showing the low prevalence and reduced growth potential affected by the packaging concept (data not shown).

3.2.1. Microbiological ecology

Among the 335 isolates collected from L&H agar, 12 genera were identified, of which six were found in the raw material only (not inoculated) (Fig. 2). The initial microbiota of the control samples was not consistent but taking all three samples into account, *Photobacterium* spp. (66%), *Flavobacterium* spp. (21%), *Janthinobacterium* spp. (3%), *Aliivibrio* spp. (3%), *Psychrobacte*r spp. (2%) and *Shewanella* spp. (2%) were identified (Fig. 2). All of these, except *Janthinobacterium* spp. are marine bacteria often found in fresh marine fish products (Ina-Salwany et al., 2019; Kuuliala et al., 2018; Møretrø, Moen, & Langsrud, 2016; Sivertsvik et al., 2002). *Janthinobacterium* spp. are known from non-marine habitats but can sporadically be detected in arctic seawater (Alonso-Sáez et al., 2014). The initial microbiota observed in the present study was moreover similar to what reported by Lerfall et al. (2018) but the distribution was different indicating environmental variations. Among inoculated samples, the inoculated bacteria were found to be the dominant taxa. However, other bacterial taxa were also identified supporting samples to have a background microbiota. Independent of packaging atmosphere, *Photobacterium* spp. was identified as the dominating genus among controls (***Control N_2*** and ***Control O_2***) at the end of storage. The same pattern was also observed in samples inoculated with *P. phosphoreum* strain HS254 (***Photo N_2***, ***Photo O_2***), *Shewanella* sp. strain HS012, or a mix of both (***Mix N_2***, ***Mix O_2***) (with some exceptions where a mix of *Shewanella* spp. and *Lactococcus* spp. was observed, Fig. 2). Generally, taking all samples under consideration, it seems that *P. phosphoreum* is identified as the dominant genus, with some exceptions among samples inoculated with *Shewanella* sp. strain HS012. Identification of *P. phosphoreum* as the dominant genus also supported results obtained by the proliferation of PC (Fig. 1).

3.3. Metabolites

A significant effect of the experimental design was found on the IMP conversion to HxR and further to Hx (GLM: $P < 0.001$). The content of IMP (Table 3) was not found to be affected by the fixed factors "gas composition" or "inoculum" (GLM: $P > 0.426$ and > 0.991, respectively). However, a significant reduction of IMP as a storage time function indicates a conversion of IMP to HxR and Hx. Although a significant main effect of storage time on the IMP concentration (GLM: <0.001), no such effects were observed on the group level (Table 3, $P > 0.095$–0.306). The content of HxR (Table 3) was significantly affected by the experimental design showing the fixed factor "gas composition" and the co-factor

Table 3
Average content (mmol/100g ± SD) of Inosine monophosphate (IMP), Inosine (HxR), and Hypoxanthine (Hx) in initial samples (n = 4) and samples after five- and 11-days cold storage (n = 3, 4 °C). A rise in H-value is indicative for decomposition, as described by Luong et al. (1992).

Parameter	Day	Experimental group								P-value [b]
		Control N_2	Shew N_2	Photo N_2	Mix N_2	Control O_2	Shew O_2	Photo O_2	Mix O_2	
IMP (mmol/100g)	Initial [a]	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	-
	5	n.d.	n.d.	0.002 ± 0.002	n.d.	0.002 ± 0.004	0.01 ± 0.02	0.03 ± 0.02	0.05 ± 0.08	>0.535
	11	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.02	0.05 ± 0.05	n.d.	0.01 ± 0.03	>0.101
	P-value [b]	>0.095	>0.095	>0.100	>0.095	>0.140	>0.244	>0.140	>0.306	
HxR (mmol/100g)	Initial [a]	0.45 ± 0.07^{x}	0.45 ± 0.07^{x}	0.45 ± 0.07^{x}	0.45 ± 0.07^{x}	0.45 ± 0.07	0.45 ± 0.07	0.45 ± 0.07^{x}	0.45 ± 0.07	-
	5	$0.3 \pm 0.1^{ab,x}$	$0.46 \pm 0.06^{a,x}$	$0.15 \pm 0.08^{b,y}$	$0.3 \pm 0.2^{ab,y}$	0.42 ± 0.09^{a}	0.43 ± 0.05^{a}	$0.47 \pm 0.04^{a,x}$	0.47 ± 0.04^{a}	= 0.005
	11	n.d.	n.d.	n.d.	n.d.	0.40 ± 0.06^{a}	0.35 ± 0.04^{a}	$0.26 \pm 0.09^{a,y}$	0.2 ± 0.2^{ab}	>0.203
	P-value [b]	<0.001	<0.001	<0.001	= 0.002	>0.719	>0.120	= 0.013	= 0.043	
Hx (mmol/100g)	Initial [a]	0.09 ± 0.01^{y}	0.09 ± 0.01^{z}	0.09 ± 0.01^{z}	0.09 ± 0.01^{y}	0.09 ± 0.01^{y}	0.09 ± 0.01^{y}	0.09 ± 0.01^{y}	0.09 ± 0.01^{y}	-
	5	$0.21 \pm 0.07^{b,y}$	$0.22 \pm 0.02^{b,y}$	$0.46 \pm 0.07^{a,y}$	$0.3 \pm 0.2^{ab,xy}$	$0.22 \pm 0.09^{b,xy}$	$0.14 \pm 0.01^{b,y}$	$0.14 \pm 0.04^{b,y}$	$0.15 \pm 0.04^{b,y}$	= 0.002
	11	$0.6 \pm 0.1^{ab,x}$	$0.60 \pm 0.03^{a,x}$	$0.6 \pm 0.0^{a,x}$	$0.6 \pm 0.2^{ab,x}$	$0.27 \pm 0.08^{bc,x}$	$0.26 \pm 0.06^{c,x}$	$0.35 \pm 0.08^{abc,x}$	$0.3 \pm 0.1^{abc,x}$	= 0.001
	P-value [b]	<0.001	<0.001	<0.001	= 0.009	= 0.022	= 0.002	= 0.001	= 0.009	
H-Value	Initial [a]	14 ± 2^{z}	14 ± 2^{z}	14 ± 2^{z}	14 ± 2^{z}	14 ± 2^{y}	14 ± 2^{y}	14 ± 2^{y}	14 ± 2^{y}	-
	5	$40 \pm 15^{ab,y}$	$33 \pm 5^{b,y}$	$75 \pm 13^{a,y}$	$50 \pm 27^{ab,y}$	$33 \pm 5^{b,x}$	$25 \pm 3^{b,y}$	$22 \pm 6^{b,y}$	$23 \pm 6^{b,y}$	= 0.001
	11	$100 \pm 0^{b,x}$	$100 \pm 0^{b,x}$	$100 \pm 0^{b,x}$	$100 \pm 0^{b,x}$	$39 \pm 11^{a,x}$	$40 \pm 9^{a,x}$	$58 \pm 15^{a,x}$	$63 \pm 31^{a,x}$	<0.001
	P-value [b]	<0.001	<0.001	<0.001	<0.001	= 0.003	= 0.001	= 0.001	= 0.017	

"n.d." indicate amounts lower than the detection limit of the specific compound.
[a] The initial concentration represents an average (n = 4) of the raw material measured before inoculation and packaging.
[b] Different lowercase superscripts within each row (abc) and within each parameter and column (xyz) indicate significant differences ($P < 0.05$) between groups by one-way ANOVA and Tukey's pairwise comparison test.

"storage" to be the main discriminants (GLM: $P < 0.001$, $F = 27.008$ and 93.672, respectively). Independent of inoculum, faster conversion of HxR to Hx was observed for samples packaged in CO_2/N_2 compared to those packaged in CO_2/O_2. Moreover, a quicker reduction of HxR was observed in samples inoculated with *P. phosphoreum* (***Photo*** $\boldsymbol{O_2}$) or a mix of both (***Mix*** $\boldsymbol{O_2}$), compared to other groups packaged in CO_2/O_2 (***Control*** $\boldsymbol{O_2}$ and ***Shew*** $\boldsymbol{O_2}$) (Table 3).

As a result of the depleted HxR content of those samples stored in CO_2/N_2, an increase in the Hx concentration was observed (GLM: $P < 0.001$, Table 3). All samples packaged in CO_2/N_2 ended with higher Hx concentrations than those packaged in CO_2/O_2 at the end of storage (day 14). Faster conversion of Hx in samples inoculated with *P. phosphoreum* (***Photo*** $\boldsymbol{O_2}$) indicates the potential of *P. phosphoreum* to catalyze the transformation of HxR to Hx. The H-value, commonly used as a quality index, was significantly affected by the fixed factor "gas composition", showing the fastest increase in samples packaged in CO_2/N_2 (Table 3). The H-value did moreover correlate significantly with levels of HxR ($r = -0.936$, $P < 0.001$), Hx ($r = 0.978$, $P < 0.001$), PC ($r = 0.777$, $P < 0.001$), and APC ($r = 0.557$, $P < 0.001$).

The TMA content (Table 4) followed the same pattern as showed for Hx (Table 3, $r = 0.937$, $P < 0.001$). In the present study, higher TMA content was found in samples stored in an MA consisting of CO_2/N_2 compare to those held in CO_2/O_2 (GLM: $P < 0.001$). To prevent the anaerobic respiration of TMAO by O_2 depleted microorganisms, O_2 is often included in the packaging atmosphere of certain species such as, e.g., codfish (Ashie, Smith, & Simpson, 1996; Dalgaard, 1995). Moreover, Jakobsen et al. (2020) demonstrated that the TMA concentration in salmon fillets during storage was significantly affected by storage time and temperature, packaging atmosphere and inoculation with *Aeromonas salmonicida.*

A positive correlation between bacterial growth (PC and APC) and depletion of headspace O_2 ($r = 0.774$, $P < 0.001$, and $r = 0.479$, $P > 0.001$, respectively), were observed of samples packaged in CO_2/N_2 (Table 2). Lack of headspace O_2 was more apparent in samples

Table 4
Average content (mmol/100g ± SD) of Dimethylamine (DMA), Trimethylamine (TMA), Alanine (Ala), Tyrosine (Tyr), Valine (Val), and Lactate in initial samples (n = 4) and samples after five- and 11-days cold storage (n = 3, 4 °C).

Parameter	Day	Experimental group								P-value [b]
		Control N_2	Shew N_2	Photo N_2	Mix N_2	Control O_2	Shew O_2	Photo O_2	Mix O_2	
DMA	Initial [a]	0.05 ± 0.02	0.05 ± 0.02^{y}	0.05 ± 0.02^{y}	0.05 ± 0.02^{y}	0.05 ± 0.02	0.05 ± 0.02^{z}	0.05 ± 0.02	0.05 ± 0.02	-
	5	0.2 ± 0.1^{ab}	0.26 ± 0.04$^{a, x}$	0.16 ± 0.04$^{ab, xy}$	0.22 ± 0.09$^{ab, x}$	0.09 ± 0.05^{b}	0.16 ± 0.01$^{ab, x}$	0.10 ± 0.02^{ab}	0.10 ± 0.04^{ab}	= 0.019
	11	0.3 ± 0.2	0.3 ± 0.2^{x}	0.22 ± 0.08^{x}	0.16 ± 0.06^{xy}	0.10 ± 0.03	0.10 ± 0.02^{y}	0.09 ± 0.03	0.11 ± 0.04	>0.074
	P-value [b]	= 0.080	= 0.013	= 0.006	= 0.022	0.204	<0.001	= 0.060	>0.080	
TMA	Initial [a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
	5	0.6 ± 0.5$^{ab, y}$	0.6 ± 0.1$^{ab, y}$	2.2 ± 0.2$^{a, x}$	1.1 ± 0.7$^{b, y}$	0.03 ± 0.01$^{c, y}$	n.d.	0.03 ± 0.03^{c}	0.01 ± 0.02^{c}	<0.001
	11	2.3 ± 0.6^{x}	2.5 ± 0.3$^{a, x}$	1.97 ± 0.06$^{ab, y}$	2.5 ± 0.6$^{a, x}$	0.4 ± 0.4$^{b, x}$	0.6 ± 0.5^{b}	1±1^{ab}	1±1^{ab}	= 0.005
	P-value [b]	<0.001	<0.001	<0.001	= 0.001	= 0.041	= 0.031	>0.059	>0.097	
Ala (mmol/100g)	Initial [a]	0.40 ± 0.04^{y}	0.40 ± 0.04^{x}	0.40 ± 0.04	0.40 ± 0.04	0.40 ± 0.04	0.40 ± 0.04^{y}	0.40 ± 0.04^{x}	0.40 ± 0.04	-
	5	0.5 ± 0.1^{xy}	0.29 ± 0.03^{y}	0.4 ± 0.1	0.5 ± 0.2	0.41 ± 0.08	0.56 ± 0.04^{x}	0.30 ± 0.03^{y}	0.4 ± 0.1	>0.056
	11	0.60 ± 0.05$^{a, x}$	0.39 ± 0.05$^{abc, x}$	0.56 ± 0.03^{ab}	0.3 ± 0.1^{c}	0.4 ± 0.1^{bc}	0.46 ± 0.06$^{abc, xy}$	0.43 ± 0.02$^{abc, x}$	0.4 ± 0.1^{abc}	= 0.004
	P-value [b]	= 0.039	= 0.050	= 0.014	= 0.015	= 0.048	>0.201	>0.810	= 0.005	
Tyr (mmol/100g)	Initial [a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
	5	n.d.	n.d.	0.00 ± 0.01	0.00 ± 0.01	n.d.	n.d.	n.d.	n.d.	>0.559
	11	0.02 ± 0.01^{a}	0.01 ± 0.00^{ab}	0.00 ± 0.01^{ab}	0.00 ± 0.01^{ab}	n.d.	n.d.	n.d.	0.00 ± 0.01^{ab}	= 0.025
	P-value [b]	= 0.080	= 0.013	= 0.003	<0.001	>0.173	>0.528	-	-	
Val (mmol/100g)	Initial [a]	0.03 ± 0.01^{y}	0.03 ± 0.01	0.03 ± 0.01^{y}	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01^{y}	0.03 ± 0.01^{y}	0.03 ± 0.01^{y}	-
	5	0.04 ± 0.01^{xy}	0.04 ± 0.01	0.03 ± 0.01^{y}	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01^{xy}	0.05 ± 0.00^{xy}	0.04 ± 0.00^{y}	>0.052
	11	0.07 ± 0.03$^{a, x}$	0.04 ± 0.01^{ab}	0.06 ± 0.01$^{ab, x}$	0.02 ± 0.01^{b}	0.05 ± 0.02^{ab}	0.06 ± 0.01$^{ab, x}$	0.06 ± 0.02$^{ab, x}$	0.05 ± 0.01$^{ab, x}$	= 0.025
	P-value [b]	<0.001	<0.001	= 0.039	= 0.050	<0.001	>0.055	>0.317	= 0.012	
Lactate (mmol/100g)	Initial [a]	5.01 ± 0.06^{x}	5.01 ± 0.06^{x}	5.01 ± 0.06^{x}	5.01 ± 0.06^{x}	5.01 ± 0.06	5.01 ± 0.06	5.01 ± 0.06	5.01 ± 0.06	-
	5	4.0 ± 0.3^{y}	4.3 ± 0.3^{y}	3.7 ± 0.7^{y}	4.3 ± 0.4^{x}	4.5 ± 0.6	4.6 ± 0.9	4 ± 1	4.8 ± 0.5	>0.661
	11	3.8 ± 0.6$^{ab, y}$	3.1 ± 0.3$^{ab, z}$	3.9 ± 0.3$^{a, y}$	1.7 ± 0.6$^{b, y}$	4±1^{a}	5.1 ± 0.6^{a}	4.4 ± 0.9^{a}	4.0 ± 0.9^{a}	= 0.002
	P-value [b]	= 0.004	<0.001	= 0.006	<0.001	>0.449	>0.480	>0.484	>0.105	

"n.d." indicate amounts lower than the detection limit of the specific compound.

[a] The initial concentration represents an average of four samples of the raw material measured before inoculation and packaging.

[b] Different lowercase superscripts within each row (abc) and within each parameter and column (xyz) indicate significant differences ($P < 0.05$) between groups by one-way ANOVA and Tukey's pairwise comparison test.

inoculated with *P. phosphoreum* (***Photo*** $\boldsymbol{N_2}$) or a mix of both inoculums (***Mix*** $\boldsymbol{N_2}$) compared to other groups (***Shew*** $\boldsymbol{N_2}$ and ***Control*** $\boldsymbol{N_2}$). A reduction of O_2 was also seen as a function of storage in the headspace of samples packaged in CO_2/O_2 (Table 2). This reduction was most prominent in groups inoculated with *Shewanella* sp. (***Shew*** $\boldsymbol{O_2}$ and ***Mix*** $\boldsymbol{O_2}$), and the opposite of what was seen in samples held in CO_2/N_2. Moreover, these observations could indicate a faster conversion of TMA in groups showing depleted O_2 levels (not shown by metabolite analyses due to fewer sampling points available).

Both TMA and DMA are formed from the same substrate, TMAO, but through different mechanisms (Castell, Neal, & Smith, 1970; Fraser Owen & Sumar, 1998; Huss, 1995). TMA is formed by reduction of TMAO by spoilage microbiota such as, e.g., *P. phosphoreum* and *S. putrefaciens* (Boskou & Debevere, 1998; Debevere & Boskou, 1996; Gram & Dalgaard, 2002; Gram & Huss, 1996; Sivertsvik et al., 2002), of which DMA through endogenic mechanisms (Castell et al., 1970). In the present study, the DMA content was affected by the experimental design (GLM: $P < 0.001$), the fixed factor "gas composition" ($P < 0.001$), and the co-factor "storage" ($P < 0.001$). The factor "inoculum" did not affect the sample DMA content ($P > 0.389$). However, at the end of storage, the DMA content was generally higher in samples packaged in CO_2/N_2 than in those packaged in CO_2/O_2.

Lerfall et al. (2018) discussed biogenic amines in saithe as affected by packaging gas and concluded that significant amounts of cadaverine were formed when saithe were packaged in an MA consisting of CO_2/N_2. They suggested, moreover, a link between the production of cadaverine and the growth of *S. putrefaciens*. Other biogenic amines were, however, only detected at low concentrations. In the present study, biogenic amines were not measured due to signals overlaying in NMR spectra and the observed inhibited growth of *Shewanella* sp. strain HS012. However, the significance of biogenic amines in seafood spoilage is highly relevant (Bulushi, Poole, & Dykes, 2009) and should not be neglected.

Accumulation of free amino acids (Ala, Tyr, and Val) and lactate (Table 4) also indicated bacterial activity and spoilage. A significant correlation between bacterial growth (PC and APC), Ala, Tyr, Val, and lactate was observed in the present study. The different levels of correlation between the metabolites and the bacterial growth indicate that different mechanisms are involved. Observed correlations for PC and APC to the different metabolites were: Ala: $r = 0.218$, $P > 0.079$, and $r = 0.292$, $P = 0.017$, respectively; Tyr: $r = 0.465$, $P < 0.001$, and $r = 0.303$, $P = 0.013$, respectively; Val: $r = 0.321$, $P = 0.009$, and $r = 0.409$, $P = 0.001$, respectively; and lactate: $r = -0.583$, $P < 0.001$, and $r = -0.257$, $P = 0.035$, respectively. The content of Ala and Val was not affected by either the fixed factor "gas composition" or "inoculum" (GLM: $P > 0.668$; 0.107, and $P > 0.581$; 0.202, respectively). The content of Tyr and lactate was, however, affected by "gas composition" ($P = 0.004$ and $P = 0.001$, respectively), but not by "inoculum" ($P > 0.801$ and $P > 0.424$, respectively). All metabolites were, however, affected by the co-factor "storage" ($P < 0.05$), showing increased levels of Ala, Tyr, and Val and decreasing amounts of lactate as a function of storage.

3.4. Physiochemical parameters

The muscle pH (Table 5) was affected by the experimental design (GLM, $P < 0.001$) showing the fixed factor "gas composition" to give a higher F-value compared to "inoculum" ($F = 17.55$ and 2.99, respectively). The significant drop in average muscle pH from the whole fish (measured 24 h post-mortem, 6.6 ± 0.2) to fillets (measured 48 h post-

Table 5

Muscle pH, fillet drip loss (DL, g/100g of initial weight), and firmness (F60%, N) in modified packaged saithe as affected by inoculum, packaging gas mixture, and storage. Presented values are an average of four samples per group (n = 4) if otherwise not stated.

Parameter	Day	Experimental group								P-value [b]
		Control N_2	Shew N_2	Photo N_2	Mix N_2	Control O_2	Shew O_2	Photo O_2	Mix O_2	
Muscle pH	2	6.1 ± 0.07^{b}	$6.3 \pm 0.1^{a, xy}$	6.2 ± 0.1^{ab}	$6.2 \pm 0.1^{ab, xy}$	$6.1 \pm 0.06^{ab, y}$	6.1 ± 0.05^{b}	6.1 ± 0.1^{b}	$6.1 \pm 0.7^{b, y}$	= 0.011
	5	6.2 ± 0.1	6.2 ± 0.08^{y}	6.4 ± 0.2	6.2 ± 0.08^{xy}	6.1 ± 0.05^{y}	6.2 ± 0.1	6.3 ± 0.3	61 ± 0.1^{y}	>0.201
	7	6.3 ± 0.1^{ab}	$6.5 \pm 0.2^{a, x}$	6.4 ± 0.2^{ab}	$6.4 \pm 0.1^{ab, x}$	$6.1 \pm 0.06^{b, y}$	6.1 ± 0.1^{b}	6.3 ± 0.2^{ab}	$6.2 \pm 0.09^{ab, xy}$	= 0.005
	9	6.3 ± 0.1^{ab}	$6.5 \pm 0.04^{a, x}$	6.4 ± 0.2^{ab}	$6.3 \pm 0.2^{ab, xy}$	$6.2 \pm 0.1^{b, xy}$	6.2 ± 0.05^{b}	6.3 ± 0.1^{ab}	$6.2 \pm 0.03^{b, xy}$	= 0.016
	11	6.2 ± 0.07^{ab}	$6.4 \pm 0.04^{a, xy}$	6.2 ± 0.2^{ab}	$6.4 \pm 0.2^{a, xy}$	$6.2 \pm 0.2^{ab, xy}$	6.1 ± 0.07^{b}	6.2 ± 0.05^{ab}	$6.2 \pm 0.07^{ab, xy}$	= 0.008
	14	6.2 ± 0.1^{cd}	$6.4 \pm 0.07^{a, xy}$	6.2 ± 0.1^{bcd}	$6.1 \pm 0.1^{d, y}$	$6.4 \pm 0.02^{ab, x}$	6.3 ± 0.05^{abcd}	6.4 ± 0.08^{ab}	$6.3 \pm 0.08^{abc, x}$	<0.001
	P-value [b]	>0.131	= 0.017	<0.196	= 0.023	= 0.005	= 0.030	>0.094	= 0.003	
DL (g/ 100g)	2	5.1 ± 0.5^{y}	4.2 ± 0.4^{y}	6 ± 2^{z}	5 ± 1^{y}	7 ± 3^{y}	6 ± 2^{y}	6 ± 1^{z}	5 ± 2^{y}	>0.678
	5	10 ± 3^{xy}	8 ± 3^{xy}	6 ± 1^{z}	9 ± 4^{xy}	12 ± 3^{xy}	8 ± 2^{y}	11 ± 2^{yz}	11 ± 5^{xy}	>0.273
	7	11 ± 4^{xy}	9 ± 3^{xy}	10 ± 3^{yz}	8 ± 2^{xy}	12 ± 3^{xy}	14 ± 2^{x}	13 ± 4^{xy}	14 ± 5^{x}	>0.081
	9	$12 \pm 3^{ab, xy}$	$11 \pm 6^{ab, x}$	$8 \pm 1^{b, yz}$	$12 \pm 5^{ab, xy}$	$16 \pm 4^{a,x}$	$13 \pm 3^{ab, x}$	$15 \pm 2^{ab, xy}$	$15 \pm 3^{ab, x}$	>0.051
	11	$13 \pm 4^{abc, x}$	$10 \pm 2^{c, xy}$	$12 \pm 2^{abc, xy}$	$10 \pm 4^{bc, xy}$	$18 \pm 3^{a, x}$	$16.4 \pm 0.5^{ab, x}$	$17 \pm 1^{a, x}$	$15 \pm 3^{abc, x}$	= 0.001
	14	$14 \pm 3^{ab, x}$	$10.1 \pm 0.8^{b, xy}$	$15 \pm 2^{ab, x}$	$14 \pm 3^{ab, x}$	$17 \pm 4^{a, x}$	$16 \pm 2^{ab, x}$	$15 \pm 3^{ab, xy}$	$15 \pm 3^{ab, x}$	>0.069
	P-value [b]	= 0.016	>0.060	<0.001	= 0.043	= 0.001	<0.001	<0.001	= 0.005	
F60% (N)	Initial [a]	16 ± 4^{y}	16 ± 4	16 ± 4	16 ± 4	16 ± 4	16 ± 4^{y}	16 ± 4^{y}	16 ± 4^{y}	-
	2	17 ± 5^{y}	14 ± 2	20 ± 4	21 ± 6	18 ± 5	20 ± 7^{xy}	21 ± 7^{xy}	16 ± 4^{y}	>0.570
	5	22 ± 5^{xy}	18 ± 1	16 ± 4	28 ± 6	29 ± 10	20 ± 10^{xy}	21 ± 5^{xy}	21 ± 3^{xy}	>0.058
	7	22 ± 3^{xy}	21 ± 6	18 ± 2	22 ± 4	25 ± 12	25 ± 5^{xy}	27 ± 10^{xy}	30 ± 12^{xy}	>0.620
	9	31 ± 8^{x}	24 ± 6	19 ± 4	19 ± 8	30 ± 3	23 ± 5^{xy}	23 ± 8^{xy}	24 ± 4^{xy}	>0.071
	11	22 ± 5^{xy}	20 ± 7	20 ± 7	22 ± 8	21 ± 5	32 ± 7^{x}	22 ± 3^{xy}	35 ± 18^{x}	>0.141
	14	20 ± 4^{y}	22 ± 4	16 ± 4	22 ± 4	29 ± 6	20 ± 13^{xy}	32 ± 9^{x}	25 ± 4^{xy}	>0.061
	P-value [b]	= 0.001	= 0.034	>0.482	= 0.040	= 0.003	= 0.024	= 0.009	= 0.006	

[a] The initial F60% value represents an average of 12 samples of the raw material measured before inoculation and packaging.

[b] Different lowercase superscripts within each row (abcd) and within each parameter and column (xyz) indicate significant differences ($P < 0.05$) between groups by one-way ANOVA and Tukey's pairwise comparison test.

mortem, 6.35 ± 0.21) and further to the second day post packaging (four days post-mortem, 6.14 ± 0.12) did indicate the fish to be in an "*in rigor*" state at the point of processing. The effect of the factor "gas composition" did moreover support the results reported by Lerfall et al. (2018), showing significant higher pH during storage in samples packaged in CO_2/N_2, compared to those packaged in CO_2/O_2 (6.19–6.38 and 6.08–6.23, respectively, $P < 0.001$). However, the opposite pattern was observed at day 14, where the highest pH was observed in samples packaged in CO_2/O_2 (6.34 ± 0.08 versus 6.21 ± 0.17, $P = 0.012$). Samples inoculated with *Shewanella* sp. strain HS012 and packaged in an atmosphere of CO_2/N_2 (***Shew*** $\boldsymbol{N_2}$) had a rapid increase in muscle pH (0.3 pH units) between day five and day seven, followed by a relatively high pH throughout storage (6.43–6.51). The equivalent group stored in CO_2/O_2 (***Shew*** $\boldsymbol{O_2}$) did not show this increase. This observation indicates other factors affecting the system, e.g., differences in microbiological growth among groups (Fig. 1A) or changes in the microbial ecology during storage (Fig. 2). The fish samples' microbiological ecology presented in ***Shew*** $\boldsymbol{O_2}$ was initially a mix of *Shewanella* sp*., P. phosphoreum,* and others. During storage, however, the microbiota changed to 100% *P. phosphoreum* (Fig. 2), reflecting the inhibitory effect of CO_2 (>60% CO_2) on *Shewanella* spp. (Gram & Dalgaard, 2002; Sivertsvik et al., 2002).

In the present study, the drip loss was reported to be up to 18% of the initial weight after 14 days of cold storage, which was following levels earlier reported for MA packaged saithe fillets (Lerfall et al., 2018). However, only minor differences in drip loss were observed between groups (Table 5), showing the fixed factors "gas composition" and "inoculum" to have a negligible impact throughout 14 days of cold storage.

The sample firmness was significantly affected by the experimental design (GLM, $P < 0.001$), showing the fixed factor "gas composition" (CO_2/N_2 or CO_2/O_2) as the main discriminant ($F = 8.073$, $P = 0.005$). In contrast, no effects were found related to the factor "inoculum". Due to the large variation observed within groups, only minor effects were found on the group level (Table 5). However, by analyzing for main effects, a significant increase in muscle firmness was observed as a function of the co-factor "storage" (GLM: $F = 15.048$, $P < 0.001$). Increased firmness (F60%, N) can be associated with the high drip loss reported ($r = 0.341$, $P < 0.001$) (Table 5). Neither the drip loss nor the fillet firmness (F60%, N), however, correlate with the muscle pH measured in the same samples ($r = -0.094$, $P > 0.196$ and $r = 0.040$, $P > 0.585$, respectively).

4. Conclusion

The saithe fillets packaged in CO_2/N_2 (67% CO_2) on average, gave faster conversion of HxR to Hx, and thereby an increase in the H-value (reduced freshness), faster increase in DMA and TMA content, the highest level of the amino acid Tyr, and the lowest level of lactate. Independent of starting biota and packaging gas composition (CO_2/N_2 or CO_2/O_2, the initial CO_2 concentration of 67%), the main SSO after 14 days of cold storage was *P. phosphoreum*. Moreover, a high initial content of *P. phosphoreum* can be challenging due to its tolerance against CO_2 and thereby rapid loss of fillet freshness of MA packaged saithe. High initial levels of *Shewanella* spp. can be outcompeted by using an MA consisting of a proper level of CO_2 (>60%) and even better in combination with an excess of O_2 available.

CRediT authorship contribution statement

Jørgen Lerfall: Writing first draft, data analysis, conceptualisation, revising, and edition. **Elena Shumilina:** Experimental work, data analysis, writing, and editing. **Anita Nordeng Jakobsen:** writing, data analysis, conceptualisation, and revising.

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