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Effects of Ice and Seawater Storing Conditions on the Sensory, Chemical and Microbiological Quality of the Mediterranean Hake (*Merluccius merluccius)* During Post-Catch Handling and Distribution

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***Abstract*—**Changes in the sensory, chemical and microbiological quality of the Mediterranean hake during post-catch handling and distribution were investigated. 115 fish samples were seasonally received during three stages of the transfer route from the sea to the consumer and two storage methods were recorded, seawater and ice storage. Microbiological evaluation revealed higher status for the ice stored samples regarding heterotrophic bacteria (2.68 log cfu/g and 1.92 log cfu/g at 22ºC and 37°C respectively) and psychrotrophic counts (3.20 log cfu/g), with statistically significant differences among storage methods. Sensory evaluation also revealed higher status for the ice stored samples with a mean quality index of 0.17 and a spoilage time estimated at 30 hours, in contrast to seawater storage, which varied from 0.28 to 0.3, and a 14-hour estimated spoilage. Detected pathogens were identified mainly in the seawater stored samples, posing questions on the quality of the product reaching the seafood markets.

Keywords*—Merluccius merluccius*, Microbiological quality, Psychrotrophic bacteria, Sensory evaluation.

I. Introduction

MEDITERRANEAN hake (*Merluccius merluccius*) is a widely distributed fish species in the Mediterranean basin, living in a depth range between 25-100m. It is commonly sold for consumption at retail fish stores or seafood markets, covering a large part of the annual demand in Greece and the maintenance of its high quality standards is a major concern to industry and consumers worldwide.

Fish is one of the most highly perishable food products [1]. According to the HACCP concept, there is no quality control for fresh fish except for time – temperature – tolerance. However, long – term storage of fish, even in low temperatures, is not recommended due to biochemical deterioration [2]. During handling and storage, quality deterioration rapidly occurs, as a result of bacteriological activity leading to an induced enzymatic activity and a limited shelf life [3], [4]. Shelf life is determined as the period of time under defined conditions of storage for which a food product remains safe for use, meaning that it should retain the desired sensory, chemical, functional or microbiological characteristics during this period. Although spoilage of foods can be caused by both chemical reactions and physical damage, the major hazard is microbial growth and metabolism [5]. Spoilage process refers to production of off-odor and off-flavor, slime formation, large visible pigmented or non-pigmented colonies, production of gas and discoloration [6].

On board handling, storage and transportation have also been mentioned as important factors that influence the numbers and types of bacteria on the raw material [7]. Taking into account the specific microbial ecology of marine waters, quality deterioration of fish is caused by Gram-negative, fermentative and psychrotolerant bacteria, such as Vibrionaceae and *Pseudomonas* spp. and *Shewanella* spp., respectively [5], [6], [8], [9]. The spoilage potential of *Shewanella putrefaciens* is a result of its ability to reduce trimethylamine oxide and produce volatile sulfide from sulfur-containing amino acids [10]. Moreover, certain Salmonella types may also be part of the indigenous microbial flora, especially given that aquatic birds spread salmonellae, as well as other human pathogens in the environment [9].

Faulty rearing, harvesting and processing practices can result in cross-contamination of fish with food borne pathogenic bacteria [7]. Maintaining the high quality of fresh fish during the period of post-catch and distribution seems a real challenge. What is of crucial importance is the time lapse before icing and the exposure to ambient temperatures, as these conditions encourage proliferation of microorganisms [1]. Delayed icing is a common practice and in some cases, proper icing may not be accomplished even at the landing centers.

In an effort to prevent rapid deterioration of fish quality, the European Commission issued the directive 91/493/EU [11], which indicates all actions that should take place during post-catch handling and distribution in order for the fishery products to meet the standards set by European regulation [12], [13]. In Greece, ice storage is not strictly used at all stages of transport, as many fishermen preserve fish in boxes containing only seawater, exposed at ambient temperatures and sunlight for hours, during transport to the port.

The present study was undertaken in order to investigate the potential sensory, chemical and microbiological quality

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deterioration of fish samples, obtained from different transport stages in an extended area of the Ionian Sea (Western Greece), using the two most common preservation methods followed by fishermen, ice and seawater storage. Previous literature on sensory, chemical and microbiological properties of fish is mainly related with species such as *Tuna viscera* [14], *Sparus aurata* [3], [4], [15] and *Dicentrarchus labrax* [15], with only few articles focusing on *Merluccius merluccius* [16]-[18]. Moreover, it is the first time that this kind of study is performed in the particular area and the overall goal was to compare and suggest proper storage conditions in order for fresh fish to maintain high quality standards and avoid spoilage throughout the course to consumers.

II. Materials and Methods

A. Sample Collection

Ten seasonal sampling campaigns took place in the region of Western Greece, covering the coastal zones of Achaia, Ilia and Etoloakarnania prefectures (Fig. 1). Samples were received from three transport stages, the first immediately after catchment, the second after a storage period of 5-6h on the boat and the third in the seafood markets (storage period of 12-13h). A total of 115 hakes were collected and preserved according to FAO regulation [19]. Each sample was preserved in 4°C and transferred to the lab for analysis, immediately after the end of the campaign. All samples received from Achaia and Etoloakarnania were stored from the fishing personnel in boxes containing seawater, whereas all samples taken from Ilia prefecture were immediately stored on ice, layered under, around and on top of the fish.

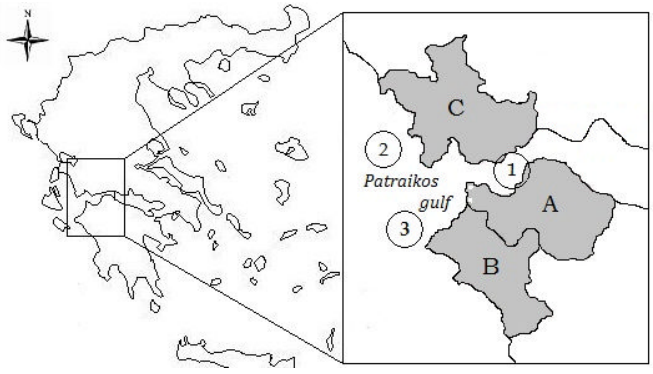

Fig. 1 Study area. Samples were collected from three zones (1) Achaia – Seawater I (2) Etoloakarnania - Seawater II and (3) Ilia - Ice storage

B. Sensory Evaluation

Fishes from each stage of every storage method were scored from three trained panelists, according to characteristics concerning general appearance, eyes and gills, using the Quality Index Method developed by Baixas-Nogueras et al., 2003 [17]. Panelists were asked to score surface appearance, flesh firmness, clarity, pupil and shape of the eyes, color and smell of the gills using a 0-1 scale, where 0 represented best quality and higher score indicated poorer quality.

C. Chemical Analysis

Total volatile base nitrogen (TVB-N) was determined using the Antonackopoulos and Vyncke method [20], while trimethylamine nitrogen (TMA-N) was measured by applying the method of AOAC [21]. Determination of histamine was performed by the use of histmanine ELISA kits from IBL-Hamburg according to the manufacturer's instructions. All values were expressed as mg/100g of fish flesh.

D. Microbiological Analysis

Each sample was tested for the presence and enumeration of psychrotrophic bacterial species (*Pseudomonas* sp. & *Vibrio* sp.), H_2S producing bacteria (*Shewanella putrefaciens*), *Enterobacteriaceae* (*Salmonella* sp.) and heterotrophic plate count (HPC) at 22°C and 37°C according to International Standards Organization (ISO) techniques. Flesh samples were cut aseptically into slices in sterile containers. A sample of 25g of skinless fish flesh was obtained aseptically from each fish, transferred to a stomacher bag (Seward Medical, London, UK) and was homogenized for 2 min in a stomacher (Lab Blender 400; Seward Medical) with 225mL of Peptone Saline Solution (Oxoid). Pseudomonads were determined on cetrimide fusidin cephaloridine agar (supplemented with selective supplement SR 103, Oxoid, Basingstoke, UK). The surface-plate method was applied and plates were incubated at 30°C for 48h. For the isolation of *Vibrio* sp., the homogenate was firstly incubated at 30°C for 24h and then the surface-plate method was also performed on TCBS agar (Oxoid). Plates were incubated at 30°C for 24h. Determination of H_2S-producing bacteria (typical of *Shewanella putrefaciens*) was permormed using Triple Sugar Iron Agar (TSI - Oxoid) and plates were incubated at 30°C for 72h. Black colonies formed by the production of H_2S were enumerated after 2–3 days of incubation. For *Salmonella* sp., enrichment steps were performed. Firstly, the homogenate was incubated at 37°C for 24h, and then portions of 1mL and 100μL were mixed with 10mL of Selenite Cystine broth (Oxoid) and Rappaport – Vassiliadis broth (Oxoid), respectively. Second incubation followed at 37°C for Selenite Cystine broth and 42°C for Rappaport – Vassiliadis broth for 24h. After enrichment steps the surface-plate method was applied on Salmonella – Shigella agar (Oxoid) and XLD agar (Oxoid) and all plates were incubated at 37°C for 24h.

For the enumeration of HPC the pour plate count method was chosen, using 2ml of the homogenate and mixing with melted Plate Count Agar (Oxoid), tempered at 44°C. Two sets of plates were prepared for all samples. One set was incubated aerobically at 37°C for 48h and the other set at 22°C for 72h. All plates were examined visually for typical colony types and morphological characteristics associated with each growth medium. In addition, the selectivity of each medium was checked. All colonies were counted as colony-forming units (cfu) per g of the fish sample. The isolated microorganisms were identified depending on their biochemical characteristics and using standardized identification systems API 20E and API 20NE (BioMérieux, 69280 Marcy-l'Étoile, France).

E. Statistical Analysis

Chemical and microbiological data of the different storage methods were statistically analyzed using the Analysis of Variance (ANOVA) of the SPSS 14.0 statistical package in order to check for variation between samples. Means were separated by the least significant difference test at P<0.05.

III. Results

A. Sensory Analysis

Fig. 2 shows sensory changes of hakes under the two recorded storing conditions (ice and seawater) in the different sampling areas. All samples at the present study were acceptable for consumption with high quality indices as values were lower than 0.36. Nevertheless, those retained in seawater presented a more rapid deterioration rate, reaching the seafood stores with a mean quality index at 0.28 and 0.3, in contrast to the ice stored samples, which presented an index of 0.17. Although the observed differences were not statistically significant according to the ANOVA test (P>0.05), a more gradual decrease of the sensory quality status was obvious from catchment to distribution stages for the seawater storage. Flesh firmness, clarity, pupil and shape of the eye, as well as the color of the gills were the main sensory parameters influenced by quality degradation. Spoilage time regarding sensory data for each sampling area is estimated by applying a linear regression between the mean quality index values (mean values between seasons) and time in hours (Fig. 3).

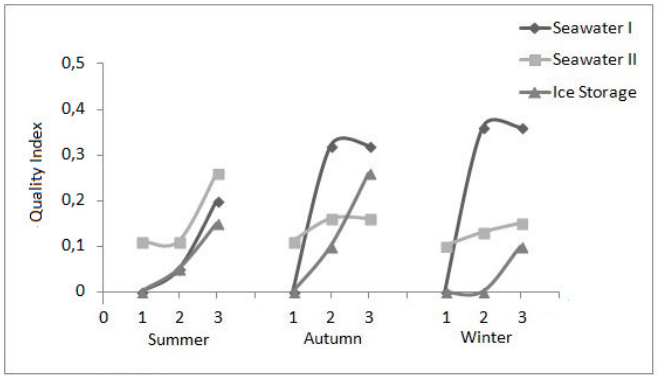

Fig. 2 Quality Index of fish samples according to sensory evaluation The three sampling stages are depicted by the numbers on x-axis 1: Immediately after catchment, 2: After 5-6h of storage, 3: Reaching the consumer after almost 12-13h of storage

B. Microbiological Analysis

Microbiological analysis revealed that the ice stored samples underwent lower deterioration especially during the summer period, when seawater temperature was high, thus favoring proliferation of microorganisms. Heterotrophic bacteria (Figs. 4 and 5) were lower for the consumption stages of the ice storage method, reaching mean values of 2.68 log cfu/g and 1.92 log cfu/g at 22°C and 37°C respectively, while the consumption samples of the seawater storage reached higher values, exceeding 3.17 log cfu/g during autumn and summer seasons. Moreover, a more gradual increase in heterotrophic plate counts was observed during the three transport stages for the seawater stored samples, with the differences being statistically significant (P<0.05) for the summer samples.

Counts of psychrotrophic bacteria also presented lower mean values for the ice stored samples, reaching 3.24 log cfu/g in the consumption stage of the summer season, in contrast to the higher values (almost 3.6 log cfu/g) in the seawater stored samples. Microbiological variation detected in fish samples is shown in Table I. Several bacterial species were identified, with some of them being significant for public health, such as *Pseudomonas aeruginosa*, *Vibrio alginolyticus* and the rarely occurring *Ochrobactrum anthropi*. Colonies of *Vibrio* sp., *Pseudomonas aeruginosa*, *Ps. cepacia* and other species were isolated mainly from samples received during summer and preserved in seawater.

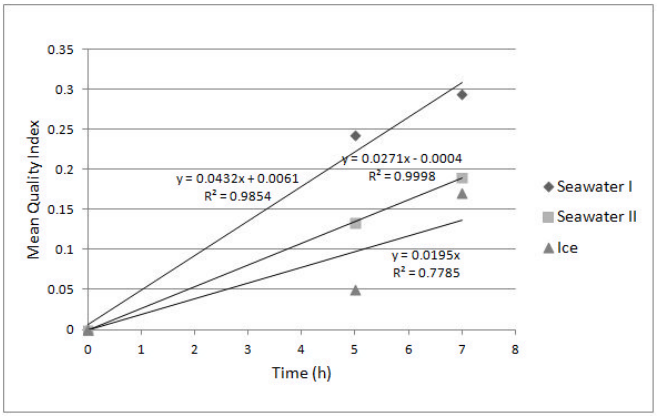

Fig. 3 Graphical representation of the mean quality index through time (hours) combined with linear regression for each sampling area For each case, the quality index is the mean seasonal value

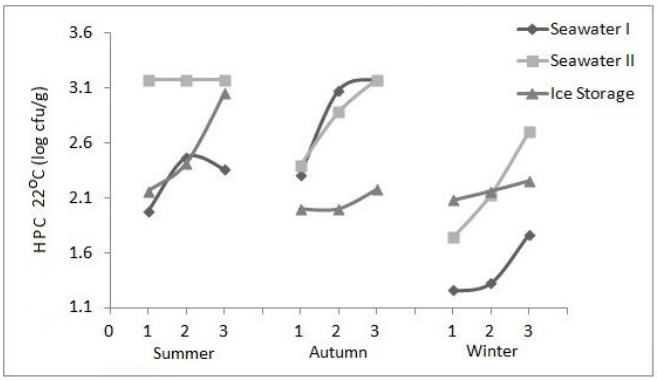

Fig. 4 Heterotrophic Plate Counts (HPC) at 22°C expressed as logcfu/g (colony forming units) Three sampling stages are depicted on x-axis 1: Immediately after catchment, 2: After 5-6h of storage, 3: Reaching the consumer after almost 12-13h of storage

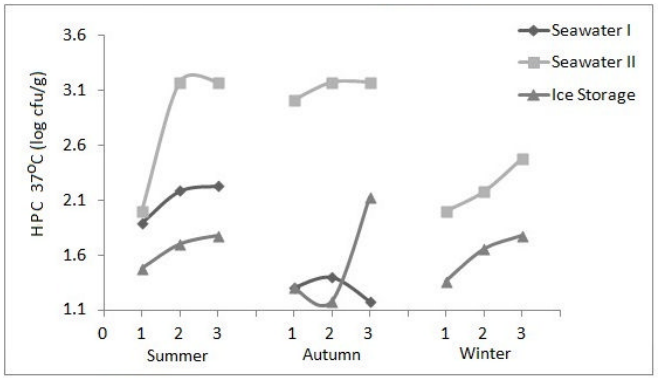

Fig. 5 Heterotrophic Plate Counts (HPC) at 37°C expressed as logcfu/g (colony forming units) Three sampling stages are depicted on x-axis 1: Immediately after catchment, 2: After 5-6h of storage, 3: Reaching the consumer after almost 12-13h of storage

TABLE I
BACTERIAL SPECIES IDENTIFIED IN THE COLLECTED SAMPLES

Sampling Method	Sampling Stage	Season	Bacterial Species
Seawater I	1	Summer	*Vibrio fluviallis, V. vulnificus, Pseudomonas aeruginosa*
	2		No identification
	3		*Vibrio fluviallis, Pseudomonas aeruginosa, Proteus vulgaris*
Seawater I	1	Autumn	*Pseudomonas cepacia*
	2		*Pseudomonas cepacia*
	3		*Pseudomonas cepacia*
Seawater I	1	Winter	No identification
	2		No identification
	3		No identification
Seawater II	1	Summer	*Pseudomonas cepacia*
	2		*Pseudomonas cepacia*
	3		*Pseudomonas cepacia*
Seawater II	1	Autumn	*Pseudomonas aeruginosa, Pseudomonas putida*
	2		
	3		
Seawater II	1	Winter	No identification
	2		No identification
	3		*Ochrobactrum anthropi*
Ice	1	Summer	*Aeromonas hydrophila*
	2		*Aeromonas hydrophila*
	3		*Aeromonas hydrophila*
Ice	1	Autumn	No identification
	2		No identification
	3		No identification
Ice	1	Winter	No identification
	2		No identification
	3		No identification

Three Sampling Stages are Depicted;1: Immediately After Catchment, 2: After 5-6h of Storage, 3: Reaching the Consumer After Almost 12-13h

C. Chemical Analysis

Chemical analysis did not reveal any statistical differences between samples of the different storage methods. Nevertheless, ice stored samples presented lower histamine formation than those stored on seawater boxes (Table II), while in autumn, where the seawater temperature is higher, histamine values were higher. Concerning TMA-N and TVB-N values, there seems to be no specific norm from witch safe conclusions could be made.

TABLE II
CHEMICAL ANALYSIS RESULTS: HISTAMINE FORMATION (mg/100g), TMA-N (mg/100g), TVB-N (mg/100g) IN THE DIFFERENT SAMPLING STAGES

Sampling Method	Sampling Stage	Season	Histamine	TVB-N	TMA-N
Seawater I	1	Summer	7.97	0.03	1.52
	2		16.99	0.34	1.52
	3		18.93	0.56	1.33
Seawater I	1	Autumn	3.88	0.06	1.36
	2		4.51	0.14	1.37
	3		7.90	0.98	1.61
Seawater I	1	Winter	0.00	0.89	0.12
	2		0.00	0.01	0.28
	3		4.71	0.15	0.89
Seawater II	1	Summer	0.00	0.42	1.00
	2		0.00	0.87	1.36
	3		34.48	0.67	1.44
Seawater II	1	Autumn	0.00	0.42	0.96
	2		0.00	0.98	1.22
	3		26.68	1.68	1.35
Seawater II	1	Winter	0.00	0.56	0.84
	2		0.00	0.67	0.86
	3		6.57	0.69	1.10
Ice	1	Summer	5.45	0.90	1.21
	2		7.17	1.70	1.54
	3		8.60	1.90	1.88
Ice	1	Autumn	4.36	0.76	1.32
	2		7.17	1.54	1.54
	3		6.26	1.20	1.79
Ice	1	Winter	0.00	0.15	0.92
	2		0.00	0.34	1.12
	3		8.15	0.39	1.23

1: Immediately After Catchment, 2: After 5-6h of Storage, 3: Reaching the Consumer after Almost 12-13h of Storage

IV. DISCUSSION

Differences were observed in the sensory and microbiological quality between ice and seawater storing conditions, being statistically significant for microbiological data. The ice stored samples reached the consumption stage less deteriorated at all aspects of quality. Although all samples that reached the seafood markets were acceptable for consumption, sensory and microbiological data showed higher quality degradation for samples retained in seawater.

Regarding sensory data, no sample reached the limit of acceptability (0.60-0.65) during the stages of post-catch handling and distribution. As indicated by other studies, the abovementioned limit is reached after at least 8 days of ice storage at -18°C [3], [17]. The fact that differences observed were not statistically significant is explained due to the short time between catchment and distribution (almost 12 – 13 hours) and is expected to become significant after a greater period of storage. Estimated spoilage was calculated at 30 hours for the ice stored (0°C) samples (the time expected to reach the limit of 0.60), 22 hours for the seawater stored (ambient temperature) samples of Etoloakarnania (Seawater II) and 14 hours for the seawater samples of Achaia prefecture (Seawater I), resulting in a very limited time for the fishing

personnel to handle and distribute the product before consumption in order to meet the European quality standards. As a consequence, seawater storage could lead to undesirable results, regarding the final product quality.

According to microbiological analysis, all fish samples were suitable for consumption as total counts were lower than the acceptable limit, which varies between 10^6cfu/g [22] and $3x10^6$cfu/g [23] in aerobic plate count analysis. Apart from the acceptable numbers of total bacterial counts, results show significant differences between the two storage conditions. Microbial populations showed faster growth in seawater storage than on ice, leading to more rapid quality deterioration, findings which are in accordance to several studies on other species [24]-[27].

Bacterial variation detected raises concerns for human health, taking into account the fact that pathogenic species were included. *Ochrobactrum anthropi* has been reported of provoking septicemia, bactremia and endocarditis specifically for immunodeficient patients [28]-[31], while *Vibrio alginolyticus* was described as the cause of otitis media, in a child handling pressure equalizing tubes [32]. Moreover, severe *Vibrio vulnificus* infections are chronic hepatic disease or immunodeficiency in patients [33], [34].

Furthermore, predominant bacterial species identified, belonged to the genus of Pseudomonas, which was strongly correlated to psychrotrophic bacteria counts, TMA-N and TVB-N concentrations, a fact that is also supported by other studies, which report certain correlations among biogenic amines, *Pseudomonas* sp. and psychrotrophic bacteria. Contents of cadaverine and putrescine have shown the best correlation with *Pseudomonas* sp. and psychrotrophic bacteria, respectively. In addition, *Pseudomonas* sp. is responsible for the decarboxylation of amino acids lysine and ornithine, leading to the formation of cadaverine and putrescine and degradation of fish quality [3], [35].

Spoilage estimation could not be applied in microbiological data due to the limited sampling time from catchment to distribution (almost 13 hours). Moreover, a linear regression would result in faulty conclusions, as bacterial growth is described by an exponential function and not linear.

Regarding chemical analysis, seawater stored samples during summer and autumn exceeded the histamine limit of 20mg/100g proposed by European regulation 2073/2005 [12], revealing more rapid chemical deterioration compared to ice storage, as in accordance to Chong et al. [36] for the Indian mackerel. In contrast, all samples presented TMA-N and TVB-N values much lower than the limits proposed [18], implying that both methods do not influence the formation of these volatile amines.

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