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Microbiological spoilage and volatiles production of gutted European sea bass stored under air and commercial modified atmosphere package at 2 °C

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ABSTRACT

Microbiological, sensory, TVB-N and TMA-N changes and Volatile Organic Compounds (VOCs) detection using the SPME/GC–MS technique, were performed to evaluate potential chemical spoilage indices (CSI) of gutted sea bass (*Dicentrarchus labrax)* stored at 2 °C under air and in modified atmosphere packaging (MAP CO_2: 60%, O_2: 10%, N_2: 30%). Shelf-life, determined by sensory evaluation, of gutted sea bass stored at 2 °C under air and MAP was 9 and 13 d respectively. *Pseudomonas* and H_2S producing bacteria were among the dominant spoilage microorganisms under both storage conditions, while Lactic Acid Bacteria (LAB) and *Brochothrix thermosphacta* were co-dominant with *Pseudomonas* and H_2S producing bacteria under MAP. The traditional CSIs such as TVB-N and TMA-N were increased substantially only at the late stages of storage or after rejection of the products, making them unsuitable for freshness/spoilage monitoring throughout storage. A substantial number of VOCs attributed to microbiological action or chemical activity, were detected including alcohols, aldehydes, ketones, organic acids and esters. The level of microbial origin VOCs such as ethanol, 2-ethyl-1-hexanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 3-methylbutanal, 2-methylbutanal and some ethyl esters increased during storage, suggesting their potential as CSIs.

1. Introduction

European sea bass (*Dichentrarchus labrax*) is one of the main marine fish species farmed in Greece and other Mediterranean countries. Its white flesh and low fat content make it popular among the aquacultured species. Gutted sea bass is considered as a value-added seafood product with increasing demand in the international seafood market (Dias Aquaculture SA, Greece, personal communication).

Gutted sea bass, as all fresh seafood products, is very perishable and the main mechanism of its quality deterioration is microbial spoilage (Gram and Huss, 1996). A fraction of the initial microbiota of fish, known as specific spoilage organisms (SSOs) or ephemeral spoilage organisms (ESO), which is favoured by storage conditions (e.g., atmosphere, temperature), prevails over the rest of the microbiota, reaching high populations and producing several metabolites (chemical spoilage indices-CSIs). The latter are responsible for the off-flavors/odours which results in their organoleptic rejection (Dalgaard, 2003; Doulgeraki et al., 2012; Gram and Huss, 1996; Nychas et al., 2008).

Various sensory, microbiological and chemical methods have been employed to assess the fish and seafood quality (Oehlenschläger, 2014). Sensory methods require trained assessors and are expensive, difficult to standardize and unsuitable for utilization as routine procedure by the seafood industry (Dainty, 1996; Dalgaard, 2003). It is also known that the microbial growth is the main cause of fresh seafood quality deterioration and the currently used microbiological methods are retrospective, expensive and time consuming (Dainty, 1996; Dalgaard, 2003). Thus, there is the need for the development of rapid indirect monitoring of microbial

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growth by determining the activity of the microorganisms based on the determination of the products of microbial metabolism (Dainty, 1996; Ellis and Goodacre, 2001).

Various compounds such as TMA, TVB-N, sulphuric compounds, aldehydes, ketones, esters, etc., are produced by various microorganisms during fish spoilage (Dalgaard, 2003; Gram and Huss, 1996). The most commonly used chemical methods for monitoring the microbial activity in fish are the determination of TVB-N and TMA. In most fish species however, these two parameters increase only at the late stages of storage making them suitable only as acceptance/rejection criterion and not suitable for freshness indices (Oehlenschlager, 2014). Volatile organic compounds (VOCs) have been studied as potential CSIs for spoilage/freshness evaluation, considering that they usually vary significantly between the initial and rejection day of seafood. Finally, Solid Phase Micro-Extraction coupled with Gas Chromatography/Mass Spectrometry (SPME-GC/MS) has also been used to study the level of VOCs in seafood in order to evaluate the degree of seafood spoilage/freshness (Duflos et al., 2006; Edirisinghe et al., 2007; Joffraud et al., 2001; Jonsdottir et al., 2008; Jorgensen et al., 2001; Leduc et al., 2012; Noseda et al., 2012; Parlapani et al., 2014a; Soncin et al., 2008; Wierda et al., 2006).

A suitable compound for the assessment of spoilage should: (a) be a microbial metabolite produced by the main spoilage microorganisms (b) be initially absent or at low levels in food (c) increase during storage (d) show good correlation with microbial growth, sensory score and remaining shelf-life (Jay, 1986). To determine among microbial metabolites those fulfilling the aforementioned requirements, an initial investigation has to focus on the VOCs profile throughout storage in order to detect the compounds that are present at the early stages of storage and increase until the end of shelf-life.

To our knowledge, there is no study concerning the microbiological spoilage analysis and investigation of VOCs production of chilled stored gutted sea bass under air and MAP. The aims of this work were to (a) determine the microbiological changes and shelf-life (b) monitor the TVB-N and TMA-N changes and (c) determine the VOCs profile using SPME-GC/MS and find out any potential CSIs of gutted sea bass spoilage/freshness. Such investigation is expected to provide valuable information regarding the spoilage and quality monitoring of sea bass.

2. Materials and methods

2.1. Fish handling and storage

Two different batches of gutted sea bass of approx. 400 g packaged in polystyrene boxes (Sirap Gema S.p.A. Italy) under air or MAP, were provided by DIAS Aquaculture SA (Attica, Greece) on May of 2011. The MAP gases concentrations were CO_2: 60%, O_2: 10%, N_2: 30%, which comprise one of the most commonly commercial gases composition used by the Hellenic seafood industry. The MAP film was the BDF 8050F (Cryovac-Sealed Air Ltd, Athens, Greece). Samples were transferred to our laboratory 6 h after the packaging into insulated boxes with melted ice. The samples were stored in an incubator operating at 2 °C.

2.2. Sensory analysis and determination of shelf-life

Sensory evaluation was carried out by five panelists, who evaluated the appearance of skin, outer slime and eyes and the odour of flesh. The sensory attributes were evaluated using a 1 to 5 descriptive hedonic scale with 5 as the highest quality score (skin: bright, shining, iridescent, eyes: convex, black pupil, translucent cornea, slime: transparent and aqueous, odours: fresh, seaweedy, shellfishy) and 1 the lowest (skin: dull, discoloured, wrinkled, eyes: concave to sunken, grey pupil, milky and red cornea, slime: brown very mucus and viscous, odors: putrid, sour, amines, sulphides, feacal). A score of 3 was considered as the average score for minimum acceptability.

2.3. Microbiological analysis

All microbiological media were purchased by LAB M (Lancashire, UK), except for STAA which was supplied by Biolife Italiana srl (Milano, Italy). Iron Agar (IA) was prepared as described by Gram et al. (1987) by mixing the following ingredients: peptone 20 g/L, meat extract 3.0 g/L, yeast extract 3.0 g/L, ferric citrate 3.0 g/L, sodium thiosulphate 0.3 g/L, NaCl 5 g/L, L-cysteine 0.6 g/L, agar 14 g/L and adjusting the pH to 7.4.

Twenty five (25) g from the dorsal area of each fish were placed into stomacher bags with 225 ml sterile MRD (Maximum Recovery Diluent, 8.5 g/L NaCl, 1.0 g/L bacteriological peptone) and homogenized for 1 min, using a Stomacher (Bug Mixer, Interscience, London, UK). Then, volumes of 0.1 ml from the serial dilutions in MRD were spread on the surface of culture media in order to enumerate a) *Pseudomonas* spp. on cetrimide-fucidin-cephaloridine agar (CFC), incubated at 25 °C for 48 h and (b) *Brochothrix thermosphacta* on streptomycin sulphate, thallus acetate, cycloheximide (actidione) agar (STAA), incubated at 25 °C for 72 h. Samples of 1 ml of serial dilution in MRD were used for the pour plate technique for enumeration of a) Total viable counts (TVC) on Iron Agar, incubated at 25 °C for 72 h, (a) H_2S producing bacteria (presumable *Shewanella*) on Iron Agar by counting only black colonies, after incubation at 25 °C for 72 h, (b) Enterobacteriaceae on Violet Red Bile Glucose agar (VRBGA), incubated at 37 °C for 24 h and (c) Lactic Acid Bacteria (LAB) on Mann, Rogosa, Sharpe agar (MRS) after incubation at 25 °C for 72 h. The results were expressed as mean log cfu/g ± standard deviation of 4 replicates (2 replicates from each batch of fish).

2.4. Determination of TVB-N and TMA-N

The chemicals were supplied by Sigma-Aldrich (Steinheim, Germany). Ten (10) g of flesh were homogenized in trichloracetic acid (TCA) 60 g/L and filtered through Whatman No.1 filter paper in a 100 ml volumetric flask. Forty (40) ml in duplicates were used for TVB-N analysis using the steam-distillation procedure according to Vyncke et al. (1987) and the remaining 10 ml were used for the spectrophotometric determination of TMA using picric acid, according to Dyer (1945). The results were expressed as mean mg N/100 g ± standard deviation of 4 replicates (2 replicates from each batch of fish).

2.5. VOCs determination by headspace SPME-GC/MS analysis

A modified procedure of the method described by Iglesias et al. (2009) was used. In particular, a total amount of 50 g of fish flesh was obtained from 4 different packages (2 from each batch) and pooled. Five (5) g of the pooled fish in duplicates were transferred into a 20 mL glass vial. The vial was sealed hermetically using a mininert valve (Sigma Aldrich) and their contents were magnetically stirred for 15 min at 40 °C. Then, the SPME fibre (DVB/CAR/PDMS 50/30 μm) was exposed to the headspace for additional 30 min, under the same conditions. The length of the fibre in the headspace was kept constant. Before each analysis, the fibre was exposed to the injection port for 10 min to remove any volatile contaminants.

Analysis was performed on an Agilent 7890A gas chromatograph coupled to an Agilent 5973C mass spectrometer. Helium was

used as a carrier gas at a constant flow rate of 1 mL/min. The injection port was equipped with a liner (0.75 mm i.d.) suitable for SPME analysis. It was operated in splitless mode for 1 min at 250 °C. Separation of compounds was performed on an HP-5MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness, Agilent). Oven temperature was initially maintained at 40 °C for 5 min, then was raised to 150 °C at a rate of 4 °C/min, and finally was raised to 250 °C at a rate of 30 °C/min and held for 5 min. The interface temperature was set at 280 °C. The mass spectrometer was operated in electron impact mode with the electron energy set at 70 eV and a scan range of 29–350 *m/z*. The temperature of MS source and quadrupole was set at 230 and 150 °C, respectively.

Identification of the compounds was performed by comparing: (i) the linear retention indices (LRI) based on an homologous series of even numbered n-alkanes (C8–C24, Niles, Illinois, USA) with those of standard compounds and by comparison with literature data, and (ii) MS data with those of reference compounds and by MS data obtained from NIST library (NIST/EPA/NIH Mass Spectral Library with Search Program, data version NIST 05, software version 2.0d). Amdis software (version 2.62, http://chemdata.nist.gov/mass-spc/amdis/) was used for the deconvolution of mass spectra and identification of target components.

The amount of volatile compounds was expressed in arbitrary unit of the peak area of deconvoluted component multiplied by 10^{-6}.

2.6. Statistical analysis

Differences of means in viable counts, TVB-N, TMA-N and sensory score were statistically tested by performing t-tests or Analysis of Variance followed by Tukey's significant difference test, using STATISTICA 6.0. A probability level of $P \leq 0.05$ was considered statistically significant. Statistical testing for difference of means in VOCs data (area under the chromatographic peak) was not applicable, for the reason that the measurements were conducted in duplicates and the determination of their concentration did not take place, since our aim was to monitor which of the detected compounds were produced from the early stage of storage and had the tendency to increase until the end of shelf-life, in order to identify which of them are realistic candidates for CSI. However, the VOCs data were subjected to Principal Component Analysis (PCA), using STATISTICA 6.0. Initially the total variance of the VOCs data was explained by 7 Principal Components (PCs), with the cumulative variance of the first two to be 69%. The variables (peak areas) for which the communality values of the first three PCs were higher or equal to 0.7 were considered as significantly explaining the variance of the data set, while the rest variables were removed. Then, a second PCA was performed revealing that the total variance of the VOCs data were explained again by 7 PCs but the cumulative variance of the first two PCs was 84.8%.

3. Results and discussion

3.1. Microbiological, sensory and shelf-life evaluation

Initially, fish freshness was excellent. The fresh characteristics were diminished gradually with time. More specifically, the fresh characteristics remained in good quality (total sensory score above 4) for 3 and 5 d under air and MAP respectively. Finally, the shelf-life of gutted sea bass stored at 2 °C under air and MAP was 9 and 13 d, respectively (Fig. 1).

Storage under elevated CO_2 extends shelf-life of fish compared to aerobic storage (Noseda et al., 2014). Shelf-life of fish and fishery products is depended on the storage conditions such as temperature and atmosphere, level of initial microbial contamination and handling like gutting, filleting, addition of antimicrobials and other factors that may affect growth of spoilage microorganisms. Consequently, several shelf-life durations have been reported in the scientific literature. Shelf-life of whole sea bass stored in ice varies from 11 to 19 d (Cakli et al., 2007; Kyrana and Lougovois, 2002; Paleologos et al., 2004; Papadopoulos et al., 2003), while gutted sea bass in ice varies from 8 to 14 d (Paleologos et al., 2004; Papadopoulos et al., 2003). Shelf-life of sea bass fillets stored at 4 °C, under air and MAP with the same gaseous composition with our study, found to be 8 and 12 d respectively (Kostaki et al., 2009). Our shelf-life determination is in accordance with these studies.

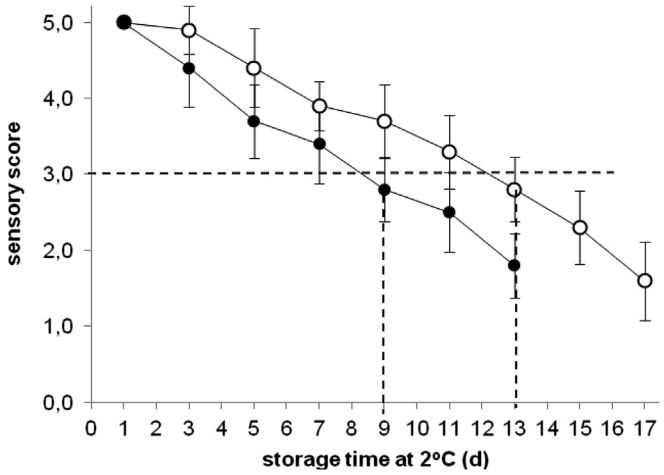

Fig. 1. Sensory score of gutted sea bass stored under air (●) and MAP (○) at 2 °C. The dashed lines show the time of organoleptic rejection.

The TVC initially (day 1) were about 3 logs cfu/g. At the same level were the populations of *Pseudomonas* and H_2S producing bacteria ($p > 0.05$), while LAB, *B. thermosphacta* and Enterobacteriaceae were about 1 log cfu/g lower (Fig. 2a,b). At the time of organoleptic rejection, TVC had reached the level of 7.5 and 7 log cfu/g for sea bass stored under air and MAP respectively.

MAP affected not only the growth rate but also the final populations of spoilage bacteria. Bacteria grew faster under aerobic conditions, while the increase of CO_2 and O_2 reduction of MAP inhibited the bacterial growth and changed the microbial spoilage by suppressing mostly the Gram negatives and favouring the Gram positives (Fig. 2a,b). Primarily *Pseudomonas* spp., and secondarily H_2S producing bacteria were the dominant spoilage microorganisms under air (Fig. 2a). Indeed, at the end of shelf-life, *Pseudomonas* spp., and H_2S producing bacteria reached populations as high as 7.2 and 6.7 log cfu/g respectively, while *B. thermosphacta* and LAB were about to 3.5 logs cfu/g and Enterobacteriaceae down to 2.5 logs cfu/g (Fig. 2a). Under MAP, the dominant microorganisms were again *Pseudomonas* and H_2S producing bacteria, reaching at the end of shelf-life populations as high as 6.4 logs cfu/g, followed by LAB with 6, *B. thermosphacta* with 5.2 and Enterobacteriaceae with 4.6 logs cfu/g (Fig. 2b).

Pseudomonas spp. and H_2S producing bacteria are the dominant spoilage microorganisms of chilled stored fish under air caught from the warm temperate waters of Mediterranean Sea (Paleologos et al., 2004; Papadopoulos et al., 2003; Parlapani et al., 2013, 2014b; Tryfinopoulou et al., 2002). Our study is in accordance with these results. *Pseudomonas* was the dominant bacterial population followed by H_2S producing bacteria. Under elevated CO_2 and reduced O_2 of MAP, *B. thermosphacta* and LAB usually predominate by outcompeting the strictly aerobic pseudomonads (Koutsoumanis et al., 2000). However, in the present work, although *B. thermosphacta* and LAB benefited from the MAP, they were not the predominant

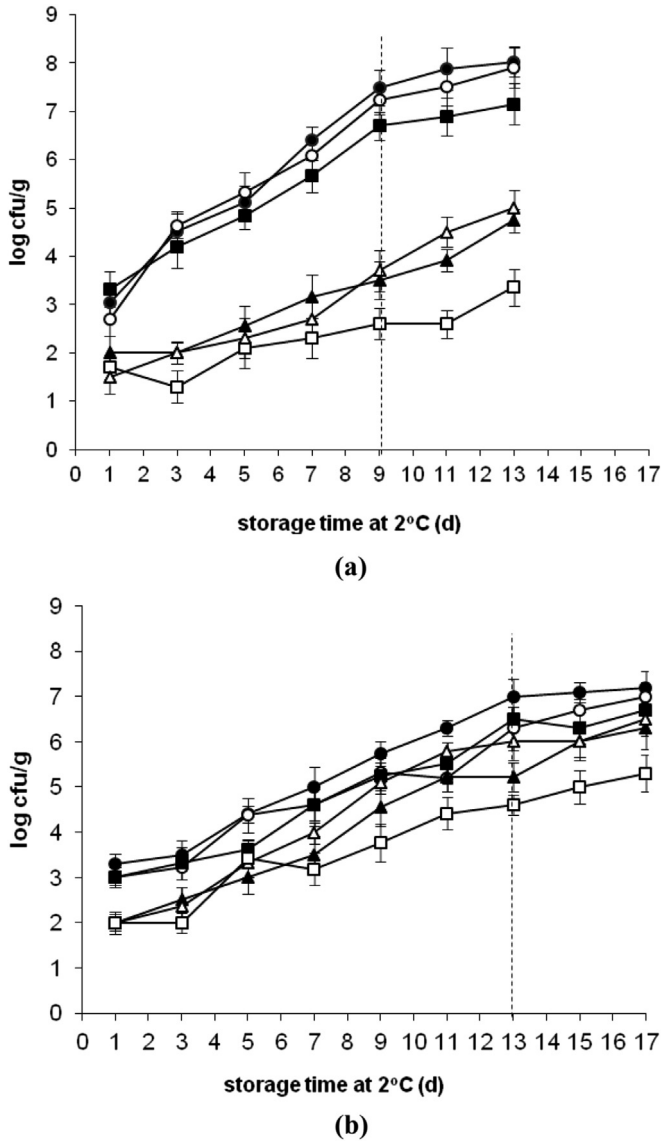

Fig. 2. Microbiological changes during storage of gutted sea bass at 2 °C under air (a) and MAP (b). TVC (●), *Pseudomonas* spp. (○), H_2S producing bacteria (■), Enterobacteriaceae (□), *Brochothrix thermosphacta* (△) and LAB (▲). Each data point and the error bars show the mean and ±st. dev. of 4 replicates. The vertical dashed lines indicate the time point of organoleptic rejection.

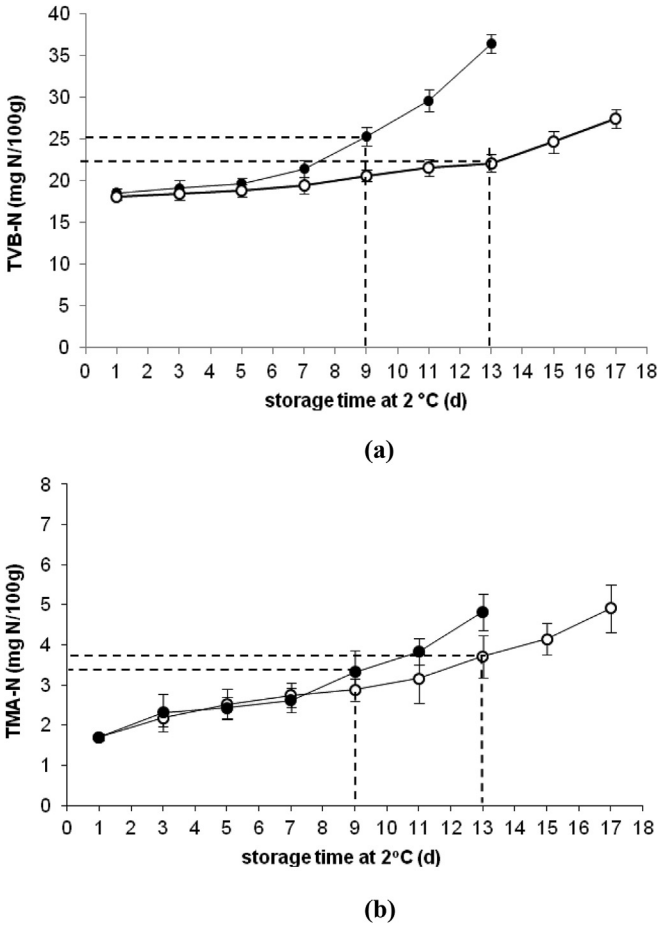

Fig. 3. TVB-N (a) and TMA-N (b) changes of gutted sea bass stored under air (●) and MAP (○) at 2 °C. Each data point and the error bars show the mean and ±st. dev. of 4 replicates. The dashed lines shows the time point of organoleptic rejection.

microorganisms and their population did not reach levels higher than *Pseudomonas* and H_2S producing bacteria. Accordingly, Kostaki et al. (2009) using the same gas mixtures with the present work, found that H_2S-producing bacteria and *Pseudomonas* spp., predominated during storage of organic aquacultured sea bass (*Dicentrarchus labrax*) caught from Greek waters, while *B. thermosphacta* and LAB populations did not reach levels higher than 6 logs cfu/g.

3.2. TVB-N and TMA-N determination

The initial amount of TVB-N was 18.1 and 18.4 mg/100 g for air and MAP respectively ($p > 0.05$). During the first 5 days of storage, TVB-N values did not change and were not different between air and MAP samples ($p > 0.05$). Subsequently, TVB-N increased and developed more rapidly under air as compared to MAP (Fig. 3a). At the organoleptic rejection time point, the concentration of TVB-N was 25.3 and 22.1 mg N/100 g for gutted sea bass stored under air and MAP, respectively. TMA-N was initially 1.7 mg N/100 g. TMA-N was not different between the two products until the 7th day of storage ($p > 0.05$) and increased substantially only after the end of shelf-life for both air and MAP products, reaching values about 4 and 5 mg N/100 g by the end shelf-life and the end of experiment respectively (Fig. 3b).

TVB-N values include the measurements of TMA, dimethylamine, ammonia and other volatile nitrogen containing compounds produced by bacterial degradation of proteins and aminoacids (Gram and Huss, 1996). The initial TVB-N values were similar to those reported in the literature for sea bass (Cakli et al., 2007; Castro et al., 2006; Kyrana and Lougovois, 2002). At the end of shelf-life TVB-N values never reached amounts as high as 30–35 mg N/100 g, which is the legislated limit (EC, 2074/2005). Similar results were also reported in the literature by other researches (Cakli et al., 2007; Castro et al., 2006; Kyrana and Lougovois, 2002; Papadopoulos et al., 2003).

It is well established that *Pseudomonas* spp. grows well on aerobically stored seafood, producing volatile ammonium containing compounds (Dainty, 1996; Gram and Huss, 1996). Indeed, TVB-N increased at the end of storage, when *Pseudomonas* reached a considerable population level of about 6 logs cfu/g (Figs. 2a and 3a). In sea bass under MAP, TVB-N development was slower. This might be attributed to the suppression of *Pseudomonas* and enhancement of LAB and *B. thermosphacta*, which produce organic acids (Drosinos and Nychas, 1997; Tsigarida et al., 2003) instead of

nitrogenous compounds. TVB-N increases only at the last stages of storage or after the organoleptic rejection making it unsuitable for CSI, as has already been reported by other researchers (Cakli et al., 2007; Castro et al., 2006; Kyrana and Lougovois, 2002).

TMA is produced by the decomposition of TMAO as a consequence of bacterial activity, mostly of *Shewanella putrefaciens* and *P. phosphoreum* that use TMAO instead of oxygen, as the final electron acceptor in respiratory metabolism (Dalgaard et al., 1993). Initial values of TMA-N in sea bass vary from 0 up to 2 mg N/100 g (Cakli et al., 2007; Kostaki et al., 2009; Kyrana and Lougovois, 2002; Papadopoulos et al., 2003). Our observed level of 1.7 mg N/100 g is within the same range of those reported by Kostaki et al. (2009). The level of TMA-N was found to be low even after a long storage time and only a slight increase was noticed near the end of shelf-life. In general TMA-N development is not pronounced in fish from Mediterranean waters as compared to other species from Northern Seas (Koutsoumanis and Nychas, 1999; Kyrana and Lougovois, 2002; Papadopoulos et al., 2003). This can be attributed to the low levels of TMAO in fish from Greek waters (Koutsoumanis and Nychas, 1999) and/or to low levels of *S. putrefaciens*. Indeed Dalgaard et al. (1993) reported that *Shewanella* population higher than 10^8 cfu/g is required to produce considerable TMA-N amounts. On the other hand, Kyrana and Lougovois (2002) determined the TMAO level in sea bass to be 22.8 mg N/100 g, which is quite lower compared to 66–75 mg N/100 g found in *Gadus morhua* (Herland et al., 2009). Usually TMA-N is produced in much higher amounts when fish is stored under low oxygen conditions as a consequence of *P. phosphoreum* and *S. putrefaciens* domination (Dalgaard et al., 1993). However, in chilled fish from Mediterranean waters *P. phosphoreum* is not a significant part of the spoilage microbiota (Carrascosa et al., 2014; Koutsoumanis and Nychas, 1999), while under MAP the population of H_2S producing bacteria (presumptive *S. putrefaciens*) which produce TMA, was reduced. These arguments can explain the low levels of TMA-N. For those reasons TMA-N cannot be used as spoilage indicator in our case.

3.3. VOCs analysis

Without taking into account terpenes, aromatic compounds and hydrocarbons which were present in trace amounts, a total of 40 VOCs were detected. The detected compounds were alcohols, aldehydes, ketones, esters and one organic acid (acetic). The VOCs profile during storage of gutted sea bass under air and MAP are presented in Table 1. The majority of these compounds (23) were detected in both atmospheres, while nine (9) were detected only under air and eight (8) only under MAP. Esters were detected only under air, while acetic acid was detected under MAP (Table 1). Most of the compounds were appeared sporadically or fluctuated during storage, while some compounds increased during storage. In particular, 3-methyl-1-butanol, 2-methyl-1-butanol and the ethyl esters of acetate, propionate and isobutyrate were increased under air, while 2-methylbutanal, hexenal, heptanal, n-decanal, 2,3-octanedione and 3,5,-octadien-2-one increased under MAP. Finally, ethanol and 3-methylbutanal increased under both atmospheric conditions (Table 1).

PCA graphical results are shown in Fig. 4. The variable plot gives a graphical representation of the relationship between the VOCs and the first two axes, the PC 1 and PC 2 axes accounted for the 57.46 and 27.3% of the total variance of the data respectively, showing that there were 3 groups (Fig 4a). In general, the group 1 contains compounds that were increased during MAP, while in group 2 the compounds were fluctuated in both storage atmospheres. Finally in group 3 the compounds were appeared to increase substantially at the late stages of storage, especially during aerobic conditions. Fig. 4b demonstrates the score plots for the first two PCs of fish samples, showing that all MAP stored samples (Fig 4b, samples M1 to M4) consist one group while the scores of air stored samples (Fig 4b, samples A1 to A4) were more scattered. Regarding the aerobic storage, the samples taken after the end of shelf life (Fig 4b sample A4) consist a separate group, presumably due to the higher values that were determined at that point of storage for most of the volatiles, in contrast to the other samples (Table 1).

It is known that different temperatures and atmospheric conditions not only select different dominant microorganisms with different metabolism but also affect both their growth rate and metabolic activity (Nychas et al., 2008). This can explain the difference on VOCs profile between air and MAP stored fish. Regardless the diversity of the initial microbiota, certain microorganisms finally predominate, mostly due to the selection imposed by the storage temperature and oxygen availability and produce the spoilage metabolites (Nychas et al., 2008). Hence, the VOCs associated with the spoilage undoubtedly are produced by the dominant spoilage microbiota, making the effect of the initial diversity to the VOCs profile a minor issue.

Numerous VOCs detected in the present study have already reported in the literature as bacterial metabolites, while others as products of chemical activity. A lot of aldehydes, ketones and alcohols, found in our study as 1-hexanol, 1-octen-3-ol, heptanal, octanal, nonanal, nonenal, decanal etc., which come from enzymatic catabolism of unsaturated fatty acids, are aroma compounds that characterize fresh fish (Leduc et al., 2012; Selli and Cayhan, 2009). Quite few compounds associated with aroma of European sea bass were reported by Leduc et al. (2012). They found that some compounds such as thiophene and 1-nonen-3-ol were diminished while others such as hexanal, 1-octen-3-one and dimethyl-trisulfide increased during storage. For this reason Leduc et al. (2012) suggested the former as potential quality markers while the latter as potential spoilage markers. However, none of these compounds were detected in our case. It is known that geographical origination, seasonal variations, different storage conditions and different feeding affect the development of volatiles (Alasalvar et al., 2005; Gram and Huss, 1996; Grigorakis et al., 2009).

Compounds such as 1-penten-3-ol, 1-octen-3-ol, 2,3 pentanedione, and cis-4-heptanal, which were also found in our study, are involved with the oxidative rancidity of polyunsaturated fatty acids (Duflos et al., 2006; Iglesias and Medina, 2008). Some of the above mentioned compounds like hexenal, heptanal, octanal, nonanal and n-decanal increased during storage under MAP (Table 1). However, these compounds are not related to bacterial action, which is the primer mechanism of fresh sea bass quality deterioration and consequently further investigation as potential CSIs was not applied.

Various alcohols, aldehydes, ketones, acids and esters have been reported as products of the metabolic activity of microorganisms such as *Pseudomonas* spp., *Shewanella* spp., Enterobacteriaceae, *B. thermosphacta* and LAB during fish and/or meat spoilage. More specifically, the ethyl esters presumably are mainly related to *Pseudomonas* spp. activity (Casaburi et al., 2014; Edwards et al., 1987). Indeed, esters were found only in gutted sea bass stored under air where the aerobic conditions enhance growth and metabolic activity of pseudomonads. The profile of esters such as ethyl acetate, propionate and isobutyrate in relation to TVC are shown in Fig. 5. Ethyl propionate and isobutyrate amounts were increased after 5th day of storage, when TVC and pseudomonads population reached 10^5 log cfu/g, while an abrupt increase was noticed after the end of shelf-life (d 9), when TVC and pseudomonads population was higher than 10^7 cfu/g (Table 1, Figs. 2a and 5a,b). Ethyl acetate exhibited a slight increase initially with an

Table 1
Detected VOCs and their relative concentrations (surface × 10^{-6} under the chromatographic peak) in gutted sea bass during storage (in days) under air and MAP at 2 °C. Each value is the mean of duplicate measurement of pooled sample.

		Air (area × 10^{-6})				MAP (area × 10^{-6})			
		1	5	9	13	1	7	11	15
	Alcohols								
1	Ethanol	19.73	35.70	42.88	108.38	5.64	10.32	20.15	25.04
2	1-pentanol	0.22	0.06	0.29	0.71	0.09	0.22	0.28	0.22
3	1-hexanol	0.39		0.17	2.03	0.65	0.15	0.31	0.32
4	1-decanol						0.10		
5	1-dodecanol					0.07	0.07	0.20	
6	2-methyl-1-butanol			0.23	1.49				
7	3-methyl-1-butanol		0.06	1.11	11.12				
8	2-ethyl-1-hexanol	0.40	2.79	34.24	35.55	0.34	0.10	0.24	
9	1-penten-3-ol	39.86	15.57	30.86	26.34	2.01	4.28	3.23	3.07
10	2-penten-1-ol	0.88		0.93	0.47	0.43	0.45	0.45	0.46
11	1-octen-3-ol	3.25	1.41	2.82	4.36	0.44	1.54	1.05	1.00
12	Heptyl alcohol					0.05		0.14	
	Aldehydes								
13	2-methylbutanal	0.32	0.49	0.44	0.58		0.11	0.26	0.34
14	3-methylbutanal	0.96	1.54	7.30	8.64	0.18	0.38	2.35	2.61
15	2-pentenal	0.13	0.21	0.44		0.10			
16	Hexenal	50.00	24.11	38.33	4.58	4.42	7.03	8.27	8.30
17	Heptanal	0.26	1.76	0.42	0.27	0.25	0.22	0.49	0.53
18	Octanal		0.70			0.44	1.10	1.56	1.67
19	Nonanal	1.59	2.85	3.21	1.79	3.38	5.45	6.40	6.89
20	2-decenal, (E)					0.34	0.41	0.46	0.50
21	n-decanal	0.09	0.15	0.29	0.19	1.21	1.77	2.60	2.88
22	trans-2-hexenal	0.17		0.14		0.19	0.16	0.17	
23	Trans-2-octenal	1.21	2.80	5.90	9.31	2.03		2.76	
24	Trans-2-nonenal					0.54	0.34		
25	Cis-4-heptenal	3.01	0.22	2.35		1.09	1.51	0.80	0.64
26	Cis-6-nonenal				1.66				
27	Trans,trans-2,4-heptadienal	0.49	0.18	0.84		0.34	0.20	0.22	0.19
	Ketones								
28	Acetone	1.61	5.12	2.84	0.87	2.06	16.64	0.97	0.66
29	2,3 pentedione	24.87	7.21	16.24		2.72	1.43	2.45	2.00
30	2,3-octanedione	1.93	1.07	1.55	0.29	0.32	0.32	0.55	0.54
31	6-methyl-5-hepten-2-one					0.13	0.27	0.24	0.22
32	3,5-octadien-2-one	1.37		0.78		1.43	1.60	2.67	2.70
	Esters								
33	Ethyl acetate	2.45	2.92	3.92	13.79				
34	Ethyl propionate			0.05	0.20				
35	Ethyl-2-methylbutyrate				1.32				
36	Ethyl isobutyrate			0.23	2.31				
37	Ethyl isovalerate				1.07				
38	Butanoic acid, 2-methylbutyl ester		0.05	0.02	0.10				
39	Ethyl hexanoate					0.06			
	Organic acids								
40	Acetic acid					1.53	0.26	0.19	0.22

abrupt increase after the end of shelf-life (Fig. 5c). Ethyl acetate increased in cod (Olafsdottir et al., 2005) and pangasious fillets (Noseda et al., 2012) but not from the beginning of storage, like in our case.

Production of acetic acid has mainly attributed to the metabolic activity of *B. thermosphacta* and some LAB (Joffraud et al., 2001; Laursen et al., 2006; Tsigarida et al., 2003). Indeed, in our study acetic acid was detected only in sea bass stored under MAP where *B. thermosphacta* and LAB growth was more pronounced compared to storage under air. However, acetic acid did not exhibit a consistent profile in our case, in contrast to other researchers who suggested acetic acid as spoilage marker in fresh king salmon (Wierda et al., 2006) and in MAP stored pangasious (Noseda et al., 2012) and salmon fillets (Mace et al., 2013).

Regarding alcohols, ethanol is produced both by LAB under reduced oxygen and *Pseudomonas* under air storage (Casaburi et al., 2014; Olafsdottir et al., 2005). Ercolini et al. (2009) reported that 2-ethyl-1-hexanol is produced by *Carnobacterium* in spoiled beef. Additionally, 3-methylbutanol and 2-methylbutanol are produced by *Carnobacterium* in packed shrimp (Laursen et al., 2006) and various bacteria such as *Pseudomonas*, *Shewanella* and *Brochothrix* in meat (Casaburi et al., 2014). The amounts of alcohols such as ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol and 2-ethyl-hexanol were varied during storage in relation to TVC (Fig. 6). Under air, the amount of ethanol increased gradually until d 9 (end of shelf-life), followed by an abrupt increase after that point when TVC reached high numbers (Table 1, Figs. 2a and 6a). Under MAP, the increase was smoother throughout storage (d13), presumably due to the slower development of LAB and the occurrence of other spoilage bacteria under MAP (Table 1, Figs. 2b and 6a). The profile of 2-ethyl-1-hexanol was different showing a slight increase at the beginning, a rapid increase at the middle stages and a plateau after the end of the shelf-life (Table 1, Fig. 6b). Finally, the profiles of 3-methyl-1-butanol and 2-methyl-1-butanol were similar to those of ethyl esters, presenting a slight increase from the middle stages of the storage and a rapid increase just after the end of shelf-life (d9) (Table 1, Fig. 6c,d). Primarily ethanol and 2-ethyl-1-hexanol (under air only) and secondarily 3-methyl-1-butanol and 2-methyl-1-butanol (under air only) can be considered as potential CSI candidates for gutted sea bass. Ethanol is also proposed as

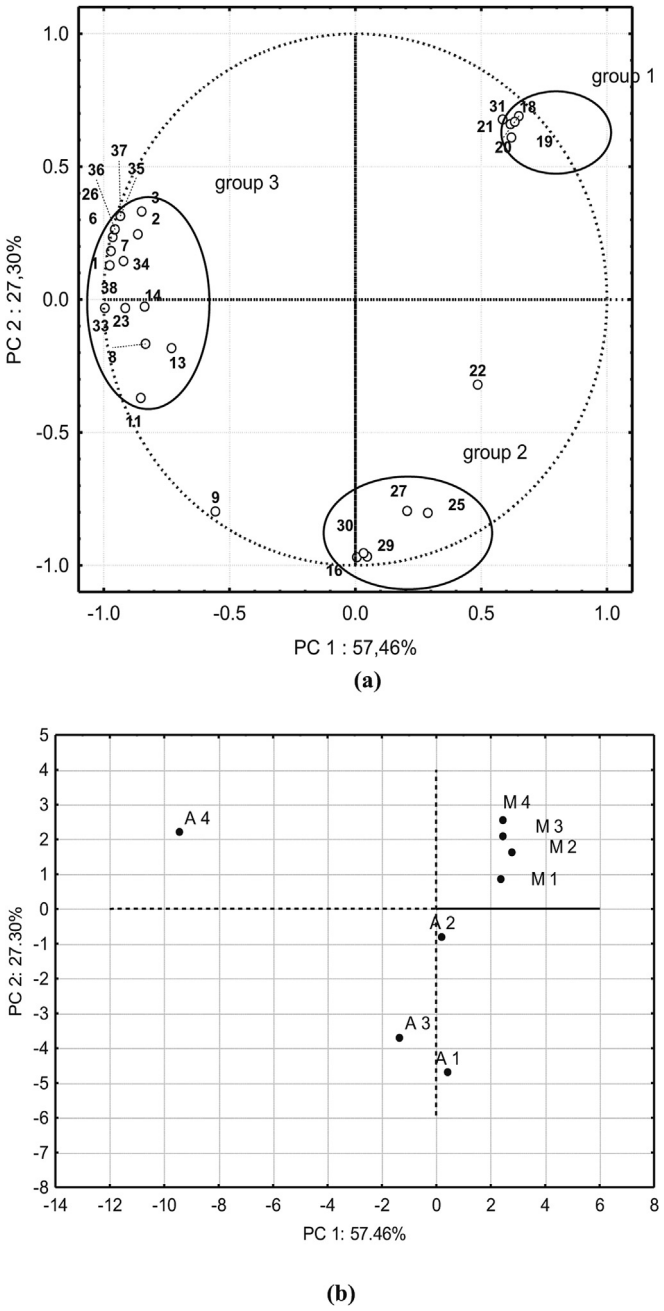

Fig. 4. Principal Component Analysis showing (a) the loading plots with the significant variables (the variable numbers corresponds to the volatile numbers of Table 1) to first two PCs and (b) score plots for the first two PCs of fish samples labelled as A: air stored, M: MAP stored and, 1 = fresh, 2 = slightly deteriorated, 3 = deteriorated, 4 = spoiled.

potential spoilage marker of pangasious fillets (Noseda et al., 2012), while its presence is also correlated very well with the electronic nose CO sensor response for chilled stored cod (Olafsdottir et al., 2005). Finally, 3-methyl-1-butanol has also been suggested as spoilage marker for ice-stored sea bream (Soncin et al., 2008) and yellowfin tuna (Edirisinghe et al., 2007).

Among aldehydes 3-methylbutanal and 2-methylbutanal have been reported as products of *Pseudomonas* spp., *Shewanella* spp. and *Brochothrix* spp. in spoiled chicken carcasses and meat (Casaburi et al., 2014) and of *Carnobacterium* species in seafood (Joffraud et al., 2001; Laursen et al., 2006). Additionally, they have

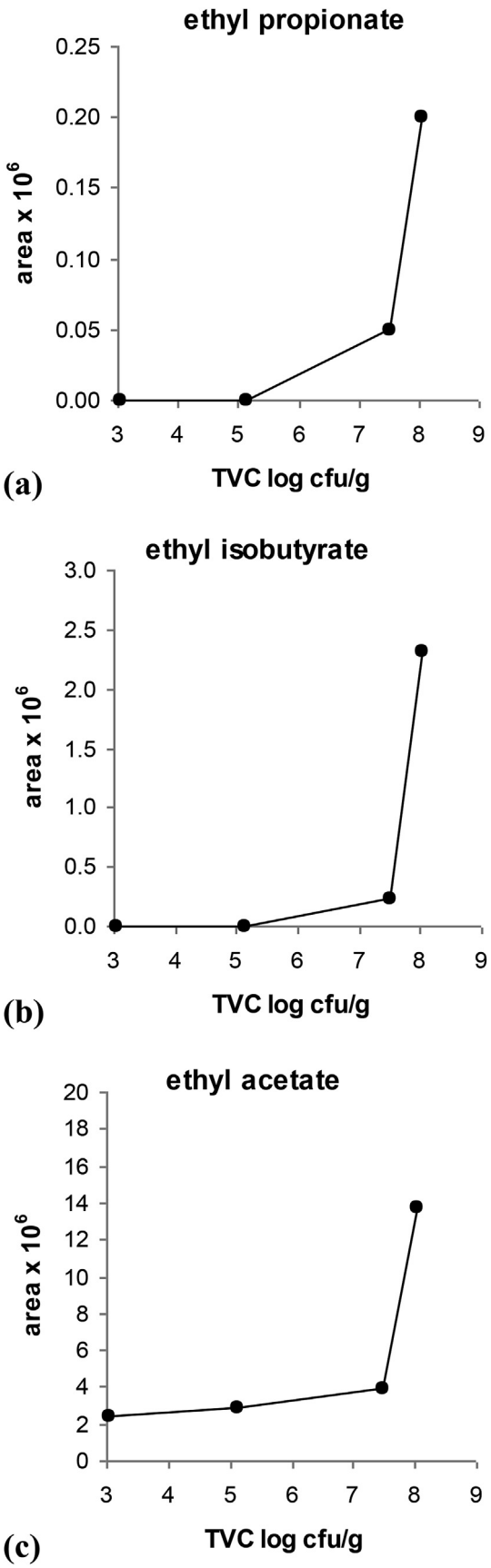

Fig. 5. Changes of selected ethyl esters in relation to TVC during storage of gutted sea bass stored at 2 °C under air (●). Each data point is the mean of duplicate measurement.

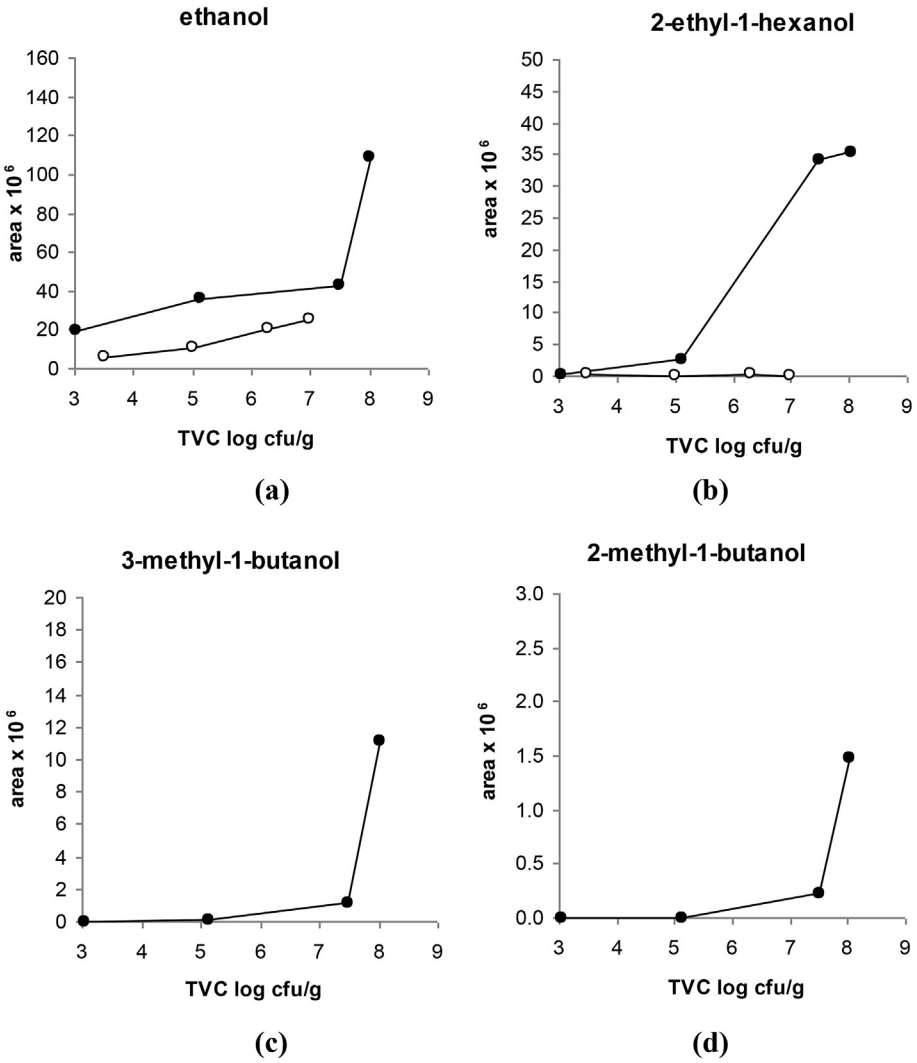

Fig. 6. Changes of selected alcohols in relation to TVC during storage of gutted sea bass stored at 2 °C under air (●) and MAP (○). Each data point is the mean of duplicate measurement.

been found in various chilled stored fish such as sea bream (Soncin et al., 2008; Parlapani et al., 2014a), cod, whiting and mackerel (Duflos et al., 2006; Olafsdottir et al., 2005), where *Pseudomonas* spp. and H_2S producing bacteria predominate. The profiles of 3-methylbutanal and 2-methylbutanal in relation to TVC during the storage of gutted sea bass are shown in Fig. 7. Very good profile was exhibited by 3-methylbutanal in both air and MAP conditions showing a gradual increase from the beginning until the end of storage (Table 1, Fig. 7a). On the contrary, 2-methylbutanal profile was excellent only under MAP (Table 1, Fig. 7b). These aldehydes have already been proposed in the literature as potential spoilage markers of whole sea bream (Soncin et al., 2008) and sea bream fillets (Parlapani et al., 2014a).

Regarding ketones, 2,3 pentanedione was reported as product of *Carnobacterium* in cold-smoked salmon (Joffraud et al., 2001), while acetone as product of *Psychrobacter* and *Pseudoalteromonas* in brown shrimp (Broekaert et al., 2013). Acetoin (3-hydroxy-2-butanone), which has been attributed to LAB (Jonsdottir et al., 2008) and *P. phosphoreum* (Olafsdottir et al., 2005) when they reach high population densities, did not detected in our case. It must be noted however that *P. phosphoreum* is a negligible part of the spoilage microbiota of chilled fish from temperate waters (Carrascosa et al., 2014; Koutsoumanis and Nychas, 1999) and the population of LAB never reached populations exceeding 6 logs cfu/g in our case.

4. Conclusions

According to the results of the current study, several volatiles such as ethanol and 3-methylbutanal under both air and MAP, 2-methylbutanal only under MAP and 2-ethyl-1-hexanol, 3-methyl-1-butanol, 2-methyl-1-butanol and ethyl esters of acetate, propionate and isobutyrate only under air, can be identified as potential CSIs candidates of gutted sea bass.

To identify microbial metabolites for monitoring fish freshness/spoilage, a lot of factors might have to be taken into account. Storage conditions such as temperature and its fluctuation, bacterial competition, variability of fish batches caught at different seasons, etc., have to be taken into account. Further work focused on the abovementioned selected VOCs is required to determine accurately their concentrations from different batches stored under various conditions and correlate VOCs concentration with freshness/spoilage status and remaining shelf-life. This can be the first step for designing bio-sensors for on-pack freshness assessment and shelf-life determination.

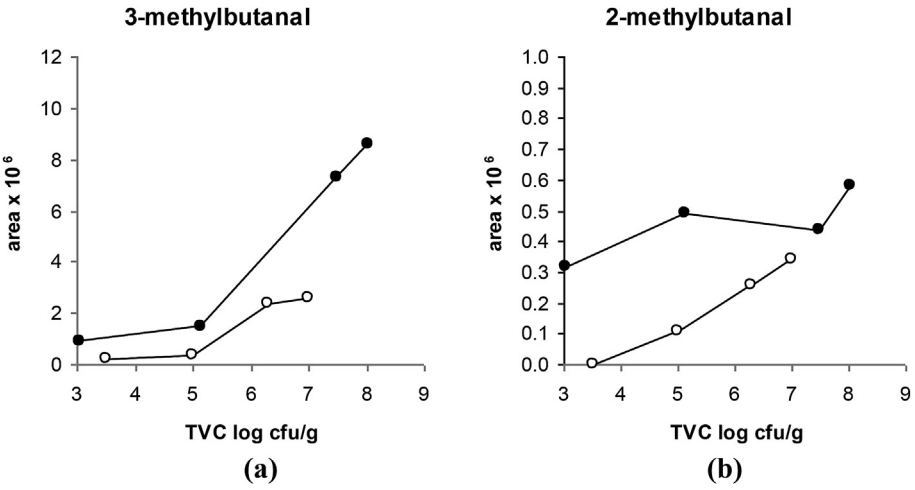

Fig. 7. Changes of selected aldehydes in relation to TVC during storage of gutted sea bass stored at 2 °C under air (●) and MAP (○). Each data point is the mean of duplicate measurement.

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