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Evaluation of Microbiological Contamination of Ice from Marketplaces in Lisbon

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“Humankind cannot gain anything without first giving something in return.
To obtain, something of equal value must be lost. That is the law of Equivalent Exchange.
(...)
But the world isn′t perfect, and the law is incomplete.
Equivalent Exchange doesn′t encompass everything, but I still choose to believe in its principle,
that all things do come at a price,
that there′s an ebb, and a flow,
a cycle,
that the pain we went through did have a reward,
and that anyone who′s determined and perseveres,
will get something of value in return, even if it′s not what they expected.”
(Maruyama & Yasuhiro, 2009)

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Resumo

Introdução e objetivos: Os mercados de peixe são populares em Portugal, até aos dias de hoje, e frequentemente procurados por locais e turistas pelo seu peixe fresco e de qualidade. Quando preparado, armazenado e/ou manuseado sob condições sanitárias deficientes, o gelo usado para refrigerar e manter os produtos pode ser contaminado com microrganismos patógenos e, portanto, tornar-se uma fonte de possíveis surtos. Não existe literatura acerca da análise do gelo de mercados em Portugal, e como tal, o nível de contaminação nestes locais é desconhecido. O objetivo desta dissertação é fornecer evidências acerca da contaminação do gelo dos mercados de Lisboa, durante toda a cadeia de produção até ao momento da utilização em banca, assim como providenciar sugestões afim de garantir a melhor qualidade possível do gelo em contacto com os produtos de consumo.

Métodos: Para avaliar o grau e – no caso da existência de contaminação, a fonte – de contaminação do gelo usado, a análise foi efetuada em gelo imediatamente após a produção (produção), em gelo armazenado (armazenamento) e em gelo exposto nas bancas (produto-contacto), num total de 54 amostras recolhidas em 18 mercados do município de Lisboa, Portugal. Foram analisados parâmetros microbiológicos (contagem de microorganismos totais a 37ºC, 22ºC e 5ºC, coliformes totais, *Escherichia coli*, *Enterococcus* spp., *Staphylococcus* spp. e *Staphylococcus* spp.coagulase-positiva e *Salmonella* spp.) e físico-químicos (amónia, condutividade, cloro residual livre, cloretos, nitratos, nitritos, pH, oxidabilidade e dureza total); aos atuais resultados foram acrescentados 34 resultados de análises previamente completadas para os mesmos mercados. Toda a recolha e análise de amostras foi conduzida de acordo com os procedimentos respetivos (Organização Internacional para a Estandardização, Standard Methods for the Examination of Water and Wastewater e Normas Portuguesas). Os funcionários das bancas foram também entrevistados. A análise de diferenças estatisticamente significativas entre estágios foi conduzida segundo o teste de Kruskal-Wallis, seguido, quando relevante, de um teste pairwise de Dunn com múltiplas comparações com correção de Bonferroni, pelo programa XLSTAT.

Resultados: Nos estágios de produção e armazenamento não foi detetada contaminação microbiana; não foram também encontradas diferenças significativas nos níveis de contaminação entre estes dois estágios, com exceção dos parâmetros de microrganismos totais a 22ºC – apresentando estes estágios valores de conformidade à legislação para água de consumo de 100% e 83.3%, respetivamente. Porém, um nível consideravelmente alto de contaminação microbiana foi identificado no estágio de produto-contacto, mais especificamente para coliformes totais (detetados em 100% das amostras, média de 548 NMP /100 mL), *E. coli* (51.7%, 1 NMP/100 mL), *Enterococcus* spp. (96.6%, 29 NMP /100 mL), *Staphylococcus* spp. (100%, 267 UFC/100 mL), *Staphylococcus* spp. coagulase-positiva (22.2%, 2 UFC/100mL) e microorganismos totais a 37ºC, 22ºC e 5ºC (82.8%, 96.6% e 96.2%, respetivamente, >300 UFC/mL), revelando um total de 100% de amostras neste estágio em não conformidade com a legislação aplicável a água de consumo. *Salmonella* spp. não foi detetada em nenhuma amostra, em nenhum dos estágios. Os questionários conduzidos aos funcionários revelaram, na sua maioria, o uso de indumentária e equipamento adequados, assim como a aceitável manutenção e limpeza da banca, mas observações no local detetaram comportamentos inadequados que podem ser fonte de contaminação cruzada.

Discussão: As amostras recolhidas no estágio de produção estão de acordo com o esperado, e em linha com os resultados das análises efetuadas pela EPAL à água do município de Lisboa. No estágio de armazenamento, verificou-se um aumento da contaminação em alguns mercados, com surgimento de amostras positivas para coliformes, *E. coli* e *Enterococcus* spp., e com valores acima do

paramétrico para contagens de microrganismos totais a 37ºC, 22ºC e 5ºC. Considerando que o gelo se deposita imediatamente após a produção, por força da gravidade, a contaminação neste estágio pode ser atribuída a más práticas de manuseio por parte dos colaboradores, assim como a falta de higiene dos utensílios que entram em contacto com o gelo. Por sua vez, as amostras do estágio de produto-contacto revelam níveis altos de contaminação microbiológica. Enquanto valores altos para contagens de microrganismos totais podem ser consideradas normais pelo contacto com o pescado, coliformes, *Enterococcus* spp. e *E.coli* são indicadores de contaminação fecal, e a presença de *Staphylococcus* spp. indica a ocorrência de contaminação cruzada, mais uma vez possivelmente causada por práticas inadequadas de higiene e manuseio.

A diminuição de focos de contaminação pode ser feita não só através do aumento ou reforço da formação dos profissionais das bancas, assim como qualquer outro funcionário que manuseie o gelo, mas também da implementação de protocolos de limpeza e manutenção das bancas e de outros artigos que entrem em contacto com o gelo e de todo o equipamento de produção e transporte (condutas de água e gelo, máquina de produção, lâminas, entre outros). Paralelamente, a melhoria do transporte do pescado de forma a garantir a melhor qualidade sanitária e evitar decomposição acelerada. Também seria benéfica a existência de monitorização frequente, semelhante à efetuada nesta dissertação, bem como a criação de legislação específica para o controlo da qualidade sanitária do gelo.

Palavras-chave: Lisboa; gelo; mercado; contaminação de gelo; segurança do consumidor; segurança alimentar.

Abstract

Introduction and objectives: Fish markets are, to this day, popular in Portugal, and frequently sought by locals and tourists for fresh, quality fish. When prepared, stored and/or handled under poor sanitary conditions, the ice used to refrigerate products can be contaminated with pathogenic microorganisms and therefore be a source of possible outbreaks. There is no literature for the analysis of market ice in Portugal, and therefore the degree of contamination in these locations is unknown. The aim of this dissertation is to provide evidence for the existence (or lack thereof) of contamination in Lisbon's market's ice, throughout the chain of production until the moment it is displayed in stalls, as well as to provide guidance and implementation suggestions in order to guarantee the best possible quality ice in contact with consumer goods.

Methods: In order to evaluate the degree – and, if contamination is detected, the source – of microbiological contamination on the ice used in these markets, analysis was performed in freshly produced ice (production stage), stored ice (storage stage) and ice in display at the stalls (product-contact stage), to a total of 54 collected ice samples at 18 markets within the municipality of Lisbon, Portugal. Microbiological (total plate counts at 37ºC, 22ºC and 5ºC, total coliforms, *Escherichia coli*, *Enterococcus* spp., *Staphylococcus* spp. and coagulase-positive *Staphylococcus* spp., and *Salmonella* spp.) and physical-chemical (ammonia, conductivity, chloride, chlorites, nitrates, nitrites, pH, organic matter content and total hardness) parameters were analysed, to which 34 previous analysis results for the same markets were added. All collection and analysis were performed in accordance to the respective procedures from the International Organization of Standardization, Standard Methods for the Examination of Water and Wastewater and Portuguese Norms. Stall personnel was also interviewed. Significant statistical difference was analysed between stages using a Kruskal-Wallis test, following a Dunn's multiple comparisons test with Bonferroni correction whenever relevant, using XLSTAT software.

Results: Production and storage stages did not present microbiological contamination; also, no statistically significant difference was found between microbiological contamination levels between these two stages, except for total microbial count at 22ºC – presenting tap water legislation conformity values of 100% and 83.3%, respectively. However, a relevantly higher microbial contamination was identified in product-contact stages, most specifically total coliforms (detected in 100% of samples, average of 548 MPN/100 mL), *E. coli* (51.7%, 1 MPN/100 mL), *Enterococcus* spp. (96.6%, 29 MPN/100 mL), *Staphylococcus* spp. (100%, 267 CFU/100 mL), coagulase-positive *Staphylococcus* spp. (22.2%, 2 CFU/mL) and TMC at 37ºC, 22ºC and 5ºC (82.8%, 96.6% and 96.2%, respectively, >300 CFU/mL), to a total of 100% of samples not in conformity to legislated parameters for tap water. *Salmonella* spp. was not detected in any of the samples at any stage. Questionnaires showed that stall employee's attire and equipment was mostly appropriate, as well as stall maintenance and cleaning, while in loco observations revealed inappropriate behaviours that may enhance cross contamination.

Discussion: Production stage samples' values are well within expected values, and in line with EPAL's results for tap water in Lisbon municipality. During storage stage, an increase in contamination was verified in some markets, with the emergence of samples positive for coliforms, *E. coli* and *Enterococcus* spp., and with total microbial counts above parametric values; bearing in mind that ice deposits immediately after production, by force of gravity, contamination at this stage can be due to poor handling practices by employees, as well as to lack of utensil hygiene. Product-contact stage samples reveal high levels of microbiological contamination – and while high total microbial counts can be considered normal (due to contact with fresh fish), coliforms, *E. coli* and *Enterococcus*

spp. are clear indicators of faecal contamination, and the presence of *Staphylococcus* spp. indicates cross-contamination, once again caused by inadequate hygiene and handling practices.

Decrease in contamination sources can be achieved not only by increasing or reinforcing stall personnel training (as well as any other employee who handles ice), but also implementing cleaning and maintenance protocols for stalls, ice handling accessories and production and transport equipment (water and ice conducts, production machine, blades, among others). Alongside, there should be improvement in the transport of fish, as to guarantee its best possible sanitary conditions and avoid accelerated decomposition. Existence of frequent monitorization, similar to that of this dissertation, would also be beneficial, as well as the creation of specific legislation for the control of sanitary quality of ice.

Keywords: Lisbon; ice; market; ice contamination; consumer safety; food safety.

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List of abbreviations, acronyms and symbols

AFDO – Association Food and Drugs Officials

ANVISA – National Agency for Sanitary Vigilance (from Portuguese, Agência Nacional de Vigilância Sanitária)

BHI – Brain Heart Infusion

BPW – Buffered Peptone Water

CAE – Portuguese Classification for Economic Activities (from Portuguese, Classificação Portuguesa de Actividades Económicas)

CDC – Center for Disease Control

CFU – Colony Forming Units

EDTA – Ethylenediaminetetraacetic Acid

EN – European Norm

EPAL – Free Waters Public Company (from Portuguese, Empresa Pública das Águas Livres)

EPIA – European Packaged Ice Association

FDA – Food and Drug Administration

FEHD – Food and Environmental Hygiene Department

HACCP – Hazard Analysis and Critical Control Point

IPIA – International Packaged Ice Association

ISO – International Organization of Standardization

M.E.V.A.G – Medium for the Study of Carbohydrates Attack Path (from French, Milieu d'Étude de la Voie d'Attaque des Glucides)

MAN – Mannitol Salt Agar

mL – millilitre

MPN – Most Probable Number

NA – Nutrient Agar

NMP – Most Probable Number (from Portuguese, Número Mais Provável)

NP – Portuguese Standard Guideline (from Portuguese, Norma Portuguesa)

RAP – Rappaport Vassiliadis Broth

SMEWW – Standard Methods for the Examination of Water and Wastewater

SSA – *Salmonella*-Shigella Agar

TMC – Total Microbial Count

TSI – Triple Sugar Iron agar

TT – Muller Kauffamnn Tetrationate-novobiocin Broth

UFC – Colony forming units (from Portuguese, Unidades Formadoras de Colónias).

XLD – Xylose Lysine Deoxycholate Agar

Y – Yeast Agar

1. Introduction

Long before cold producing electric devices, there was a need to preserve food and other produces.

Ice houses – well isolated domed or underground brick structures, fed with snow or ice from close bodies of water or that used natural mechanisms like changes in temperature to produce ice – are documented in Mesopotamia around 2000 BC, by the Chinese around 1110 BC and in Greece and Rome around 500 BC (David, 1994). These were frequently used and some of their remains survive until today, with estimated building dates of around 400 BC in Iran (Hosseini & Namazian, 2012), 1600 in Paris and Britain (David, 1994) and circa 1700 the US (Alexandria City Hall, 2015). These eventually progressed to household ice-boxes – by 1909, 81% of US families had one, more or less elaborate (Chapin, 1909) - and, more recently, to electrical refrigerators.

In Portugal, a single exemplar remains – The Royal Factory of Snow of Serra de Montejunto, built around 1740, that served the Royal Family. Before that, ice and snow were collected directly from the ground at Serra da Estrela (Furtado, 1997).

With the advent of more efficient technologies, we can easily produce ice, both in bulk in factories and in smaller quantities, at home. The International Packaged Ice Association (IPIA) estimates a production between 100 and 600 tons a day per plant, exclusively in the US (IPIA, 2017).

1.1. Legislation concerning ice

Ice can be produced from sea or freshwater and loosely subdivided in four types: block ice, flake ice, compacted blocks of ice (such as flakes) or slush ice (mixed with water) (Shawyer & Pizzali, 2003). Giving that it is produced with water and can be ingested, the U.S. Food and Drug Administration (FDA) classifies ice as food (FDA,2017) and therefore regulation is required, specifically during production and packaging - this is proven to be particularly challenging, as many businesses produce their own ice for sale. Given this, several procedures were established.

World Health Organization's (WHO) guidelines for drinking water quality are also applicable to ice intended for human consumption (WHO, 2011); these guidelines advise to treat ice meant for human consumption as any other water meant to consume and not to ingest ice unless it is known to be produced from safe, sanitary water. Association Food and Drugs Officials' Guidelines for the Inspection and Enforcement of Good Manufacturing Practices (GMP) Regulations for Handling and Manufacturing of Packaged Ice relays a series of recommendations such as control of employees clothing and personal hygiene, clear separation between different areas of work within the factory, production plant design, cleaning procedures and schedules and the quality of the water used (both for production and other activities within the area) (Association of Food and Drugs Officials [AFDO], 1988). In a more particular manner, a document was issued for food services and retail establishments, which convey the same basic recommendations, adding that "an establishment where ice is packaged for human consumption should be permitted or licensed by the state or local regulatory agency." (AFDO, 2010). The IPIA, although based in the US, regulates ice production in several factories across the world, with certified ice plants in the US, Canada, Cayman Islands, Jamaica, Mexico, Panama, Chile, the UK, Germany, Italy, Norway, Lebanon, India, Philippines and South Africa (IPIA, 2018) and, in 1998, has produced a manual of good practices (Packaged Ice Quality Control Standards) which is based in AFDO's GMP but custom-made specifically for packaged ice. The following of this quality control programme is a default requirement for an ice production establishment to obtain an IPIA membership. In 2005 in Brazil, the Agência Nacional de Vigilância

passed a regulation to control the production of bottled waters and ice, specifying that ice should be made of water microbiological and chemically acceptable for human consumption (Agência Nacional de Vigilância Sanitária, 2005); this regulation supports a 1997 decree (Agência Nacional de Vigilância Sanitária, 1997) that contains the same recommendations, even though both documents only briefly mention ice.

In Europe, the EPIA – European Packaged Ice Association – has the same purpose and goals as the IPIA. In Portugal, there is no record of any similar agency. All ice manufacturers abide by the same legislation, but all plants implement their own specific control procedures.

Concerning actual legislation, the regulation 852/2004 ensures the hygiene of foodstuffs, and specifies that "ice which comes into contact with food or which may contaminate food is to be made from potable water or, when used to chill whole fishery products, clean water." (European Parliament and Council, 2004). It also states that all ice should be produced, handled and stored in ways that prevent contamination. Italy also has its own ice association (Istituto Nazionale de Ghiaccio Alimentare – INGA).

In low-income countries, despite the existence of a few regulations, managing quality and availability of clean, disinfected water may be a challenge, as well as providing the population with knowledge of personal and food hygiene and proper machine maintenance. Chavasit and collaborators (2011) have made several recommendations directed at these countries in order to diminish bacterial load found in ice plants products.

Evaluation of the quality of ice is essential in order to verify potential health risk to consumers.

1.2. Ice as a source for outbreaks

Infections after consumption of pathogenic bacteria are quite common. According to the Center for Disease Control [CDC], foodborne pathogens are estimated to cause around 9.4 million illnesses each year just in the United States; as most are relatively mild, these situations usually go unnoticed or untreated and therefore are not associated with an outbreak or to a specific infection focus (Dewey-Mattia et al., 2018). Because it may be extremely difficult for an infected individual to realize the source of contact, it may be reasonable to assume that an undefined number of cases of infection by microbiological pathogens might be connected to contaminated ice from markets but fail to be reported. Although market ice is not directly consumed, it is in close and prolonged contact with foodstuffs – therefore, the quality of the products can be influenced by the presence of microorganisms in the ice and cross contamination might occur.

Portugal reported the first cases of drink and foodborne intoxications by Shiga toxin-producing *Escherichia coli*, *Campylobacter* spp. and *Listeria* spp. in humans in 2015. The country has one of the lowest rates of Salmonellosis (1095 confirmed cases from 2011 to 2015) but one of the highest rates of hospitalization; it is also noticeable an increasing number of cases (European Food Safety Authority [EFSA]and European Centre for Disease Prevention and Control, 2016).

The most recent report by the EFSA (2016) shows that 20 strong-evidence and weak-evidence food and waterborne outbreaks were identified in Portugal in 2015, to a total of 421 identified patients; although 97 resulted in hospitalization, there were no deaths associated. This corresponds to a reporting rate of 0.19 per 100.000 inhabitants, much lower than European Union average of 0.95 and one of the lowest among reporting EU member countries but is still an increase of over 50% when

compared to the period of 2010-2014. When evaluating human cases involved in food and waterborne outbreaks, Portugal amounts to a total of 4.06 cases per 100.000 inhabitants, still a much lower number compared to the average of 7.28 cases among EU member countries – with countries presenting rates high as 57.1 per 100.000 (Czech Republic). Several outbreaks have been identified in the EU. The most recent available data concerning food and waterborne outbreaks in the European Union is dated from 2015 and is summarized in table 1.1.

Table 1. 1 - Summary of 2015 EU member countries food and waterborne outbreaks causes. Adapted from EFSA (2016).

Bacterial	Outbreaks	Illnesses	Deaths
Salmonella	953	6616	3
***E. coli*, Shiga toxin-producing**	6	674	0
Campylobacter	387	1440	1
Listeria	14	230	4
Yersinia	13	54	0
Vibrio	4	29	0
Brucella	1	2	0
Other bacterial agents	29	337	0
Total bacterial	**1407**	**9382**	**8**
Toxins, parasitic, viral	1302	23900	8
Other agents	127	648	0
Unknown	1463	11941	1
Total	**4362**	**45874**	**17**

Ice can be produced practically anywhere with very little effort and requires very little control. Even so, it is not usually one of the main foods analysed in outbreak situations, and literature found on that matter is not extensive. In EU member countries, place of exposure was determined for 409 outbreaks in 2015 – households were the most frequent source of outbreaks (45.72%), followed by canteens, school, hospitals and other catering services (23.72%) and restaurants, street vendors, take-aways and other retailers (19.56%) (EFSA, 2016). Data concerning outbreaks in the US from 2009 to 2014 shows that a vast majority (80%) of outbreaks was known to irradiate from restaurants, caterers or banquet facilities and other commercial establishments (Dewey-Mattia et al., 2018). In all these locations, ice is frequently locally-made and commonly used in drinks and as a mean of conservation. Contrary to popular belief, the act of freezing and/or the use of alcoholic beverage; for example, addition of alcoholic beverages like scotch and tequila – minimum of 40% and 38% ethanol by volume, respectively (Scotch Whisky Association, 2009; Estados Unidos Mexicanos, 1994) – does not eliminate all microorganisms present in ice, although it greatly reduces the number of viable microbes (Dickens, DuPont & Johnson, 1985). A 2017 study corroborates these results, showing that whisky, Martini, tonic water and soda reduce bacterial load of ice, but some strains maintain residual growth (Gaglio et al., 2017).

Some case studies have successfully identified ice as the outbreak source. As early as 1940, an outbreak of dysentery in a hospital facility in Connecticut USA was identified as caused by contaminated ice from a local ice machine (Godfrey & Pond, 1940). In September 1987, several concentrated outbreaks of gastroenteritis arose in Delaware and Philadelphia, USA, and were linked to a Pennsylvania ice plant whose wells were contaminated following a flood, after the bagged commercial ice tested positive for norovirus (Cannon et al., 1991); on the same year, a small outbreak

was linked to a single *Mycobacterium* spp. contaminated ice machine in a medical ward in Vancouver, Canada (Laussucq et al., 1988).

In 1990, an outbreak of giardiasis was associated to the consumption of ice in beverages, after 75% of a dinner's attendees became ill following the ice manipulation of a staff member with asymptomatic giardiasis and another one with a *Giardia*-infected toddler, showing that ice can easily be cross-contaminated and that it is a good mean for the dissemination of bacteria (Quick et al., 1992).

An outbreak on board of a cruise ship in Hawaii, USA, in 1992, caused 202 cases of acute diarrheal illness and was closely linked to the amount of ice consumed in beverages, as the attack rate was considerably higher in passengers who consumed ice (Khan et al., 1994). *Mycobacterium* spp. was again isolated from an ice machine at a HIV-infected patients medical ward in Baltimore, USA, in 2001 (Gebo et al., 2002). Outbreaks of Hepatitis A have been linked to ice slushies, due to the use of unfiltered well water in Sichuan Province, China, in 2006 (Zhang et al., 2009), and had previously been linked to poor handling of the ice during preparation in Alaska, USA, in 1988 (Beller, 1992), causing 90 and 57 infections, respectively.

In 2015, an outbreak caused by ice produced with norovirus-contaminated un-boiled and un-filtered water triggered 200 cases of gastroenteritis among high school students in Taiwan (Cheng et al., 2017).

1.3. Ice analysis as a case study

More recently, ice used for commercial purposes has been a target of many studies.

The analysis of edible ice from fresh juices stalls in Nigeria showed very high amounts of colony forming units (CFU) and total coliforms in all samples, and of faecal coliforms and *Staphylococcus* spp. in all but one (Ukwo, Ndaeyo & Udoh, 2011). A very similar study conducted in Mumbai, India also yields the same results, both in types of microbiological findings and amounts of contamination (Durgesh, Ranjana & Varsha, 2008). In Malaysia, a study found contamination by faecal coliforms in 53% of ice samples collected in food retailers; all samples had low free residual chlorine and, therefore, were not properly disinfected and may have not been produced with clean tap water (Noor et al., 2012). Most water used in low and lower-middle income countries is collected from holes and wells and used untreated and unfiltered, as shown by Shamsuddeen et al (2010). Samples of water used for commercial production of ice had a mean aerobic plate count of $2.05x10^3$ CFU, up to 110 coliforms per mL and two presented *E. coli*, while the samples of produced ice had a mean aerobic plate count of $7.90x10^3$ CFU, up to 130 coliforms per mL and four presented *E. coli*. In Iran, 20% of samples collected from ice factories, retail businesses and travelling salespeople were contaminated with some kind of vibrion – two of which were *Vibrio cholera* 01, pathogenic to humans, extremely virulent and possibly lethal; 9% of samples tested positive for *Salmonella paratyphi*, 16% for *E. coli* and 12% for aerobic bacteria (Hamedy, Khosravi & Omidy, 2004).

A 1999 analysis of ice from retail and catering establishments in the United Kingdom sampled ice used for beverages as well as for food display. Results showed that 9% of the ice used for beverages presented coliforms, 1% *E. coli*, 1% excess enterococci and 11% excess aerobic plate count at 37ºC, as opposed to the iced used in food display, where 23% presented coliforms, 5% *E. coli*, 8% excess enterococci and 29% excess aerobic plate count at 37ºC. It was also noted that ice produced in ice plants were less contaminated than the ice that was locally produced (Nichols, Gillespie & De Louvois, 1999). This last interpretation is also present in a 2013 analysis of packaged ice in Georgia, USA, that concluded that while all packaged, plant produced ice was well within acceptable

microbiological limits, 6% of locally produced and bagged ice had unacceptable levels of CFU, 37% had unsatisfactory amounts of coliforms, 13% presented enterococci and 1% *E. coli* - furthermore, one sample presented *Salmonella* spp. (Mako et al., 2014). A similar study conducted by the Food Safety Authority of Ireland (2007) showed that in a universe of 1044 analysed samples, 27.1% did not meet the criteria for *E. coli*, coliforms and/or enterococci.

Hampikyan and collaborators (2017) detected, in Turkey, *Escherichia coli* in 6.7% collected ice and 21.9% ice chest samples, while all water samples were negative for *E. coli*. Relatively similar results were obtained for psychrophilic bacteria and enterococci – only coliforms were present in water samples, suggesting that the remaining microorganisms were a result of either poor handling or poor machine hygiene.

A Hong Kong government study of both commercial and locally produced ice further showed that, although the ice produced in retail businesses presented moderate levels of contamination by coliforms and aerobic bacteria, all commercial packaged ice samples met the microbiological and chemical criteria only if sampled directly from the plant – samples of commercial packed ice retrieved from businesses had already been contaminated to a degree similar to that of locally produced ice (Food and Environmental Hygiene Department of Hong Kong, 2005). Schmidt and Rodrick (1998) also concluded that mechanical, ice plant bagged ice is of better sanitary quality, although some of the analysed ice plant samples contained yeast, molds and other microorganisms. Settanni et al. (2017) reaffirmed that domestic ice and samples from bars and pubs were considerably more contaminated that ice plant bagged ice, although some industry ice samples tested positive for *Pseudomonas* spp., *Staphylococcus* spp., *Bacillus* spp., *Microbacterium* spp. and *Acinetobacter* spp.. Lee et al. (2017) showed, through 16S/18S rRNA targeted sequencing that there is considerably higher microbiological diversity in in situ packaging than on automatic or ice plant baggers; potential pathogens were also found in retail packaged ice. Moyer et al (1993) had obtained identical values and conclusions regarding sanitary differences between ice plant ice and in situ produced ice from retailers.

The analysis of three different brands of packaged ice in Teresina, Brazil revealed that although all samples tested negative for coliforms and faecal coliforms, several debris were found, including small pieces of plastic, cloth fibres and an insect (Carvalho & Oliveira, 2018). Other tests of commercially packaged ice presented 55.5% of samples outside of legislated values, and 51.9% tested positive for faecal coliforms (Neto et al., 2009). In Ceará and Paraná, samples collected at gas stations all presented acceptable microbiological parameters, testing negative for coliforms and *E. coli* (Gomes et al., 2012; Freire et al., 2008).

There are, therefore, obvious differences between ice samples collected from ice plants, locally/retail produced ice and ice machines. In fact, studies concerning automatic ice-machines are not plenty. Hospital machines seem to be less contaminated than community ice-machines - even though, in a 1997 study, most hospital ice machines tested positive for *Bacillus* spp, *Staphylococcus* spp and *Pseudomonas* spp, among 14 other less occurrent microorganisms (Wilson, Hogg & Barr, 1997); Burnett, Weeks and Harris (1994) also verified that hospital machines were relatively sanitary, presenting only a few organisms of medical significance and provided several considerations to diminish bacterial contamination in hospital ice machines even further. To avoid high levels of contamination in automatic machines, Moore, Brown and Hall (1953) suggested chlorination, prior to dispensing, by submerging the ice in a disinfecting solution inside self-service ice machines - a trial showed that this method was considered effortless by staff and that no changes in taste were detected by consumers, while achieving sanitary ice.

In Greece, an analysis of locally produced ice used to cool fish and seafood collected at 10 different retail establishments near the coastline showed that *E. coli* was present in 22% and a significant load of coliforms were present in 31% of samples, as well as spore forms of *Clostridium perfringens* in 35% of collected samples and *Salmonella* spp. in 4%. The ice was handled, bare handed, by employees with little training and stored in open containers; no cleaning and disinfection records were found (Gerokomou et al., 2011). In fact, poor practices while handling ice associated with high levels of contamination – this includes dirty equipment and/or scoops, exposed ice and biofilm in machines or other areas in contact with ice (Awuor et al., 2016)

A recent study of ice used to preserve fish in Igoumenitsa revealed the presence of at least one faecal coliform colony in 91.7% of samples and enterococci in 100% of samples; 282 histamine producing bacteria colonies were isolated, of which 24.1% belonged to *Pseudomonas* spp., 20.86% were from Enterobacteriaceae family and 19.42% belonged to *Staphylococcus* spp.. The microbiological load also varied according to the ice provenience (higher in seawater and commercial ice samples than in ice made from tap water) and to time of the year (Economou et al., 2016)

An analysis of a fish market shrimp stalls in Ceará, Brazil, showed that 10% of shrimp samples and 6.6% of stall swab samples tested positive for *S. aureus* (Vieira et al., 1998). A similar study, in the same location, conducted 20 years later, bore the same results, linking the presence of *Staphylococcus aureus* in the ice and food products to the presence of the microorganism in sellers' mouths, hands and noses (Albuquerque, Vieira & Vieira, 2018). Another analysis of those stalls had already revealed the presence of 15 microorganisms, including *Listeria* spp, *Vibrio* spp, *Pseudomonas* spp, *Streptococcus faecalis* and Enterobacteriaceae (Vieira, Souza & Patel, 1998). In São Paulo, 22.2% of sampled ice used to preserve fish tested positive for coliforms and 9.5% for faecal coliforms; it also presented CFU counts above legislated values and three (4.7%) samples tested positive for *Staphylococcus aureus* (Baldin, 2011). Similarly, poor microbiological and chemical conditions had been described by Giampietro and Rezende-Lago (2009).

Ayulo, Machado and Scussel (1994) isolated, within a universe of 175 samples of fish meat, *Staphylococcus aureus in* 20% of samples (of which, 60% was shellfish meat) and *E. coli* in 24% to 37.7% (depending on the used method); 8.3% of tested *Staphylococcus* strains were toxin producing (A, AB and D toxin) and one STG-producing *E. coli* strain was found.

The results point to the same conclusions- ice contamination is frequently the result of:

- Use of poor-quality water sources that may be contaminated due to floods, leakages or environmental pollution – this is especially true in lower-income countries, where the general public may have difficulties accessing safe tap water. Even when safe, controlled public supply water is available, contaminations in pipes may occur.
- Defective production, with broken or unclean ice machines or other utensils, either by negligence or unawareness.
- Poor storage, usually in buckets or other containers without lid, exposed to the environment.
- Poor handling – usually by uneducated staff with little knowledge of food safety and hygiene that may touch the ice without effective preventive measures.

As shown by Northcutt and Smith (2009), used and/or contaminated ice can be washed with potable water to diminish bacterial load while maintaining its chemical characteristics. This will be appropriate to extend the use of ice batches in products meant to consume fully cooked (poultry and fish in general, with due exceptions) but is not recommended for ice used to maintain produce that can be consumed directly from the stalls (vegetables and fruit, for example).

1.4. Ice control and analysis in Portugal

Studies concerning the quality of ice are quite rare in Portugal. In Porto, the analysis of ice (following the same current legislation) for beverages used in beach establishments showed that 26% presented microbiological values outside of what is legally accepted; one sample tested positive for *E. coli* (Mendes, 2009).

In the specific case of Lisbon fish markets, the ice is produced locally with tap water that is strictly controlled – Lisbon public water supply company, Empresa Portuguesa das Águas Livres (EPAL), works in laboratories that are accredited according to NP EN ISO/IEC 17025 (General competence requirements for trials and calibration laboratories) since 1997 (EPAL, 2015).

The company collects several monthly samples, calculated and distributed in accordance to the volume of water supplied per location, as well as to the number of residents. Water samples are collected directly from the average consumer tap, to a total of over 1200 sampling points. Parametric values are defined in Decree 306/2007 of August 27 (EPAL, 2018). Table 1.2 summarizes the results of EPAL's tap water analysis between the months of March and December 2017, while samples for this dissertation were collected. Other procedures are conducted by the company, including but not limited to metal detection, determination of disinfection sub-products and organoleptic evaluation.

Table 1.2 - Results of EPAL's tap water analysis between March and December 2017. Adapted from EPAL (2018).

Parameter	Samples		Determined Values		Parametric Value	Samples > parametric	Compliance (%)
	Predicted	% completion	Max	Min			
Coliform bacteria (cfu/100mL)	1050	100%	300	0	0	24	97.71
***E. coli* (cfu/100mL)**	1050	100%	1	0	0	1	99.90
Colonies at 22ºC (cfu/100mL)	438	100%	>300	0	No abnormal alteration [a)]	-	100
Colonies at 37ºC (cfu/100mL)	438	100%	>300	0	No abnormal alteration [b)]	-	100
Residual Chlorine (mg/L)	1050	100%	1.95	<0.15	[c)]	0	100
Ammonia (mg/L)	438	100%	<.070	-	0.50	0	100
Conductivity (µS/cm, 20ºC)	438	100%	574	112.6	2500	0	100
Nitrate (mg/L)	438	100%	2.56	1.17	50	0	100
Nitrite (mg/L)	20	100%	<.005	-	0.50	0	100
pH	438	100%	8.94	7.55	6.5 - 9.5	0	100

[a)] Recommended value: less than 100 CFU/mL; [b)] Recommended value: less than 20 CFU/mL;

[c)] Recommended concentration between 0.2 and 0.6 mg/L

There is no specific literature for the analysis of ice quality in Portuguese markets.

1.5. Fish as foodstuff

Fish is a particularly nutritious yet perishable food. Fish spoilage starts the moment it is captured and is the result of multiple chemical and microbiological alterations, which can be enhanced by mechanical damage to the flesh (Fraser & Sumar, 1998). Therefore, conservation is necessary, through processes such as freezing, salting, canning or chilling.

Bacteria are present in virtually all fish tissues. Literature suggests that microbe count on the skin is low for most species (as only a portion of the skin is colonised), mostly comprised of aerobe bacteria, and that it is correlated to the amount of pollution present in the water. Gills and the digestive tract have considerably large populations that amount to 10^6 of bacteria per gram of gill and 10^8 aerobic and 10^6 anaerobic per gram of digestive tissue (Austin, 2002). Muscle tissue, considered to be sterile, has been found to harbour bacteria even in healthy fish (Toranzo et al., 1993)

Although microbial load and species depend on the species of the fish, location of catch and trophic level, freshwater fish microbiota includes, among others *Acinetobacter* spp. (skin, gut), *Aeromonas* spp. (skin, gills, gut), *Enterobacter* spp. (skin, gills, gut), *Klebsiella* spp. (gut), *Listeria* spp. (gut), *Moraxella* spp. (skin, gut), rarely *Plesiomonas* spp. (gut), *Pseudomonas* spp. (skin, gills, gut), *Staphylococcus* spp. (gut) and *Vibrio* spp. (skin). Marine fish microbiota includes, among other, *Acinetobacter* spp. (skin, gut), *Alcaligenes* spp. (skin, gills, gut), *Aeromonas* spp. (gut), *Clostridium* spp. (gut), *Enterobacter* spp. (skin, gut), *Moraxella* spp. (gut), *Mycoplasma* spp. (gut), *Pseudomonas* spp. (skin, gut) and *Vibrio* spp. (skin, gut) (Fraser & Sumar, 1998; Austin, 2002; Novotny et al., 2004; Egerton et al., 2018).

Pseudomonas spp. (Toranzo et al., 1993), *Vibrio* spp. (including occasional cases of *V. cholerae*) (Grimes et al., 1993), *Streptococcus* spp. and other genus have also been reported in organs of healthy fish (Novotny et al., 2004). All of these genera include species pathogenic to humans (Egerton et al., 2018). Presence of *Staphylococcus* spp., *Vibrio* spp., enterococci and coliforms in large amounts are not common but indicate passage through polluted or contaminated waters (Geldreich & Clarke, 1966; Pal & Gupta, 1992). It is, therefore, necessary to thoroughly clean the fish before displaying it and after gutting and preparing it – to avoid cross contamination between fishes and fish tissues –, but also to ensure proper conservation before selling – as to slow bacterial growth.

1.6. Parameters

1.6.1. *Salmonella* spp.

Salmonella is a genus of the family Enterobacteriaceae of rod-shaped, non-spore forming, Gram-negative bacteria. The genus includes two species (S. *enterica* and S. *bongori*); *S. enterica* is further subdivided into six subspecies (CDC, 2018b). There are over 2.500 known serotypes of *Salmonella* spp. (Hammack, 2012).

Salmonella is responsible for two illnesses, depending on the serotype:

- Typhoid fever (*Salmonella enterica typhi* and *S. paratyphi*). The symptoms include high fevers, diarrhoea or constipation, abdominal pain, headaches, coughing, lethargy and general discomfort that can last for four weeks. Rose coloured rashes may occur (CDC, 2018a). Although the disease can be mild, more serious cases can present blood poisoning (septicaemia) and consequentially endocarditis,

as well as septic arthritis and cholecystitis (Chaignat, 2005). The mortality rate can be as high as 10% for more severe cases (Hammack, 2012). Development of an altered mental state is associated with high mortality rate (Chaignat, 2005)

- Nontyphoidal salmonellosis (other serotypes), with symptoms like diarrhoea, vomiting, fevers and general discomfort that can last up to one week (Braam, 2005b) and can be caused by as little as one cell (depending on the person and infected strain) (Hammack, 2005). In this case, the mortality rate is low, but serious dehydration and electrolyte imbalance due to diarrhoea and vomiting may be life threatening, especially in risk groups like the elderly, infants, the immunocompromised, etc (Hammack, 2012). In some cases, nontyphoidal salmonella has been known to cause septicaemia and/or bacteraemia after managing to pass the gastrointestinal barrier, as well as meningitis, osteomyelitis, pyoderma, septic arthritis, cholecystitis, endocarditis, pericarditis, pneumonia, or pyelonephritis (Braam, 2005b).

Sources of infection are several: the intestinal tract of vertebrates (including humans), still and pond water, or produce or water contaminated with untreated sewage. Raw or poorly cooked meat is also a risk (Braam, 2005b). *S. enteritidis* can be found inside eggs, and other species have been isolated from the outside of shells. *S. typhi* has been found on oysters, fruit and raw milk (Chaignat, 2005). Cross contamination and human asymptomatic carriers are well documented (Hammack, 2012).

1.6.2. Total microbial counts (5ºC, 22ºC and 37ºC)

CFU counts aim to estimate the number of viable microorganisms (bacteria, yeasts, molds, etc.). This relies on the fact that every colony that was able to grow in culturable medium was founded by a single cell, and therefore allows us to estimate the number of bacteria in a sample, considering the dilution factor (Lee, 2008).

1.6.3. *Staphylococcus* spp.

Staphylococcus spp. is a genus of ubiquitous Gram-positive, catalase-positive, spherical bacteria. They can present themselves alone, in pairs, chain, or clustered. The genus includes coagulase positive (mostly pathogenic) and coagulase negative (mostly non-pathogenic) species, to a total of over 30 species. Many of these (including *S. aureus*) are enterotoxin producing, therefore being able to cause food poisoning – *S. aureus*, particularly, can cause food poisoning, pneumonia, bacteraemia, TSS (toxic shock syndrome) and general wound infections (Hait, 2012).

Staphylococcus spp. food poisoning is caused by ingestion of enterotoxins and is relatively common but with low mortality rate (Toyofuku, 2005). Less than 1 microgram of enterotoxin can cause the intoxication – which correlates with 100.000 *S. aureus* viable cells per gram of food (Hait, 2012). The symptoms, that last from a few hours to a couple of days, are usually acute and include vomiting, cramping, diarrhoea and, in some cases, dehydration, low body temperature and low blood pressure (Toyofuku, 2005).

Staphylococcus spp. is frequently found in all kinds of food: meat, eggs, salads, baked goods, creams and filings, dairy and many foods derived of these (Hait, 2012). Although cooking in high heat may destruct many viable cells, the bacteria is hard to eliminate - as are its' enterotoxins, which are quite heat stable and will remain effective even after submitted to high temperatures (during confection, for example) (Toyofuku, 2005). In fact, *S. aureus* is mesophilic and extremely resistant. The species can proliferate in temperatures between 7°C and 47.8°C, being 35°C the optimal temperature, and pH between 4.5 and 9.3, optimal between 7.0 and 7.5 – conditions very similar to

those of the human body (Hait, 2012). Given to their ubiquitous nature, *Staphylococcus* spp. can be found in the air, equipment and almost every surface, even when thoroughly cleaned. *Staphylococcus* spp. species are present in the nose, mouth, throat, hair and skin of over half of the healthy population, and therefore there are many unaware carriers that can be sources of cross contamination - by direct contact with lesions or by coughing and sneezing near surfaces or directly into the food (Hait, 2012).

1.6.4. Total coliforms and *Escherichia coli*

Coliform bacteria (from the family Enterobacteriaceae) are rod-shaped, non-spore forming, lactose-fermenting Gram-negative bacteria (motile or not). They are commonly used as an indicator of water and food quality – although they are not universally pathogenic, they indicate contamination and probable presence of other faecal bacteria (although they don't necessarily indicate the presence of faecal matter).

Escherichia coli is a Gram-negative, rod-shaped, coliform bacteria. It is one of the most common species in the human gut, being part of the normal, healthy intestinal flora (Feng, 2012). Nevertheless, there are six recognized pathogenic groups, five of which are proven to be responsible for foodborne intoxications and known to be the cause of several outbreaks: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAggEC) (Braam, 2005a). Another group, diffuse-adherence *E. coli* (DAEC) exists but is not yet confirmed to be diarrheic.

- Enterotoxigenic *E. coli* (ETEC): unlike other groups, ETEC is highly motile. They produce many virulence factors, including heat-labile and heat-stable toxins (Braam, 2005a). They are capable of causing gastroenteritis with cramping and diarrhoea that can last for some days, and that can be dangerous in low-income countries, where these bacteria cause most deaths. Sources of contamination include contaminated water and food, usually after poor manipulation (Feng, 2012); direct person-to-person transmission is rare (Braam, 2005a).

- Enteropathogenic *E. coli* (EPEC): unlike other groups, they present the locus for enterocyte effacement (LEE) pathogenicity island, which, together with other genes, allows the bacteria to adhere to intestinal epithelial cells. Usually causes infantile diarrhoea, usually in very young children, as adults require a very high number of viable cells to become infected: the disease has a low death rate but can last for up to 120 days and can cause dehydration and malabsorption. Low income countries have more reported outbreaks and higher mortality rates than other countries (Braam, 2005a). Sources of contamination include raw meat and food contaminated with faecal matter (Feng, 2012).

- Enterohemorrhagic *E. coli* (EHEC): this group is included in the 200 to 400 serotypes group of Shiga toxin-producing *Escherichia coli* (STEC), being EHEC serotypes the ones that cause major health issues. The prototypic strain for this group is O157:H7, responsible for around 75 to 90% of EHEC infections. Nevertheless, foodborne intoxication due to other strains (O111, O26, O121, O103, O145 and O45) are emerging (Braam, 2005a). This group is characterized by the production of several subtypes of Shiga toxin (similar to that of *Shigella dysenteriae*) and by the presence, like EPEC, of LEE (locus for enterocyte effacement). As little as 10 to 100 cells of O157:H7 (other EHEC require slightly higher numbers of cells) can cause from mild diarrhoea and discomfort to haemorrhagic colitis – with vomiting, severe cramps and bloody diarrhoea, which can lead to potentially fatal haemolytic uremic syndrome (acute renal failure),

which has a 3 to 5% mortality rate –, and thrombotic thrombocytopenia purpura. Progression to more serious states of the disease are suggested to depend on the type of toxin and can be aggravated by antibiotics (Feng, 2012). When mild, symptoms last for about a week (CDC, 2018c). Several foods have been associated to EHEC, but ground beef and ground beef products have been frequently implicated (Feng, 2012), given that cattle are a common reservoir (Braam, 2005a). Other foods like raw milk, yogurt, fruit juice, sausages and cheeses have been sources of outbreaks and, more recently, bagged and fresh vegetables like lettuce, spinach and alfalfa. Other EHEC infections were cause by water (potable and recreational) and by contact with animals (Braam, 2015a). Person-to-person transmission is also well documented (Feng, 2012).

- Enteroinvasive *E. coli* (EIEC): unlike other groups, EIEC is capable of invading the epithelial cells of the colon, due to a 37 kilobase region on a virulence plasmid. Infection is usually self-limiting and can cause a scenario of mild diarrhoea, vomiting, cramps, fever and bloody stool with mucus which usually lasts around week (Braam, 2005a). Mortality rate is very low – USA CDC reports zero deaths from EIEC. There are no specific foods frequently implicated in EIEC infections, but camembert cheese, tofu and guacamole have been the cause of three outbreaks (Feng, 2012).

- Enteroaggregative *E. coli* (EAggEC): this group is particularly associated with persistent diarrhoea episodes in children from low-income countries, although it is also associated with HIV-positive adults in higher-income countries. The bacteria present a virulence plasmid that allows it to adhere to enterocytes (intestinal epithelium cells responsible by absorption), creating a thick biofilm; most strains also produce toxins (Braam, 2005a). This groups have been found in ice samples (Falcão, Falcão & Gomes, 2004).

1.6.5. Enterococci

Enterococcus is a genus of ovoid Gram-positive, catalase-negative bacteria (Fisher & Phillips, 2009). Nearly 30 species have been proposed to the genus – although the most commonly retrieved from samples is *E. faecalis*. Recently, there have been reports of more frequent multi-drug resistant *Enterococcus* that can be foodborne (Zhang, 2012).

Enterococci are extremely resistant, almost ubiquitous. With an optimal growth temperature of around 35 to 40ºC, they have been reported to survive temperatures between 5ºC and 65ºC (Fisher & Phillips, 2009) and proliferate from 10ºC to 45ºC (Zhang, 2012). The genus can also bear high pH values and is able to grow in a 6.5% NaCl broth (Fisher & Phillips, 2009). They are opportunistic pathogens but are also commensal bacteria in animal gastrointestinal tracts – although they can be found in other parts of the body –; and are very common in insects, plants, soil and water (Zhang, 2012).

Infection due to ingestion presents symptoms similar to those of *Staphylococcus* food poisoning - vomiting, cramping, diarrhoea, fever, and general discomfort. Foodborne diseases caused by *Enterococcus* are not common in healthy individuals, as this genus is particularly relevant in hospital context. Dangerous bacteraemias though, caused by this genus – particularly *E. faecalis* – have a mortality rate as high as 50%. They can also cause endocarditis and urinary, intra-abdominal, pelvic, nosocomial and neonatal infections (Zhang, 2012). Enterococci have been found in meat products (sausages, pies) and dairy products (including pasteurized and evaporated milk), usually because of under-processing or unsanitary preparation of food (Zhang, 2012).

There is no literature concerning the sanitary quality of ice in Portuguese fish markets. Similarly, there is no specific legislation or established parameters for the analysis of ice. Considering that, the information about ice sanitary quality is scarce.

2. Objectives

The main objectives of this dissertation are:

- To determine the existence and degree of microbiological contamination of ice from stalls of fish markets in Lisbon, Portugal.

- To understand the source and location of contamination, along the ice production and handling chain.

- To provide advice as suggestions as to how to avoid and minimize contamination.

3. Methodology

All figures, unless stated otherwise, are property of the author.

3.1 Collection and processing

3.1.1. The laboratory

The samples were analysed in Lisbon Municipality Energy, Environment and Green Structure Department, Bromatology and Water Laboratory. This laboratory, active since 2002, is responsible for the evaluation of potable, underground, superficial and ornamental waters, wastewater and food from municipal canteens and schools. The building itself is comprised of two laboratories: one dedicated to physics and chemistry and one intended for microbiology analysis.

3.1.2. Sampled locations

The sample dimension consisted in 18 fish markets within the municipality of Lisbon, Portugal, as seen on figure 3.1. Samples were collected between the months of March and December 2017 in 21 trips; when markets were significantly close to each other, sampling of the two locations was done in the same trip. Visits were planned to accommodate market schedules and costumers' inflow, and therefore sampling was not regular, but always collected on Tuesdays (11), Wednesdays (8) and Thursdays (2), between 10h00m and 12h00m. The sampled markets are situated in areas of great populational density and are a common place of shopping for many, both locals and tourists. Markets were also selected based on whether there was an ice producing machine or not – there are several other markets within Lisbon Municipality that do not locally produce but acquire ice from other sources.

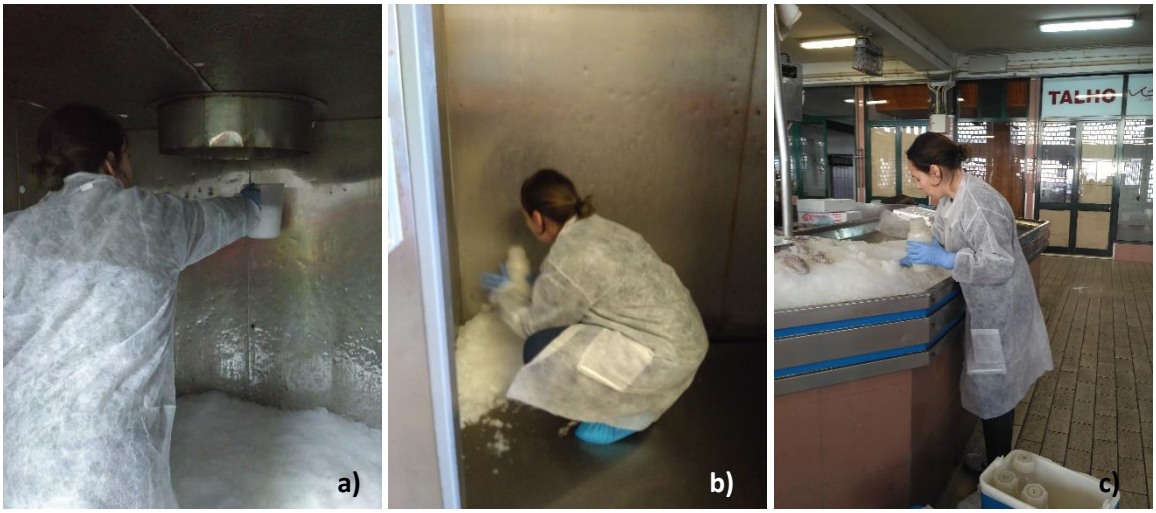
Only one stall was analysed in each market.

Figure 3.1 – General location of sampled markets.

3.1.3. Sample collection procedures

At each market, three samples were collected to a total of 88 collected samples in three different stages: production (N=29), storage (N=30), and product-contact (N=29). The collection process is briefly illustrated in figure 3.1 a), b) and c), respectively.

- Ice collected immediately after production (production): ice was produced in a block and a sharp, automatic blade carved out flakes that would immediately fall into the storage area. As all the ice machines were on top of the storage tank, the bottles were positioned right below as to collect the falling ice without contact with other surfaces. As, in some machines, the flakes would fall in a very disperse way, a disinfected wide-mouth jar was used to collect the ice more efficiently, as needed.
- Stored ice (storage): ice would stack in the storage area, which was an isolated metal compartment. The bottles were dragged across the floor and walls of the storage tank as to collect ice in contact with all surfaces.
- Ice displayed in fish stalls (product-contact): fish was removed, and the top layer of ice was discarded. Samples were collected without touching the surface and avoiding major fish debris.



Figures 3.2 –Sample collection: **a)** production, **b)** storage, **c)** product-contact. Source: Eng. Sílvia Costa.

Each sample consisted of three 1000mL translucid polypropylene, wide mouth, screw cap, autoclaved bottles (Item no 215-5685, VWR International, USA) previously prepared with 1mL of sodium thiosulfate, full of ice; as to prevent sample shortage, an extra two 2000mL translucid borosilicate 3.3 glass, narrow mouth, screw cap bottles (Item no 215-1596, VWR International, USA) were collected exclusively for physical-chemical analysis.

After collection, all bottles were firmly closed and transported directly to the lab in iceboxes. Use of ice or ice packs for sample conservation was discarded.

As for the sample handler, long sleeved, latex free, spunbond meltblown spunbond disposable lab coats (Item no 113-1134, VWR International, USA) were used along with disposable, non-sterile, powder-free nitrile gloves (Item no 112-2371, VWR International, USA) and disposable, non-sterile, low density polyethylene shoe covers (Item no 113-8281, VWR International, USA). All material was disposed after collection and gloves were disinfected with 70% ethanol before use.

Along with the samples, information about the locations was collected (mainly concerning ice handling, storage and operation conditions, amount of ice produced, established cleaning procedures, among others – the information varied according to the sampled location) in order to better understand possible contamination sources. Identification codes were attributed to each market, sample and sample information, as to guarantee privacy and unbiased analysis.

Container selection and sample identification, transport, reception and storage followed ISO 5667-3:2012. Sampling, inactivators of disinfectants and sterilization of containers followed ISO 19458:2006.

All the analysis followed the general recommendations of ISO 7218:2007.

The ice was received in the lab and allowed to thaw at room temperature, or in a 22ºC water bath during colder months, as room temperature was quite low.

3.1.4. Questionnaires

After sampling, stall employees were given the option to answer a simple questionnaire regarding stall maintenance, ice handling and their personal health. No personal data other than employees' sex was collected, and no employee was identified. A sample questionnaire is available in Annex 2. Questionnaires were answered following an informed consent.

Questions included the frequency and manner of stall cleaning, process of ice handling and transport; employees were also inquired as to any sort of education or course that could be relevant to their trade and any health issues (more specifically, skin, nail, respiratory and gastrointestinal complains) that could be a source of contamination, as well as to if they were smokers or had any specific allergies.

In loco observations were also added to each questionnaire – whether the employees wore appropriate clothing, gloves and apron, the appearance of their hair and nails and any behavior that may be inappropriate when in contact with the foodstuffs. Stall location within the market was also recorded, as well as the presence of air-conditioning.

All employees were free to refuse to answer the questionnaire, fully or partially. Fourteen markets presented questionnaires, out of 18 analyzed markets; every stall employee was offered a questionnaire, resulting, in some cases, in more than one answered questionnaire per market.

3.2 Microbiological parameters

3.2.1. *Salmonella* spp.

For the analysis of the presence of *Salmonella* spp., an adaptation of ISO 19250:2010 was followed.

Five hundred mL of undiluted sample was filtered (47 mm Ø and 0.45 µm-pore sterile membrane GN-6 Metricel; Pall Life Sciences, USA) and all the used filters used for each sample were added to 250mL of Buffered Peptone Water (BPW; Merck KGaA Germany) (Figure 3.5) and incubated at 36±2ºC for 18±2h as a pre-enrichment.

Following that, two enrichment mediums were inoculated with the former solution: 0.1mL was added to 10mL of Rappaport Vassiliadis Broth (RAP; VWR International, USA) and incubated at 41.5º±1ºC for 24±3h, and 1mL was added to 10mL of Muller Kauffamnn Tetrationate-Novobiocin Broth (TT; IDEXX Laboratories, USA) and incubated at 37º±1ºC for 24º±3h.

Each of the former inoculated mediums was streaked out by means of a sterile loop both on Xylose Lysine Deoxycholate agar (XLD; Merck KGaA Germany) and *Salmonella* Shigella agar (SSA; Merck KGaA Germany), previously dried, and incubated at 36±2ºC for 24±3h (Figures 3.6 and 3.7).

If characteristic colonies developed after incubation (red with glossy black centres, completely red or completely black in XLD and smooth, colourless and opaque, sometimes with black centres - for H_2S producing strains - in SSA), at least five individualized colonies of each plate were streaked on Nutrient Agar (NA; Merck KGaA Germany) to grow, incubated at 36º±2ºC for 24º±3h (Figure 3.8). Subsequently, each one was inoculated on a Triple Sugar Iron slant (TSI; Merck KGaA Germany) by stabbing to the bottom of the tube and streaking the slant and incubated at 36º±2ºC for 24º±3h (Figure 3.9). All colonies that tested positive (development of a yellow butt and red slant) were tested for urease with a Urea Indole test (VWR International, USA) and incubated at 36º±2ºC for up to 24h. As *Salmonella* spp. are always urease negative, all colonies that tested negative for the urea test (no visible change in colour) underwent an API® 20E (bioMérieux, France) test for confirmation, following manufacturer's instructions.

As there is no tolerance for the presence of *Salmonella* spp, all results were shown as '*Salmonella* present/absent'.

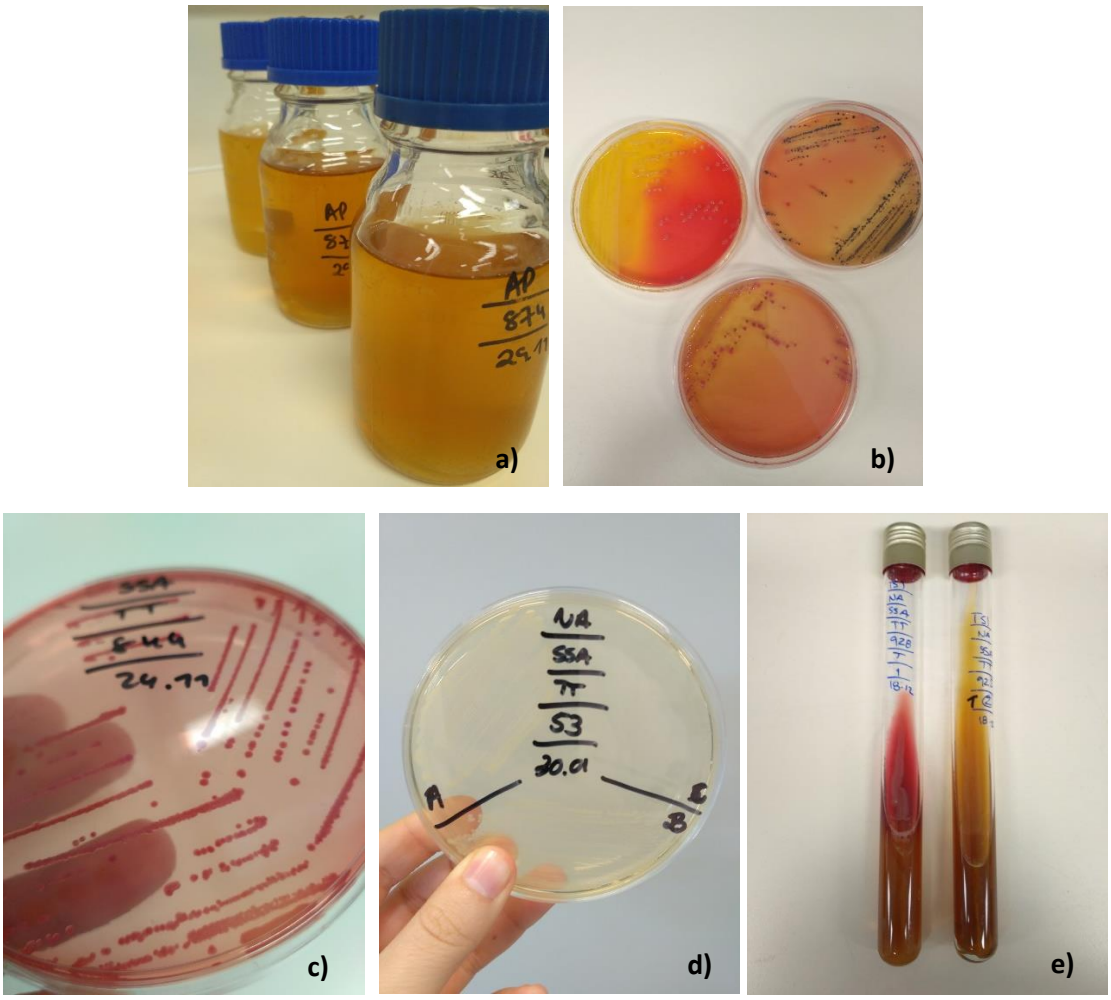


Figure 3.3 a), b), c), d) and e) – Several steps for the analysis for *Salmonella*.

3.2.2. Total microbial counts (5ºC,22ºC and 37ºC)

For total microbial counts, ISO 6222:1999 was followed.

Following the agar inclusion technique, a 1mL aliquot of undiluted sample was inoculated in a Petri dish, followed by the adding of 15-20 mL of molten yeast agar (Y; VWR Chemicals, USA); duplicates were produced for each sample. Inoculation and medium were mixed carefully in order to separate possible bacteria and to allow for individualized colonies, allowed to cool and incubated in three different conditions: 36º±2ºC for 44h±4h, 22º±2ºC for 68h±4h - as it is necessary to evaluate the present of bacteria growth at low temperatures, incubation was also performed at 5º±2ºC for 10 days (Figure 3.4).

After incubation, all plates were counted. If no colonies were observed, the results were shown as 'not detected in one millilitre'. If more than 300 colonies were counted, the results were shown as '> 300'. Plates that showed counts of over 300 CFU with confluent growth any time before the end of incubation were immediately counted as '>300' and disposed.

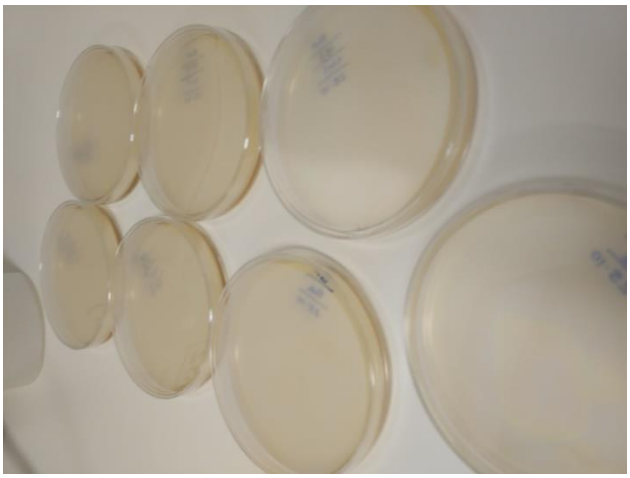


Figure 3.4 – Petri dishes inoculated with samples for analysis of total microbial count.

3.2.3. ***Staphylococcus* spp.**

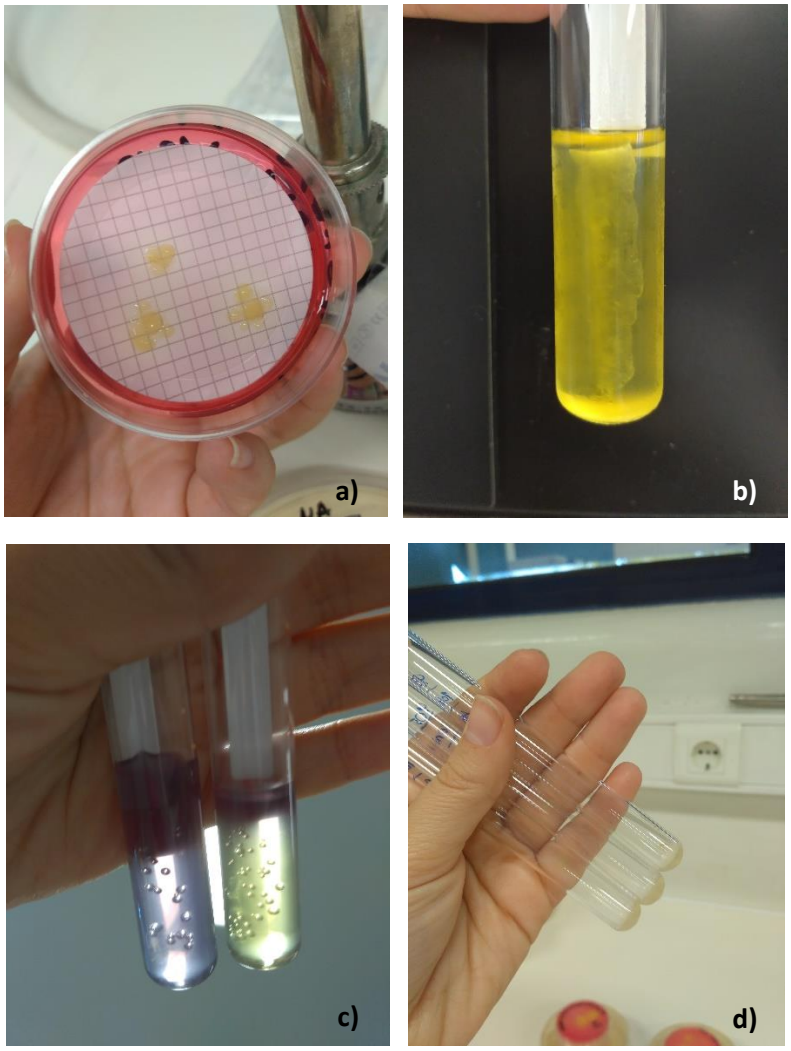
For the analysis of the presence of *Staphylococcus* spp., NP 4343:1998 was followed.

A 100mL aliquot of each water sample was filtered through a cellulose ester membrane (47 mm Ø and 0.45 µm-pore size GN-6 Metricel; Pall Life Sciences, USA). Because of the development of a large number of colonies with confluent growth, some samples were also diluted in sterile water, to a dilution factor of 1:10. The membrane was placed over a selective Chapman's Mannitol Salt Agar (MAN; Merck KGaA, Germany) and incubated inverted at 37ºC ± 1°C for 48h ± 4h (Figure 3.5 a)).

If characteristic colonies developed (small yellow, white, orange or pink colonies), all were counted, and three individualized colonies of each colour were streaked on Nutrient Agar (NA; Merck KGaA, Germany) to grow and incubated at 37±1ºC for 24º±4h. After incubation, a catalase test was performed by adding a drop of 3% hydrogen peroxide (VWR Chemicals, USA) directly to the streaked colonies on NA; colonies that displayed effervescence were considered catalase positive. A respiratory test was conducted on all catalase positive colonies, by inoculation of a M.E.V.A.G medium (Tritium Microbiologie BV, Netherlands), according to manufacturer's instructions, and incubation at 37±1ºC for 24º±4h (Figure 3.5 b) and c)).

All colonies that tested positive on M.E.V.A.G (displayed change of colour from blueish-purple to yellow) were enriched on Brain Heart Infusion Broth (BHI; Merck KGaA, Germany) and incubated at 37±1ºC for 24 hours. After that, a coagulase test was conducted by adding a drop of the inoculated

BHI broth to three drops of previously hydrated rabbit plasma with EDTA (Merck KGaA, Germany). Formation of clot was checked after incubation at 37±1ºC for 4h and 24h (Figure 3.5 d)).



Figures 3.5 – a) Filtration membrane in MAN medium, **b)** a positive M.E.V.A.G. test, **c)** two negative M.E.V.A.G. tests and **d)** three positive coagulase tests.

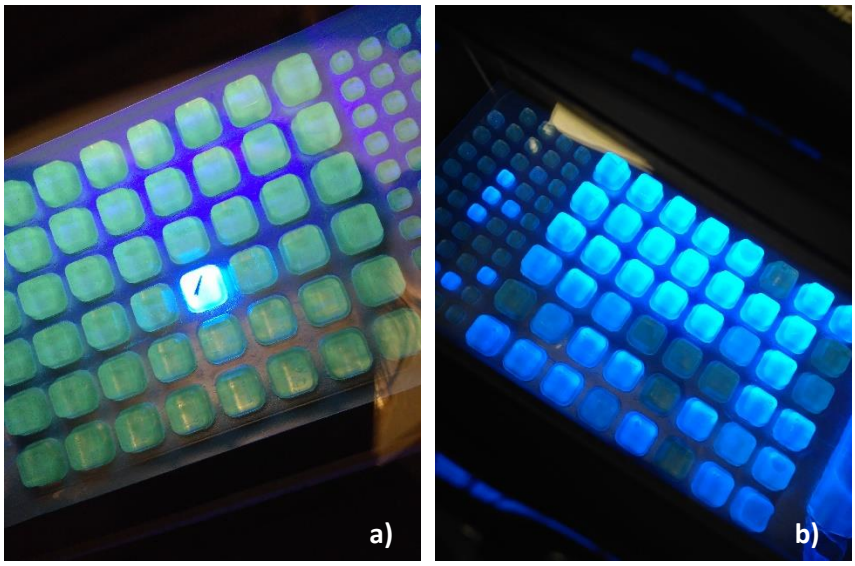
3.2.4. Total coliforms and *Escherichia coli*

For total coliform count, ISO 9308-2:2012/part. 2 (most probable number method) was followed.

Manufacturer's instructions were followed: a snap pack of Colilert-18 dehydrated medium (IDEXX Laboratories, USA) was added to a 100mL aliquot of undiluted sample and carefully shaken to dissolve medium and ensure proper mixing. The mix was aseptically poured into a Quanti-Tray/2000, that was then sealed with a Quanti-Tray Sealer 2X (IDEXX Laboratories, USA). Trays incubated at 36±2°C for 20±2h.

After incubation, wells that displayed a change of colour to yellow are considered positive (Figure 3.6 a)). All yellow wells were also checked for any degree of fluorescence under ultraviolet light, and those who presented it were considered positive for *E. coli* (Figure 3.6 b)).

Using the manufacturer's statistical tables, the most probable number (MPN) of coliform bacteria and *E. coli* were determined.



Figures 3.6 – a) Positive coliform (yellow) and **b)** positive *E. coli* wells (luminescent) in two total coliform tests.

3.2.5. Enterococci

For enterococci count, Most Probable Number Method was followed.

Manufacturer's instructions were followed: a snap pack Enterolert-DW of dehydrated medium (IDEXX Laboratories, USA) was added to a 100mL aliquot of undiluted sample and carefully shaken to dissolve medium and ensure proper mixing. The mix was aseptically poured into a Quanti-Tray/2000, that was then sealed with a Quanti-Tray Sealer 2X (IDEXX Laboratories, USA). Trays incubated at 41±0.5°C for 24±2h. After incubation, wells that displayed a change of colour from blue to green are considered positive (Figure 3.7).

Using the manufacturer's statistical tables, the most probable number (MPN) of enterococci was determined.

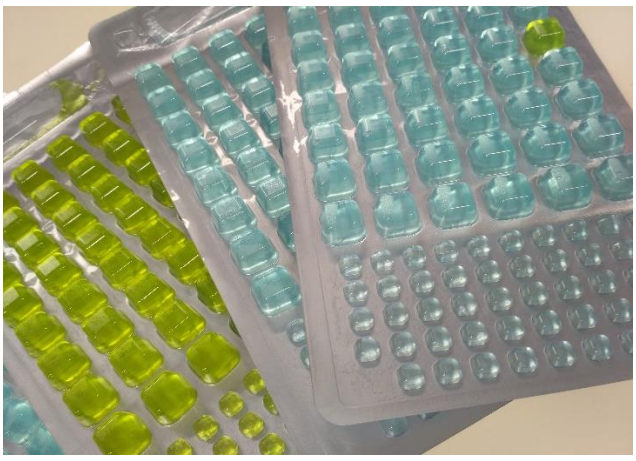


Figure 3.7 – Positive (green) and negative (blue) wells in *Enterococcus* tests.

3.3 Physical-chemical parameters

All physical-chemical parameters were evaluated in the laboratory.

3.3.1. Temperature and pH

Temperature and pH were measured according to SMEWW 4500-H^+ B.

Following manufacturer's instructions, the potentiometer (Orion™ 3-Star, Thermo Fisher Scientific, USA) was calibrated with three buffer solutions (pH 4, 7 and 10), electrodes were rinsed, dried and dipped in an aliquot of undiluted sampled; pH count was allowed to stabilize, and the final values of temperature and pH were measured.

3.3.2. Nitrite (NO_2^-), nitrate (NO_3^-) and ammonium (NH_3)

For these parameters, SMEWW 4500-NO_2^-, SMEWW 4500-NO_3^- E and SMEWW 4500-NH_3 F methods were followed, respectively.

Nitrite was determined by the Colorimetric Method. Samples were filtered with a filtrating membrane (0.45 µm-pore size GN-6 Metricel; Pall Life Sciences, USA) in order to remove suspended solids that might interfere with the readings. pH was adjusted a range of 5-9 with HCl 1N or NH_4OH 1N, as necessary. Colour reagent was added to a 50mL aliquot of sample and mixed thoroughly. Absorbance was measured at 543 nm against a distilled water blank and value was directly computed against a standard absorbance curve.

Nitrate was determined by the Cadmium Reduction Method. Samples were filtered with a membrane (0.45 µm-pore size GN-6 Metricel; Pall Life Sciences, USA) in order to remove suspended solids that might interfere with the readings; pH was adjusted a range of 7 – 9 with diluted HCl or NaOH, as necessary. Sample was reduced to by adding 75 mL NH_4Cl- EDTA solution to 25mL aliquot of sample. This mix was transferred to a reduction column previously prepared with Cd-Cu granulate and water and activated, and the flow of 7 to 10 ml/minute was collected, after discarding the first 25 mL. 2 mL of colour reagent was immediately added to the previously collected 50mL and mixed thoroughly. Absorbance was measured at 543 nm against a distilled water blank and value was directly computed against a standard absorbance curve.

Ammonium was determined by the Phenate Method. Residual chlorine was removed from the sample with a dechlorinating agent ($Na_2S_2O_3.5H_2O$, previously prepared at 3.5mg/mL); pH was adjusted to approximately 7. 25 mL of previously prepared borate tamponing solution was added, and pH was again adjusted to approximately 9.5 with NaOH 6N. The mix was then distilled with a flow of 6-10mL per minute with the extremity of the discharge tubed submerged in a receptor acidic solution. At least 200mL of distilled product was recovered in a 500mL Erlenmeyer with 50mL of H_2SO_4 0.04N. The discharge tube was then removed from the solution and the distillation was allowed for another 1 to 2 minutes. The sample was diluted to 500mL with water and neutralized with NaOH 1N. 25mL of this sample was pipetted to a 50mL Erlenmeyer and 1mL of phenol solution, 1mL of sodium nitroprusside and 2.5mL of oxidizing solution (100mL alkaline citrate and 25mL sodium hypochlorite) were added, with careful homogenization in between. The Erlenmeyer was covered with parafilm and let to rest at 22-27ºC under diffused light for at least one hour. Absorbance was measured at 640 nm and value was directly computed against a standard absorbance curve.

3.3.3. Chlorine (Cl) and chloride (Cl^-)

For these parameters, SMEWW 4500-Cl_2 G and NP 423:1966 methods were followed, respectively.

Total chlorine and free chlorine analysis were completed according to the Colorimetric Method. The pH of samples was adjusted to a range of 4-8 if needed, with NaOH or H_2SO_4. Turbid samples were filtered. A 5mL aliquot of prepared sample was pipetted to a test tube with a clean, readable barcode and a shallow microspoon of blue Cl_2-1 reagent (Merck KGaA, Germany) was added. The tube was closed and vigorously shaken until complete dissolution. The mix was allowed to rest for 3 minutes and the developed colour was read with a photometer (Spectroquant NOVA 60, Merck KGaA, Germany), providing the value of free chlorine. The tube was then opened and two drops of Cl2-2 reagent added. After mixing, the developed colour was measured with a photometer (Spectroquant NOVA 60, Merck KGaA, Germany), providing the value of total chlorine.

Chloride was analysed according to Mohr Volumetric Method. Turbid samples were filtered (0.45 µm-pore size GN-6 Metricel; Pall Life Sciences, USA) and pH was adjusted to a range of 6.5-10.5 if needed, with NaOH or H_2SO_4. A 100mL aliquot was pipetted to a 250mL Erlenmeyer and 10 drops of previously prepared potassium chromate indicator solution were added. After that, a solution of silver nitrate 0.1N was titrated, until the indicator turns to yellow. The titrated volume was noted as Va (mL). The same procedure was followed with 100mL of distilled water as to obtain Vb (mL), the titrated volume for the blank. Chloride value is calculated by

$$f \times [(Va - Vb)\ x\ 35.5] = \frac{mgCL^-}{L} \text{ (3.1)}$$

Where f is the sample's dilution factor.

3.3.4. Conductivity

Conductivity was measured in accordance with NP EN 27888:1996, following the Conductimetric Method.

The conductometer (CDM210, Radiometer Copenhagen) was calibrated using the manufacturer blank solution (1408µS/cm), according to manufacturer's instructions. Temperature was measured and, following a standard value list for the given temperature, the conductometer temperature coefficient was made to vary until it matched the standard value, and K value was obtained. The samples were again read, and conductivity values were obtained.

3.3.5. Total hardness

Total hardness was determined by the EDTA Titrimetric Method, in accordance to NP 424:1966.

A 50mL aliquot of sample was measured to a 250mL erlenmeyer. 1mL of previously prepared NH_4^+/NH_3 tamponing solution and 6 drops of Eriochrome Black T indicator solution (HIMEDIA, India) were added. Within 5 minutes, the sample was titrated with previously prepared EDTA solution until its red colour turned blue. The final colour had to be persistent. The titrated volume was noted as Va.

Total hardness is calculated by

$$\frac{(1000 \times Va)}{50} = mg\ CaCO_3/L \text{ (3.2)}$$

3.3.6. Organic matter content

Organic matter content was determined by the Kübel Tiemann's method, in accordance to ISO 8467:1993

Sample was allowed to heat to room temperature and homogenised. A 100mL aliquot of sample was pipetted to an Erlenmeyer and 10mL of previously prepared sulphuric acid solution 1.84g/mL was added. Sample was mixed and 5mL of previously prepared potassium permanganate solution was added, mixing again. Three glass pearls, free of organic matter, were added as to avoid projections. The mix was brought to ebullition for 10 minutes while continuously stirring, to ensure that all organic matter inside the Erlenmeyer is destroyed. The mix was removed from heat and a solution of sodium oxalate was added, drop by drop until any colour is gone. Following that, a solution of potassium permanganate was added drop by drop until a slight pink tone persisted for about 30 seconds. Ten mL of sodium oxalate solution was added, and the Erlenmeyer stirred. The mix was titrated with a previously prepared potassium permanganate solution until a slight pink tone persisted for about 30 seconds. The titrated volume was noted as Va.

Subsequently, 100mL of sample was pipetted to the previous Erlenmeyer, after its contents were discarded. Ten mL of sulphuric acid solution 1.84g/mL was added, as well as the previously titrated volume (Va). The Erlenmeyer was brought to ebullition for 10 minutes, after which it was removed from heat and 10mL of sodium oxalate was added. The mix was titrated with a previously prepared potassium permanganate solution until a slight pink tone persisted for about 30 seconds. The titrated volume was noted as Vb.

If the sample was diluted, a blank was also prepared, following the same procedure as above (Vb), using distilled or deionized water. The blank titrated volume was noted as Vbr

Organic matter content was calculated by

$$8 \times \frac{Vb-Vbr}{Va} \times f = mgO_2/L \quad (3.3)$$

Where f is the dilution factor.

4. Results

It is to be noted that *Staphylococcus* spp. parameter was only measured for the last 36 samples (13 production samples, 13 storage samples and 10 production samples). Results classified as 'Damaged' include samples with confluent growth (indicating the presence of microorganisms but making it impossible to obtain a count).

All detailed microbiological and physical-chemical results are compiled in Annexes 1a through 3b. Statistical analysis was conducted from those results. A summary is represented in tables 4.1 and 4.2 below.

Table 4.1 – Summary of samples' conformity by stage.

	Conformity	**No conformity**	**N**
Product-contact	0 (0.0%)	29 (100.0%)	29
Storage	23 (76.7%)	7 (23.3%)	30
Production	25 (86.2%)	4 (13.8%)	29
	48 (54.55%)	40 (45.45%)	

Table 4.2 – Summary of samples' conformity by stage and type of analysis.

	Microbiology		**Physical-chemistry**	
	Conformity	**No conformity**	**Conformity**	**No conformity**
Product-contact	0 (0.0%)	29 (100.0%)	7 (24.1%)	22 (75.9%)
Storage	25 (83.3%)	5 (16.7%)	28 (93.3%)	2 (6.7%)
Production	29 (100.0%)	0 (0.0%)	25 (86.2%)	4 (13.8%)
	54 (61.4%)	34 (38.6 %)	60 (68.2%)	28 (31.8%)

Given that there is no specific legislation for ice, parametric values for potable water were used. Microbiological parameters were classified as Acceptable/Not Acceptable or Detected/Not Detected for legislated and unlegislated parameters, respectively. Physical-chemical parameters were classified as Acceptable/Unacceptable – two parameters are not legislated but have recommended values that samples should meet.

Samples were grouped into three stages – production, storage and product-contact.

4.1. Microbiology

4.1.1. Production samples

An extended table of results for all production samples tested microbiological parameters can be found on Annex 1a. Table 4.3 summarizes the percentage of acceptable, unacceptable and damaged or not performed samples by parameter with legislated parametric values. Table 4.4 summarizes samples where there was detection (or lack thereof) of microorganisms for parameters without legislated parametric values for tap water.

Table 4.3 – Classification of legislated microbiological parameters for production samples.

	E. coli	**Coliforms**	***Enterococcus***	**TMC 37ºC**	**TMC 22ºC**
Acceptable	29 (100.0%)	29 (100.0%)	27 (100.0%)	29 (100.0%)	29 (100.0%)
Not acceptable	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Damaged/NP	0	0	2	0	0

NP – not performed

Table 4.4 – Classification of unlegislated microbiological parameters for production samples.

	Total *Staphylococcus* spp.	***Staphylococcus* spp. Coagulase +**	**TMC 5ºC**	***Salmonella***
Detected	1 (7.7%)	0 (0.0%)	5 (19.2%)	0 (0.0%)
Not detected	12 (92.3%)	13 (100.0%)	21 (80.8%)	25 (100.0%)
Damaged/NP	16	16	3	4

NP – not performed

All 29 samples tested negative for Coliforms and *E. coli*. Similarly, all 27 tested samples (two not performed) showed no counts for *Enterococcus* spp.. Tests for *Staphylococcus* spp. were only performed for the 13 more recent samples. Only one sample tested positive for *Staphylococcus* spp. but negative for Coagulase Positive *Staphylococcus* spp..

Values for Total Microbial Counts were quite low for all temperatures. Twenty-one samples presented no CFU count at 37ºC; the remaining eight had exact counts ranging from 1 to 2 CFU/mL. Twenty-two samples also presented no counts at 22ºC, while seven had counts between 1 and 20. As for counts at 5ºC, from 26 performed tests (three not performed), only five samples presented CFU counts, all with 1 CFU/mL. *Salmonella* spp. detection test was performed in 25 samples. All tested negative for the presence of *Salmonella* spp..

Figure 4.1 shows the proportion between acceptable/not acceptable and detected/not detected (and also not performed or damaged) results in each tested parameter.

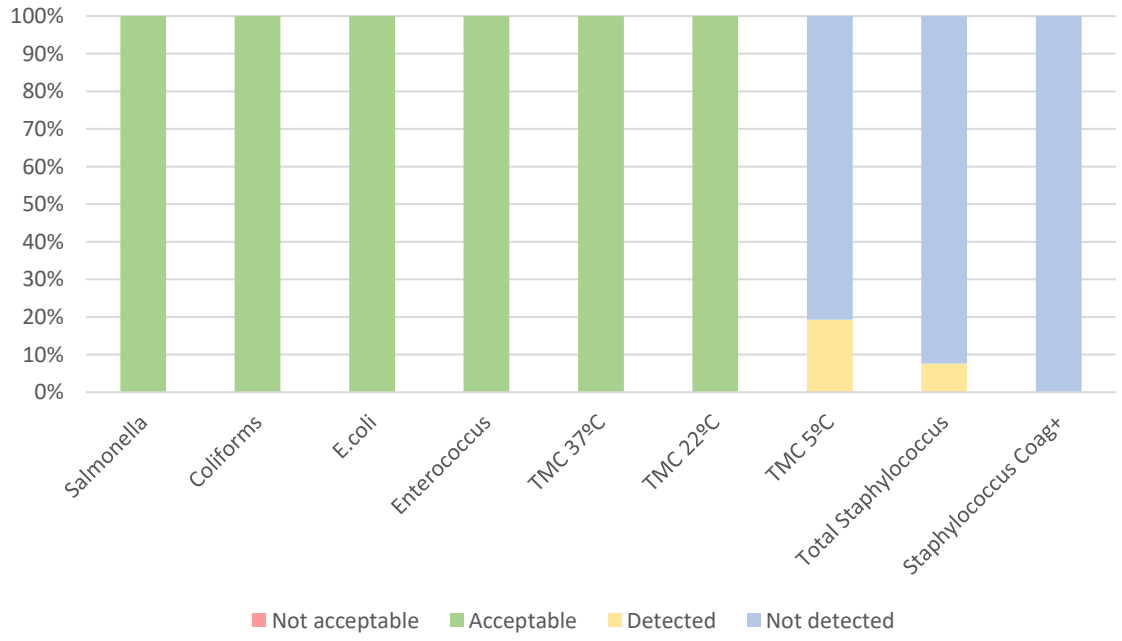


Figure 4.1. – Stacked percentage of production sample results.

4.1.2. Storage samples

An extended table of results for all storage samples tested microbiological parameters can be found on Annex 2a. Table 4.5 summarizes the percentage of acceptable, unacceptable and damaged or not performed samples by parameter with legislated parametric values. Table 4.6 summarizes samples where there was detection (or lack thereof) of microorganisms for parameters without legislated parametric values for tap water.

Table 4.5 – Classification of legislated microbiological parameters for storage samples.

	E. coli	**Coliforms**	***Enterococcus***	**TMC 37ºC**	**TMC 22ºC**
Acceptable	28 (93.3%)	26 (86.7%)	27 (90.0%)	26 (86.7%)	24 (80.0%)
Not acceptable	2 (6.7%)	4 (13.3%)	3 (10.0%)	4 (13.3%)	6 (20.0%)
Damaged/NP	0	0	0	0	0

NP – not performed

Table 4.6 – Classification of unlegislated microbiological parameters for storage samples.

	Total *Staphylococcus*	***Staphylococcus* Coagulase +**	**TMC 5ºC**	***Salmonella***
Detected	3 (23.1%)	1 (7.7%)	10 (37.1%)	0 (0.0%)
Not detected	10 (76.9%)	12 (92.3%)	17 (62.9%)	26 (100.0%)
Damaged/NP	17	17	3	4

NP – not performed

All but four (26) storage samples tested negative for Coliforms. The four samples that had positive results showed counts of 2, 15, 48 and 108 coliforms per 100mL. Similarly, 28 samples tested negative for *E. coli*. The remaining two presented the values of 1 and 7 bacteria per 100mL.

Three samples tested positive for *Enterococcus* spp., presenting values of 1, 4 and 6 counts for enterococci.

Tests for *Staphylococcus* spp. were only performed for the 13 more recent samples. In this case, none of those samples presented confluent growth. The three samples positive for *Staphylococcus* spp. presented values ranging of 2, 3 and 5 colonies per 100mL; one sample tested positive for Coagulase Positive *Staphylococcus* spp. (two colonies).

Values for Total Microbial Counts were yield mixed results. Nineteen samples presented no CFU count at 37ºC and two had counts of over 300 CFU/mL; the remaining nine had exact counts ranging from 1 to 94 CFU/mL. Eleven samples also presented no counts at 22ºC, while four had counts bigger than 300 CFU/mL; the remaining 15 showed counts ranging from 1 to 174. As for CFU counts at 5ºC, from 27 performed tests (three not performed), only one sample presented a value bigger than 300 CFU/mL; nine had counts ranging from 1 to 127 and 17 presented no CFU/mL.

Salmonella detection test was performed in 26 samples. All tested negative for the presence of *Salmonella*.

Figure 4.2 shows the proportion between acceptable/not acceptable and detected/not detected (and also not performed or damaged) results in each tested parameter.

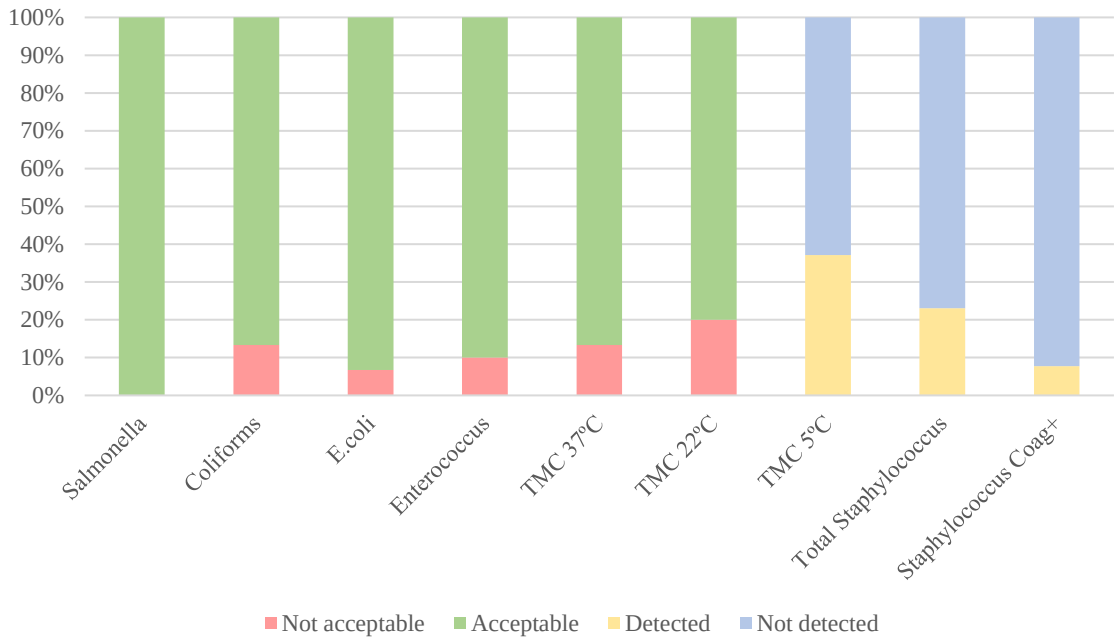


Figure 4.2. – Stacked percentage of storage sample results.

In this stage, all the unacceptable samples failed at least two microbiological parameters; even so, the majority (83.3%) passed all parameter tests. Table 4.7 summarizes the parameters the samples failed.

Table 4.7 – Summary of unacceptable storage samples by failed microbiological parameters.

Acceptable samples	25 (83.3%)
Unacceptable samples – Coliforms and *E. coli*	1 (3.34%)
Unacceptable samples – Coliforms, *E. coli Enterococcus* and TMC 22ºC	1 (3.34%)
Unacceptable samples – Coliforms, *Enterococcus*, TMC 37ºC and TMC 22ºC	2 (6.68%)
Unacceptable samples – TMC 37ºC and TMC 22ºC	1 (3.34%)

4.1.3. Product-contact samples

An extended table of results for all product-contact samples tested microbiological parameters can be found on Annex 3a. Table 4.8 summarizes the percentage of acceptable, unacceptable and damaged or not performed samples by parameter with legislated parametric values. Table 4.9 summarizes samples where there was detection (or lack thereof) of microorganisms for parameters without legislated parametric values for tap water.

Table 4.8 – Classification of legislated microbiological parameters for product-contact samples.

	E. coli	**Coliforms**	***Enterococcus***	**TMC 37ºC**	**TMC 22ºC**
Acceptable	14 (48.3%)	0 (0.0%)	1 (3.4%)	0 (0.0%)	0 (0.0%)
Not acceptable	15 (51.7%)	29 (100.0%)	28 (96.6%)	29 (100.0%)	29 (100.0%)
Damaged/NP	0	0	0	0	0

NP – not performed

Table 4.9 – Classification of unlegislated microbiological parameters for product-contact samples.

	Total *Staphylococcus*	***Staphylococcus* Coagulase +**	**TMC 5ºC**	***Salmonella***
Detected	12 (100.0%) [a)]	1 (11.1%)	26 (100.0%)	0 (0.0%)
Not detected	0 (0.0%)	8 (88.9%)	0 (0.0%)	27 (100.0%)
Damaged/NP	17	20	3	2

NP – not performed; [a)] three samples tested positive but presented confluent growth.

All (29) product-contact samples tested positive for coliforms. Eight samples reached Quantitray's maximum counting capacity, presenting values bigger than 2420 coliforms per 100mL of sample; the remaining 21 samples had values ranging from 3 to 1554 coliforms per 100mL. Sixteen samples tested negative for *E. coli*. The remaining 13 presented values ranging from 1 to 57 bacteria per 100mL.

Only one sample tested negative for *Enterococcus* spp.. From the remaining 28, five samples reached Quantitray's maximum counting capacity, presenting values bigger than 2420 enterococci per 100mL. The remaining 23 samples ranged between 1 and 1120 counts for enterococci.

Tests for *Staphylococcus* spp. were only performed for the 12 more recent samples. Three of those samples presented confluent growth, therefore testing positive for *Staphylococcus* spp. but preventing an exact count and the collection of individualized colonies for a coagulase test. The remaining nine presented values ranging between 10 and 534 per 100mL; one sample tested positive for Coagulase Positive *Staphylococcus* spp. (2 colonies).

Values for Total Microbial Counts were quite high for all temperatures. Twenty-four samples presented values of over 300 CFU/mL at 37ºC, while the remaining 5 had counts of 115, 232, 45, 101 and 135. Twenty-eight samples also presented values bigger than 300 CFU/mL at 22ºC, while one had a count of 170. Counts at 5ºC yield similar results – from 26 performed tests (one not performed and two damaged), only one sample presented an exact count of 29, while all other showed values bigger than 300 CFU/mL.

Salmonella detection test was performed in 27 samples. All tested negative for the presence of *Salmonella*.

Figure 4.3 shows the proportion between acceptable/not acceptable and detected/not detected (and also not performed or damaged) results in each tested parameter.

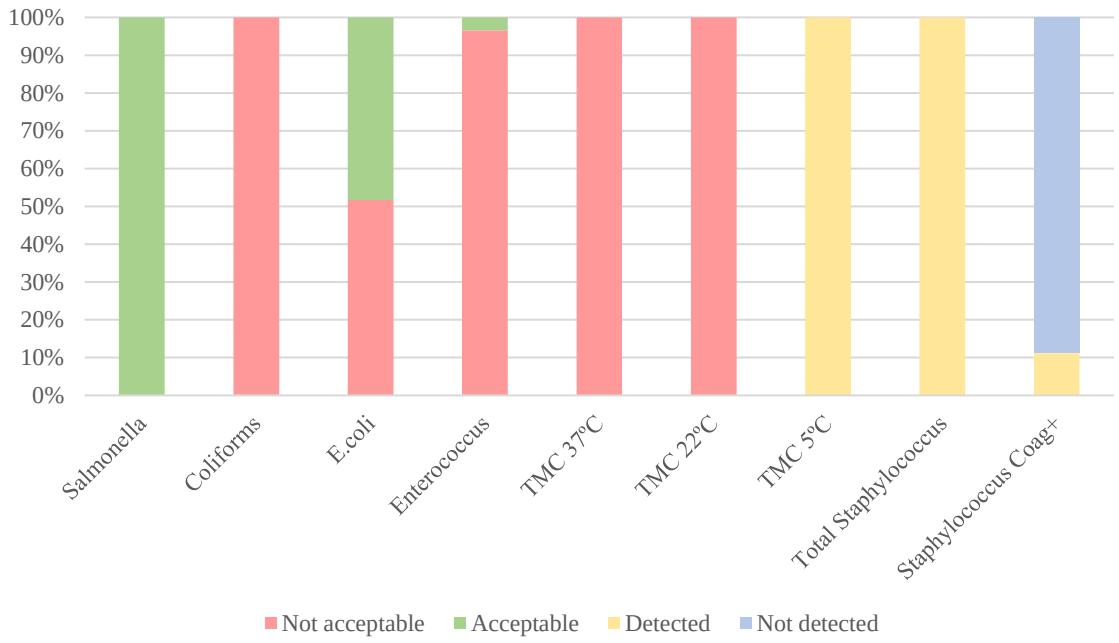


Figure 4.3 – Stacked percentage of product-contact sample results.

In this stage, all samples were considered unacceptable due to at least three failed microbiological parameters; the vast majority (96.56%) failed by four or five parameters. Table 4.10 summarizes the parameters the samples failed.

Table 4.10 – Summary of unacceptable product-contact samples by failed microbiological parameters.

Acceptable samples	0 (0%)
Unacceptable samples – Coliforms, TMC 37ºC and TMC 22ºC	1(3.44%)
Unacceptable samples – Coliforms, *Enterococcus*, TMC 37ºC and TMC 22ºC	13 (44.83%)
Unacceptable samples – Coliforms, *E. coli*, *Enterococcus*, TMC 37ºC and TMC 22ºC	15 (51.73%)

4.2. Physical-chemistry

Total chlorine and hardness, although mentioned in the legislated, do not have parametric values; there are, however, recommended and desirable values ranges: 0.2 – 0.6 mg/L for Free Residual Chlorine and 150 – 500 mg $CaCO_3$/L for Hardness.

4.2.1. Production samples

An extended table of results for all production samples tested physical-chemical parameters can be found on Annex 1b. Table 4.11 summarizes the percentage of acceptable, unacceptable and damaged or not performed samples by parameter.

Table 4.11 – Classification of physical-chemical parameters for production samples.

	Acceptable	Unacceptable	Damaged/NP
Ammonium	29 (100.0%)	0 (0.0%)	0
Conductivity	26 (89.7%)	3 (10.3%)	0
Chloride	23 (85.2%)	4 (14.8%)	2
Free residual chlorine [a]	7 (31.8%)	15 (68.2%)	7
Nitrate	29 (100.0%)	0 (0.0%)	0
Nitrite	29 (100.0%)	0 (0.0%)	0
pH	29 (100.0%)	0 (0.0%)	0
Organic matter content	22 (84.6%)	4 (15.4%)	3
Hardness [a]	0 (0.0%)	28 (100.0%)	1
	78.2%	21.8%	

NP – not performed; [a] samples presenting values below the recommended range were still considered in conformity to legislation, if all other parameters were within limit values.

If parameters without parametric values were removed (Free Residual Chlorine and Hardness), the percentage of acceptable and unacceptable samples would amount to 94.4% and 5.6%, respectively.

4.2.2. Storage samples

An extended table of results for all storage samples tested physical-chemical parameters can be found on Annex 2b. Table 4.12 summarizes the percentage of acceptable, unacceptable and damaged or not performed samples by parameter.

Table 4.12 – Classification of physical-chemical parameters for storage samples.

	Acceptable	Unacceptable	Damaged/NP
Ammonium	30 (100.0%)	0 (0.0%)	0
Conductivity	28 (93.3%)	2 (6.7%)	0
Chloride	27 (96.4%)	1 (3.6%)	2
Free residual chlorine [a]	6 (26.1%)	17 (73.9%)	7
Nitrate	30 (100.0%)	0 (0.0%)	0
Nitrite	30 (100.0%)	0 (0.0%)	0
pH	16 (53.3%)	14 (46.7%)	0
Organic matter content	24 (92.3%)	2 (7.7%)	4
Hardness [a]	0 (0.0%)	28 (100.0%)	2
	74.9%	25.1%	

NP – not performed; [a] samples presenting values below the recommended range were still considered in conformity to legislation, if all other parameters were within limit values.

If parameters without parametric values were removed (Free Residual Chlorine and Hardness), the percentage of acceptable, unacceptable and damaged/NP samples would amount to 89.4% and 10.6%, respectively.

4.2.3. Product-contact samples

An extended table of results for all product-contact samples tested physical-chemical parameters can be found on Annex 3b. Table 4.13 summarizes the percentage of acceptable, unacceptable and damaged or not performed samples by parameter.

Table 4.13 – Classification of physical-chemical parameters for product-contact samples.

	Acceptable	Unacceptable	Damaged/NP
Ammonium	15 (51.7%)	14 (42.3%)	0
Conductivity	28 (96.6%)	1 (3.4%)	0
Chloride	26 (92.9%)	2 (7.1%)	1
Free residual chlorine [a]	4 (16.0%)	21 (84.0%)	4
Nitrate	28 (96.6%)	1 (3.4%)	0
Nitrite	28 (96.6%)	1 (3.4%)	0
pH	17 (58.6%)	12 (41.4%)	0
Organic matter content	6 (23.1%)	20 (76.9%)	3
Hardness [a]	0 (0.0%)	29 (100.0%)	0
	60.1%	39.9%	

NP – not performed; [a] samples presenting values below the recommended range were still considered in conformity to legislation, if all other parameters were within limit values.

If parameters without parametric values were removed (Free Residual Chlorine and Hardness), the percentage of acceptable, unacceptable and damaged/NP samples would amount to 73.6% and 26.4%, respectively.

4.3. Comparison between stages

For comparison purposes, samples with values outside tests' window of detection were rounded – samples with results of >300 were assumed to have a result of 301and samples with results of >2419.6 were rounded up to 2420; samples below the detection threshold were slightly rounded down (<1 to 0.1; <0.06 to 0.05; <0.03 to 0.02; <0.02 to 0.01).

Samples were firstly subjected to a descriptive analysis, which is summarized in Table 4.14.

Consequentially, the data was analysed following the Kruskal-Wallis test, a non-parametric statistical test for the comparison of the distribution of three or more groups of samples. If difference was detected, a pairwise comparison was performed between each stage with Dunn's multiple comparisons test. A Bonferroni correction (with a p-value of .0167) was applied in order to protect results against type I errors (false positives). Table 4.15 lists all value results, as well as the existence (or lack thereof) of significant pairwise difference.

Significance was considered at 5%. XLSTAT statistical analysis programme was used.

Table 4.14 - Summary of the descriptive analysis of each stage.

	Production stage				**Storage stage**				**Product-contact stage**			
	Mean	**Median**	**Min**	**Max**	**Mean**	**Median**	**Min**	**Max**	**Mean**	**Median**	**Min**	**Max**
Coliforms	0.10	0.10	0.10	0.10	6.0	0.10	0.10	108.0	978.83	548.0	0.10	2420.0
E. coli	0.10	0.10	0.10	0.10	0.0	0.10	0.10	7.0	5.12	1.0	0.10	57.0
***Enterococcus* spp.**	0.10	0.10	0.10	0.10	0.0	0.10	11.0	6.0	519.14	29.0	0.10	2420.0
***Staphylococcus* spp.**	0.2	0.0	0.0	3.0	1.0	0.0	0.0	5.0	236.78	267.0	10.0	534.0
TMC at 37º	0.5	0.10	0.10	2.0	26.5	0.1	0.1	301.0	270.76	301.0	45.0	301.0
TMC at 22º	1.7	0.10	0.10	20.0	53.1	1.0	0.1	301.0	296.48	301.0	170.0	301.0
TMC at 5º	0.3	0.10	0.10	1.0	24.1	0.1	0.1	301.0	290.54	301.0	29.0	301.0
***Salmonella* spp.**	N/A											
Ammonium	0.10	0.09	0.05	0.21	0.09	0.09	0.05	0.23	1.65	0.48	0.07	9.59
Conductivity	1153.8	510.0	152.0	10900.0	1115.5	472.0	206.0	10970.0	697.5	457.0	87.0	7430.0
Chlorides	254.9	127.0	65.0	2237	247.71	108.0	48.0	3195.0	173.7	106.0	13.0	1821.0
FRC	0.10	0.10	0.04	0.4	0.103	0.10	0.03	0.6	0.09	0.07	0.02	0.3
Nitrate	2.1	1.77	0.68	5.94	2.07	1.74	0.84	6.08	4.53	1.24	0.106	5683.0
Nitrite	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.04	0.01	0.01	0.54
pH	8.0	7.9	7.50	8.8	8.79	8.95	7.6	9.9	8.52	9.0	6.8	9.7
OMC	3.6	1.64	0.63	36.0	1.99	1.49	0.65	9.76	13.76	8.3	0.96	66.9
Hardness	48.6	47.0	22.0	80.0	47.18	45.0	24.0	73.0	42.79	36.0	12.0	79.0

n/a: not applicable; FRC: free residual chlorine; OMC: organic matter content.

Table 4.15 – Kruskal-Wallis p-value results and pairwise test results for each parameter.

		Pairwise test – existence of statistically significant difference		
	p-value	**Production/ Storage**	**Production/ Product-contact**	**Storage/ Product-contact**
Coliforms	<.001	No (.445)	Yes (<.001)	Yes (<.001)
E. coli	<.001	No (.541)	Yes (<.001)	Yes (<.001)
Enterococcus	<.001	No (.554)	Yes (<.001)	Yes (<.001)
Staphylococcus	<.001	No (.566)	Yes (<.001)	Yes (<.001)
TMC at 37º	<.001	No (.458)	Yes (<.001)	Yes (<.001)
TMC at 22º	<.001	Yes (.016)	Yes (<.001)	Yes (<.001)
TMC at 5º	<.001	No (.264)	Yes (<.001)	Yes (<.001)
Ammonium	<.001	No (.832)	Yes (<.001)	Yes (<.001)
Conductivity	.093	n/a	n/a	n/a
Chlorides	.245	n/a	n/a	n/a
FRC	.050	n/a	n/a	n/a
Nitrate	.019	No (.889)	Yes (.013)	Yes (.018)
Nitrite	.002	No (.684)	Yes (.001)	Yes (.005)
pH	<.001	Yes (<.001)	Yes (.009)	No (.159)
OMC	<.001	No (.367)	Yes (<.001)	Yes (<.001)
Hardness	.438	n/a	n/a	n/a

n/a: not applicable; FRC: free residual chlorine; OMC: organic matter content. p-values for pairwise tests in brackets.

Microbiology parameters showed significantly higher values in product-contact stage, while physical-chemistry parameters presented quite homogenous results.

Salmonella was not detected at any stage.

All microbiology parameters presented a statistically significant difference between stages, while only five physical-chemistry parameters did so. In both cases, pairwise tests showed the existence of statistically significant difference between production and product-contact stages in all tested parameters; all but two parameters showed significant difference between storage and product-contact parameters. Production and storage stages were the most similar, with only two parameters presenting statistically significant difference.

4.4. Questionnaire results

4.4.1. Stalls' personnel

Fourteen out of 18 analysed market stalls were subjected to questionnaires, due to the unavailability of the remaining four. In some stalls, more than one employee was interviewed, to a total of 27 employees (in direct contact with the product) – 16 females and 11 males (59.26% and 40.74%, respectively). The great majority of employees were middle-aged – only two males and one female were young adults.

Most employees undertook some type of educational course specifically regarding their activities in municipal markets. Two employees attended classes for unrelated subjects, more precisely, a bakery and a marketing course (summarized in Table 4.16).

Table 4.16 – Summary of employees' education.

No further education	0	
Unrelated education	2	
Occupational health course 2017	7	15
Occupational health course 2016	3	
Occupational health course 2008	5	
Municipal specific course (2013-2017)	7	
Commerce association specific course (2013-2017)	3	

All interviewed employees presented appropriate working clothes (in-loco observations are summarized in Table 4.17). The vast majority used aprons, and all employees who did so claimed to wash their apron everyday – with bleach (7), multipurpose detergent (4), bleach and multipurpose detergent (2), dishwasher detergent (4), in a washing machine with clothes detergent (2), in a washing machine with water only (1), handwashing with water only (1) and with industrial cleaning detergent (1).

Table 4.17 – Summary of employees' attire and appearance.

	Clothing	**Apron**	**Gloves**	**Nails**	**Hair**
Appropriate	27 (100%)	22 (81.48%)	16 (59.26%)	23 (85.19%)	27 (100%)
Not appropriate	0 (0.0%)	5 (18.52%)	11 (40.74%)	4 (14.81%)	0 (0.0%)

About half of the interviewed employees used gloves. In loco observations revealed that almost all employees that wore gloves would, nevertheless, handle money, dirty dishrags and, less frequently, biologically grown fruit without taking the gloves off, making their use irrelevant. Employees of one of the stalls had at their disposal hand sanitizer gel, and claimed to use it frequently, but were not seen using it during the interviews.

Regardless of the use of gloves, all but four female employees had short, trimmed nails. Similarly, all employees presented their hair in an appropriate matter – all males had short hair and all females had either short or tied hair. One female had, additionally, a hair net hat.

In a general manner, employees presented few health conditions; employees answers are summarized in Table 4.18. No skin conditions were communicated other than four cases of chilblains, probably from contact with the ice; similarly, no nail infections were detected. Respiratory conditions were the most frequent, with two reports of frequent colds, one of frequent cough, a recent case of pneumonia and one of H1N1 influenza; five employees said to have a pollen allergy. The only gastrointestinal complained consisted of a case of chronic stomach ulcer. Seven employees were smokers, while one had recently quit.

Table 4.18 – Summary of employees' health situation.

	Infections or diseases					
	Skin	**Nail**	**Respiratory**	**Gastrointestinal**	**Smoker**	**Allergies**
Yes	4 (14.81%)	0 (0.0%)	5 (18.51%)	1 (3.70%)	7 (25.93%)	5 (18.51%)
No	23 (85.19%)	27 (100%)	22 (81.48%)	26 (96.30%)	20 (74.07%)	22 (81.48%)

4.4.2. Stalls

All market stalls displayed the ice and fish at least from 7h00m until 12h00m; some stalls prepared for sale earlier (earliest at 5h30m) and/or ended the working day later (latest at 16h00m) (Figure 4.4). The values presented are approximate estimates given by employees, as working hours greatly vary according to day of the week, costumer flow, availability of products, among other factors. Fish products were put on display roughly at the same time as the ice.

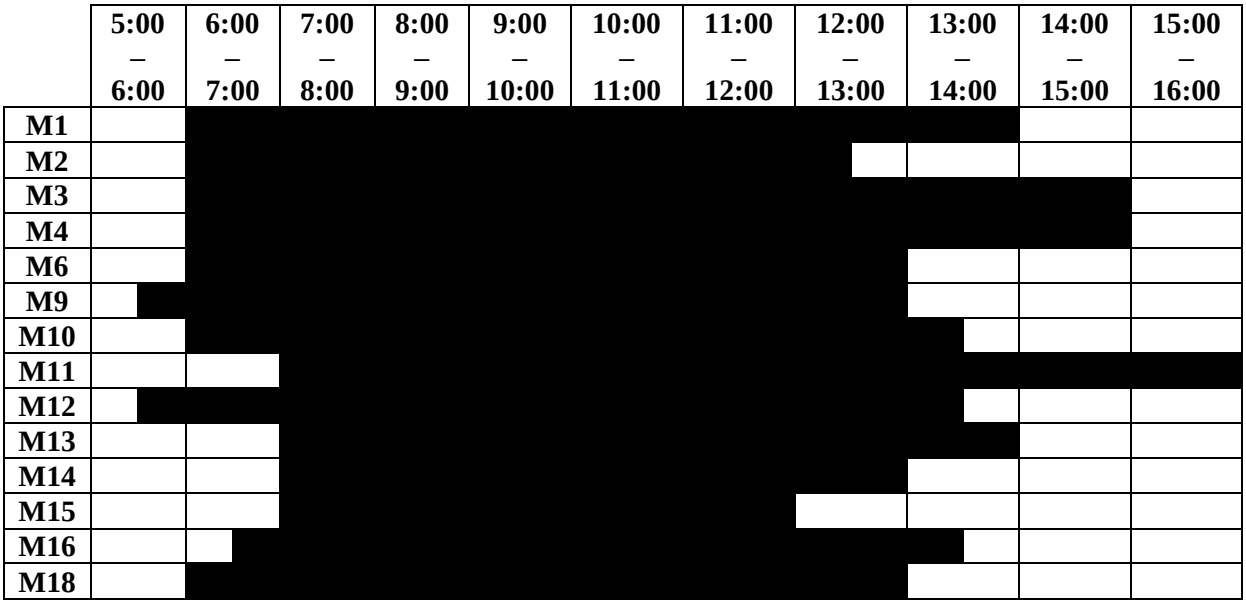


Figure 4.4. – Stalls' approximate working hours.

According to the employees, all stalls were cleaned daily, after working hours, by the employees themselves. The cleaning methods varied and are summarized in Table 4.19.

Table 4.19. – Summary of stalls' cleaning methods.

Bleach only	1
Bleach, dishwasher detergent	1
Bleach, industrial detergent	1
Bleach, multipurpose detergent	3
Dishwasher detergent only	1
Industrial cleaning plan	5
Multipurpose detergent only	2

Most stalls presented satisfactory cleaning methods. Some stalls presented high amounts of rust.

No markets had functioning air-conditioning system, and ventilation relied solely on windows and doors. Seven stalls were located away from any windows and doors, two in front of an open window, four directly in front of a main entrance and one between two doors – in the last three cases, there are major air drafts.

In all cases, the ice is produced in a closed storage area, and posteriorly stacked in boxes and carried to the stalls. In most cases, the ice is packed into the carrier boxes by a municipality employee and then put in the stalls by stalls' employees themselves, but the chain of transportation can vary (Table 4.20). To be noted that in one market, the ice is loaded into the boxes by probably untrained cleaning personnel.

Table 4.20. – Summary of ice transport methods.

Stall employees from storage area to stall	5
Municipality employee from storage to transport boxes; stall employees from boxes to stall	7
Municipality employee from storage area to stall	1
Cleaning personnel from storage to transport boxes; stall employees from boxes to stall	1

One stall noted that the transport boxes were only cleaned with water.

All water sources (both for the production of ice and for stall use) are from EPAL.

Other relevant situations:

- One stall reuses ice from the previous day whenever possible.
- One stall's bottom was plastic and presented itself quite scratched.
- One stall had installed elevated water sprinklers to maintain fish moisture.
- One employee washed fish in a water basin after gutting, without removing the used water between fish.

5. Discussion

This dissertation successfully met the objectives presented: the analysis of Lisbon markets' ice, as well as its evaluation according to current legislation for tap water.

5.1. Materials and methodology

Concerning the methodology, and given the lack of official, globally accepted methods for the analysis of ice, the tests were performed following the guidelines for potable, drinking water, bearing in mind that it is, after all, the raw material the analysed ice was made of. With the exception of *Staphylococcus* spp, *Salmonella* spp and TMC 5ªC, all the tested parameters are legislated (Decree 306/2007, August 27th) with strict parametric values, or, at least, recommended range of values, when evaluated on tap water. The analysis of *Clostridium perfringens* (legislated parameter for tap water, but not tested in the laboratory where this dissertation took place) and *Pseudomonas aeruginosa*, (legislated parameter only for bottled water) would also have been relevant, as these are both indicators for sanitary quality and contamination by poor handling (Bisson & Cabelli, 1980; Rosenberg, 2003). Of course, given the inability to analyse ice without allowing it to thaw first, it is possible that the samples suffered alteration the process of defrosting – nevertheless, thawing was conducted as smoothly as possible, avoiding major temperature shocks.

In regard to the statistical analysis, considering the relatively small sample size, the results may be limited, but the associations between parameters were shown to be significant. The biggest difficulties presented during statistical analysis were due to the fact that some samples relayed values above or below the tests' quantification limits – due to that, some results were described as 'bigger/smaller than', rather than an actual numeric value, making it impossible to use them on a statistical test. Truncation of samples' results was considered, however dismissed, as too many results would be excluded (as an example, entire parameter results, as TMC in product-contact stage); instead, values were rounded up or down as, in light of the legislation, any number higher or (in fewer cases) lower than the established parametric values is unacceptable. This, obviously, eliminates any difference between these samples (assumes, for example, that all inadequate TMC samples in product contact stage have the same microbial load), although values rounded down will have much less impact in the analysis that values rounded up.

A more appropriate solution would have been to dilute these samples until the results returned numerical values, which was impossible due to time constraints.

5.2. Analysis results

5.2.1. Production stage

Production sample results are quite homogenous and, in their majority, acceptable regarding current legislation for tap water. All tested samples were negative for *E. coli*, coliforms, *Enterococcus* spp., Coagulase Positive *Staphylococcus* spp., *Salmonella* spp. and TMC at 37ºC and 22ºC. Only one tested positive for total *Staphylococcus* spp. and five for TMC at 5º. Given that that these samples are collected immediately after production, the results should (as they are) be in line with the test results for Lisbon's municipal tap water. The presence of *Staphylococcus* spp. in one sample can be the result of contamination during analysis, but a duplicate test was impossible to perform due to lack of remaining sample. Presence of microorganisms at 5ºC can be considered normal, given that the value results were quite low (1 CFU per sample).

Tests for *Staphylococcus* spp. and Coagulase Positive *Staphylococcus* spp. were only performed on the most recent samples collected by the author for this dissertation, and therefore the previous results annexed to these do not contain results for these parameters; similarly, the two samples for *Enterococcus* spp., four for *Salmonella* spp. and three samples for TMC at 5ºC that lack results were annexed from previous results.

Concerning physical-chemical parameters, the vast majority of samples presented acceptable results, with the exception of two parameters:

- Hardness, whose results were all below the minimum for the range of recommended values. However, Lisbon's tap water generally presents low hardness (soft to medium-soft water), as measured by EPAL (n.d.) - water in the municipality shows values from 40 to 170 mg/L $CaCO_3$, averaging at 80 mg/L $CaCO_3$. There is also the addition of salts to the water immediately before production (commercial names Broxetten and Axal, among others), in order to increase ice and machine durability, which further diminishes the hardness.
- Free Residual Chlorine, with 15 samples presenting values below the minimum for the recommended range – again, EPAL (2018) regular sampling showed that there are locations where the tap water residual chlorine usually presents values below the recommended threshold.

Four samples presented high values of Chloride, while three and four, respectively, showed Conductivity and Organic Matter Content values way above the recommended threshold. All three samples with unusually high values for Conductivity had also unacceptably high results for Organic Matter Content and two for Chloride. These values can be a result from the pre-production addition of salts.

All physical-chemical parameters not tested in this stage pertain to previous analysis, similarly to the lacking microbiology results, with the exception for Organic Matter Content parameter in three cases, due to lack of analysable sample.

5.2.2. Storage stage

With few exceptions, ice samples collected from storage rooms revealed to be of acceptable quality. Only four samples presented Coliforms, two tested positive for *E. coli*, and three for *Enterococcus* spp.. Total Microbial Counts at 37ºC and 22ºC showed unrecommended numbers of microorganisms in four and six samples, respectively, and 10 samples presented one or more CFU at 5ºC. *Staphylococcus* spp. were present in three samples, being that one tested positive for Coagulase Positive *Staphylococcus* spp..

Considering that the ice immediately deposits in this location after production, one could assume that the sanitary conditions of samples would be similar to those of the production stage; however, contamination easily occurs during the loading of transport boxes – probably due to the use of dirty containers and utensils. These sources of contamination are simple to extinguish, providing that these accessories are frequently cleaned and disinfected (bleach and 70% ethanol could be easy and inexpensive, but also effective options (Al-Dabbagh et al., 2015)) and carefully stored in a clean location. It was also reported that some staff would enter the storage room without disposable shoe protection, which could be a massive contamination source – a behaviour easily corrected with specific employee training.

Several examples of poor handling were detected in several markets, with some examples presented in Figure 5.1.

Figure 5.1 – Examples of inadequate storage of accessories: **a)** bucket, **b)** transport boxes and **c)** ice shovels in direct contact with the ground.

Tests for *Staphylococcus* spp. and Coagulase Positive *Staphylococcus* spp. were only performed on the most recent samples collected by the author for this dissertation, and therefore the previous results annexed to these do not contain results for these parameters; similarly, four *Salmonella* spp. tests and three samples for TMC at 5ºC that lack results were annexed from previous results.

Most physical-chemical parameters are satisfactory, being worth of notice that almost half of all samples (14) present inadequate pH values, all above the recommended limit of 9,0.

One sample presented a high value of Chloride, and two showed Conductivity and Organic Matter Content values above the recommended threshold. Like in the former stage, both samples with high values for Conductivity had also unacceptably high results for Organic Matter Content, and one also for Chloride. Again, most likely a result from the pre-production addition of salts.

All physical-chemical parameters not tested in this stage pertain to previous analysis, similarly to the lacking microbiology results, with the exception of the Organic Matter Content parameter in four samples and Hardness in one, due to lack of analysable sample.

5.2.3. Product-contact stage

The high degree of microbial contamination was expected in this stage, not only because of microorganisms present in fish, but due to (better or worse) handling of the ice, as well as cleanliness of the stalls, transport boxes and other materials.

Total Microbial Counts revealed the presence of high numbers of microorganisms in practically all samples, to a point where all but five, one and one samples (TMC at 37ºC, 22ºC and 5ºC, respectively) went over the tests' quantification limit of 300 CFU/mL Considering, of course, that this ice was in contact with fresh fish, which, as seen in point **1.5**, can arbour several species and strains of microorganisms, these results shouldn't be alarming per se. However, the presence of coliforms (all samples), *Enterococcus* spp. (all but one sample), *Staphylococcus* spp. (all 12 tested samples) and *E. coli* (15 samples) should.

Coliforms, as *Enterococcus* spp., are indicators of overall sanitary quality, and their presence, often in high amounts (eight and five samples over the test's quantification limit of 2420 CFU/ml, respectively), indicates poor quality of the ice, pointing to contamination somewhere along the chain of transport and handling, most likely after retrieval from storage. The presence of *E. coli* further points to faecal contamination (Torturello, 2003), especially considering that most Portuguese

literature has found no trace of *E. coli* in fresh fish (Fontes et al., 2007; Pedro et al., 2010; Moita, 2016; Soares & Neves, 2017).

Simple measures could be implemented with the handlers in order to avoid contamination: among others, washing hands, thorough and frequently, and always after using the restroom; avoiding touching money, produce and unclean surfaces with hands and/or gloves; and using hand sanitizer (gel alcohol) as needed.

The presence of *Staphylococcus* spp., although not a common indicator of sanitary quality, can point to contamination by exposure, given that this genus is commonly found on humans; considering its presence on human nostrils, contamination by *Staphylococcus* spp. may increase during winter time and flu season (roughly when these samples were collected)(Bischoff et al., 2006), and its' seasonality would be interesting to understand in this context. The use of clean gloves could prevent contamination; implementation of food guards (commonly "sneeze guards") or display cases could also prove effective in diminishing *Staphylococcus* spp. contamination.

Only in this stage was there unacceptable values for Ammonia (14), Nitrates and Nitrites (one sample each); it is also the stage with the most significant number of samples with unacceptable Organic Matter Content values (20). Ammonia is a sub product of proteolytic decomposition of the fish protein tissue (Evans, 1963), and therefore expected and normal – which can also explain the increased presence of nitrates and nitrites; similarly, the organic matter content increases with the amount of organic matter present, and therefore the values are expected to be abnormal in ice that came in contact with fish and with large amounts of debris (Figure 5.4).

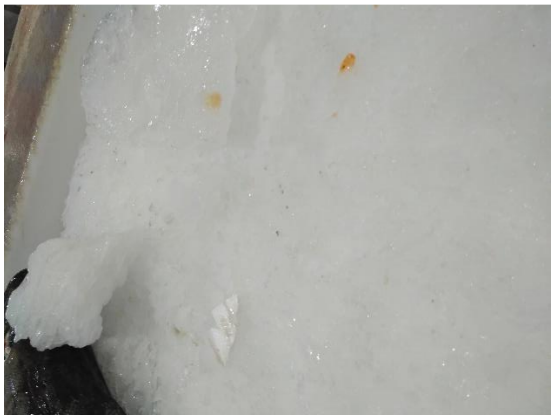


Figure 5.2 – Fish debris in ice from product-contact stage.

5.3. Questionnaires

Questionnaires can, by nature, be unreliable, as the collected information is only as truthful as the answers provided by the interviewees, giving that the answers cannot be verified; bearing that in mind, all collected information was taken with a grain of salt – and efforts were made to verify, whenever possible, inadequate situations and/or behaviours in situ, as to complement any given information. Although questionnaires were confidential – both markets and employees were given identification codes – it is known that answer biases are common (so called 'socially desirable responding'), as a way for interviewees to present themselves in a more favourable way (van de Mortel, 2008). Due to that, correlation between questionnaires and results was not studied.

Even so, questionnaires revealed overall acceptable stall cleaning measures, as well as satisfactory use of appropriate working gear (namely hair ties and aprons). Only about half of the enquired used gloves. Nevertheless, observations made during the questionnaires showed inadequate behaviours in

most stalls (more commonly, the use of gloves or bare hands while handling fish and subsequently money, dirty cloths and other materials), which would explain the high levels of microbiological contamination in product-contact stage ice. Although there is a significant discrepancy between the questionnaire answers and the results, no conclusions can be made, given the small universe of samples.

Over half (15; 55.56%) of inquired stall employees admitted having attended some type of occupational safety and health course, while 10 (37.04%) attended other specifically taught courses for fish traders and employees – it is, therefore, reasonable to assume that most are familiar with good food handling practices.

No major health situations were detected that could influence microbiological contamination, other than perhaps five cases of respiratory issues (frequent colds, frequent cough, a recent case of pneumonia and one of H1N1 influenza) and five cases of allergies – both situations can be a seasonal source of *Staphylococcus* spp. contamination due to increased coughing and sneezing (Bischoff et al., 2006). Nevertheless, employee health status should be closely monitored, especially during seasonal outbreaks.

Considering that it is important to understand potential contaminations sources, it would be useful to complement questionnaires' answers with continuous local monitoring, which would eliminate answer biases.

5.4. Correlation between stages

Correlation between stages was tested using multiple comparisons pairwise tests. Statistically significant difference was assumed to exist when $p<.05$.

With the exception of the parameter TMC at 22ºC, all microbiological parameters showed no difference between production and storage stages, but difference when comparing product-contact stage to any other of the two ($p<.001$ in all parameters) – these results point to the existence of severe focus of contamination between these two stages and product-contact stage, further stressing the thesis that poor handling and cross-contamination occurs somewhere during the usage of the ice in stalls. Only parameter TMC at 22ºC shows significant difference between all stages ($p<.016$).

Concerning physical-chemical parameters, Conductivity, Chlorine, Free Residual Chloride and Organic Matter Content showed no considerable statistical difference, and therefore pairwise tests were not conducted. Ammonia and Organic Matter Content show significant difference between product-contact stage and the remaining two – due to the fact that fish decomposition increases ammonia values and organic matter content in the ice; the same difference presents itself for the parameters Nitrates and Nitrites – pH is the only parameter that revealed significantly statistical difference between the production stage and the remaining stages, as a result of the addition of commercial salts.

5.5. Similar literature

Not much research has been found otherwise on fish market ice: a summary of results present in literature concerning this specific subject is presented below.

Table 5.1 – Summary of literature found concerning the analysis of ice from fish markets.

Reference	Vieira et al., 1997	Vieira et al., 1998	Giampietro & Rezende-Lago, 2009	Baldin, 2011	Albuquerque et al., 2018		Dissertation result
Location	Fortaleza, Brazil	Fortaleza, Brazil	São Paulo, Brazil	São Paulo, Brazil	Ceará, Brazil		Portugal
Sampling	Ice, stall	Surface, stall	Ice, stall	Ice, stall	Ice, stall	Surface, stall	Ice, stall
Coliforms (MPN/mL)	**NT**	**NT**	(100%) <0,9 - >10^3	(22,22%) 0,4 - 460	**NT**	**NT**	100%
E. coli	**NT**	**NT**	**NT**	ND	**NT**	**NT**	48.3%
***Staphylococcus* spp.**	**NT**	(6,6%) Detected	**NT**	(39,68%) Detected	(30%) Detected	(30%) Detected	100%
TMC (CFU/mL)	(100%) 10-2700 a)	**NT**	(100%) 10^4 - 10^7 a)	(61,9%) 10- $3x10^4$ b)	**NT**	**NT**	100% (45 - >300)
***Vibrio* spp.**	Detected	**NT**	**NT**	**NT**	**NT**	**NT**	**NT**
***Salmonella* spp.**	**NT**	**NT**	**NT**	ND	**NT**	**NT**	ND
Thermotolerant Coliforms (CFU/mL)	(63,4%) 4 – 1.100	**NT**	(100%) <0,9 - >10^3	(9,5%) 0,4 - 15	**NT**	**NT**	**NT**
Psychrophilic bacteria(CFU/mL)	**NT**	**NT**	(100%) 10^4 - 10^7 c)	(73%) 1 - 10^6 c)	**NT**	**NT**	100% (29 – >300)
***Listeria* spp.**	Detected	**NT**	**NT**	**NT**	**NT**	**NT**	NT
***Pseudomonas* spp.**	Detected	**NT**	**NT**	**NT**	**NT**	**NT**	NT

ND: not detected; NT – not tested. a) 35ºC; b) 37ºC; c) 7ºC.

5.6. Other general deliberations

Given that the main purpose of adding chlorine to water is the control of microorganisms, positive results for Total Microbial Count could be expected in samples with low values of Free Residual Chlorine – but in fact, all samples from production stage tested acceptably for all microbiological parameters, including the 15 that presented low amounts of chlorine. Considering that, it is reasonable to assume that low residual chlorine is not the cause of high amounts of microbial contamination; rather, that poor handling and hygiene conditions are. Implementation of a post-production chlorination procedure (similar to that employed by Moore, Brown and Hall (1953), see **1.3**) could be useful for further disinfect the ice, and avoid such proliferation of microorganisms in further stages. Still on the subject, the decrease of residual chlorine in samples from production to display is explained by the decay of chlorine by evaporation – this process is slow, as chlorine tends to evaporate slower under lower temperatures (Sheikhi et al., 2014).

The variation of pH values within and between stages can be due to the addition of different amounts and brands of pre-production salts – the increase of salt content (dissolved ions) in a solution influences the pH values (Ptizer, 1991).

Poor hygiene practices (reuse of ice, manipulation by untrained staff, use of dirty boxes and utensils) were communicated by employees and considering the increase in microbiologically unacceptable samples from production to storage to product-contact stages, these seem to be the main

source of contamination. Increased frequency of cleaning – of accessories, transport boxes and storage compartments – can help mitigate the proliferation of microbes, but the main aim should be to eliminate potential sources of contamination, which can be achieved providing further education to employees, as well as to reinforce or implement HACCP measures.

A comparison of these findings to other national researches is impossible, giving the fact that no literature on Portuguese markets has been found, besides investigations on fish quality and sanitary conditions. The presently analyzed stall samples present 100% positive results for coliforms (3 - >2420 MPN/mL), 51,7% positive for *E. coli* (1 – 57 CFU/mL), 100% positive for *Staphylococcus* (10 – 534 CFU/mL), 100% for mesophilic bacteria at 37ºC (45 - >300 CFU/mL) and 96,6% positive for psychrophilic bacteria 5ºC (29 - >300 CFU/mL) – the values are similar to the ones present in literature particularly so with those of Giampietro & Rezende-Lago (2009), with the exception for thermotolerant coliforms (that can be compared to this dissertation's tested *E. coli*) (see Table 5.1, **5.5**).

Fish markets are still major commercial locations, and reach a wide public (Ferreira, Marques & Guerra, 2015); more research would be advised, especially considering the aged population that frequents these markets, that might be particularly susceptible to foodborne infections. Although the relatively small size of the country, there are 30 companies carrying a main CAE 35302 (Portuguese Classification for Economic Activities) authorization, for the production of industrial ice – not including other companies responsible for transportation of ice and/or refrigerated foodstuffs, secondary ice production nor smaller ice producers (such as coffeeshops or bars) (Instituto Nacional de Estatística (2007), SICAE (n.d.)).

In order to further understand where most of the contaminations occur, along the handling chain, the sampling of utensil (shovels, scoops, buckets) and transport boxes' surfaces could be recommended, as well as swabs from employees' hands, gloves and in-stall utensils (knifes, hoses, cutting boards, among others).

Results were shown to be somewhat limited due to time constrains – for a broader evaluation, it is desirable to collect a higher number of samples over a longer period of time (which is being done by the laboratory, after the conclusion of this dissertation), as to detect any seasonal fluctuation in number and presence (or lack thereof) of certain microorganisms, as well as to obtain stronger average results.

Even so, and considering that these markets had never been analysed (as none other markets in Portugal have, according to found literature), collected information is satisfactory, providing clear understanding of ice quality in the municipality of Lisbon – not only for parameters legislated for tap water, but also for other sanitary indicators.

6. Conclusions

As expected, the overall sanitary quality of the ice gets increasingly worse as it moves along the handling chain; production stage samples are overall acceptable, the degree of microbiological contamination increases slightly in storage stage until all samples are deemed unacceptable in product-contact stage. All samples in the product-contact stage were deemed ‘unacceptable’, greatly due to the presence of indicators of faecal contamination.

Considering that the initial raw material is controlled tap water (of acceptable microbiological and physical-chemical quality), it is reasonable to assume that the contamination is due mainly to poor handling.

If so, behavior correction can be enough to establish a safe, clean chain of handling, with minimal contamination, providing the customer with sanitary ice and, therefore, avoiding contamination of fish. More frequent and specific courses specially directed at fish traders and cleaning staff can easily highlight the importance of sanitary control and stimulate the implementation of safe handling practices. More so, an increase in the availability of efficient cleaning products, both for stalls and handlers can help to contain the contamination. The implementation of sanitizing procedures in post-production ice and in machines and conducts can guarantee sanitary water and ice on the early stages of the production/handling chain.

Unlike some other countries, there is no overall control of ice in Portugal. This lack of centralized regulation leads to the implementation of different methods of control and precludes the employment of standardized tests and measures. Ideally, frequent analysis of ice would be desirable (for refrigeration or other purposes), particularly ice that is passible of being eaten, drank or come in contact with other foods, such as fish. Analysis should not only include the legislated parameters for drinking water, but also parameters that confirm cross contamination, such as *Pseudomonas aeruginosa*, *Staphylococcus* spp. and *Salmonella* spp..

Replication of this study in other markets in Portugal would be beneficial, since the quality of market ice in the country is completely unknown.

References

Agência Nacional de Vigilância Sanitária (1997). Portaria SVS/MS Nº 326, de 30 de Julho de 1997. *Regulamento técnico sobre as condições higiênico-sanitárias e de boas práticas de fabricação para estabelecimentos produtores/industrializadores de alimentos [Technical regulation about the sanitary conditions and good manufactoring practices for food producers/industrialiser estabelishments]*. Brazil. Retrieved from http://portal.anvisa.gov.br/documents/33916/388704/Portaria%2BSVS-MS%2BN.%2B326%2Bde%2B30%2Bde%2BJulho%2Bde%2B1997.pdf/87a1ab03-0650-4e67-9f31-59d8be3de167. Accessed in November 21st, 2018.

Agência Nacional de Vigilância Sanitária (2005). RDC Nº 274, de 22 de Setembro de 2005. *Regulamento técnico para águas envasadas e gelo [Technical regulation for bottled waters and ice]*. Brazil. Retrieved from http://portal.anvisa.gov.br/documents/33916/394219/RDC_274_2005.pdf/19d98e61-fa3b-41df-9342-67e0167bf550. Accessed in November 21st, 2018

Albuquerque, W., Vieira, R., & Vieira, G. (2018). Isolamento de *Staphylococcus aureus* do gelo, água, bancadas e vendedores de pescado da feira do Mucuripe, Fortaleza, Ceará [Isolation of *Staphylococcus aureus* from ice, water, stalls and fish vendors from Mucuripe markets, Fortaleza, Ceará]. *Revista Ciência Agronômica*, 37(3), 299-303.

Al-Dabbagh, S. Y. A., Ali, H. H., Khalil, I. I., & Hamad, M. A. (2015). A study of some antibiotics; Disinfectants and antiseptics efficacy against some species of pathogenic bacteria. *Assiut Veterinary Medical Journal*, 61(147), 210-217.

Alexandria City Hall. *Ice Well History* (2015). Retrieved from https://www.alexandriava.gov/historic/gadsbys/default.aspx?id=62354. Accessed in October 20th, 2018.

Association of Food and Drug Officials. (1988). G*uidelines for the inspection and enforcement of GMP regulations for handling and manufacturing of packaged ice*. Retrieved from http://www.foodprotect.org/issues/packets/2010Packet/attachments/III_021_e.pdf. Accessed in November 21st, 2018

Association of Food and Drugs Officials. (2010). *Guidelines for handling and producing packaged ice within foodservice and retail establishments*. Retrieved from http://www.safeice.org/uploads/1/2/9/0/12902828/afdo_packaged_ice_retail_establishments_may_12_2010.pdf. Accessed in November 21st, 2018

Austin, B. (2002). The Bacterial Microflora of Fish. *The Scientific World Journal*, 2, 558–572. doi:10.1100/tsw.2002.137

Awuor, L., Thompson, S., Thompson, B., Liberda, E.N., & Meldrum, R (2016). Microbiological quality and handling practices of ice served in selected downtown Toronto food premises. *Environmental Health Review*, 59(3), 83–87. doi.org/10.5864/d2016-017

Ayulo, A. M. R., Machado, R. A., & Scussel, V. M. (1994). Enterotoxigenic *Escherichia coli* and *Staphylococcus aureus* in fish and seafood from the southern region of Brazil. *International Journal of Food Microbiology*, 24(1), 171–178. doi:10.1016/0168-1605(94)90116-3

Baldin, J. (2011). *Avaliação da qualidade microbiológica do gelo utilizado na conservação de pescado* [Evaluation of the microbiological quality of ice used in the conservation of fish] (Master's

thesis). Retrieved from Repositório Institucional UNESP: http://hdl.handle.net/11449/94606. Accessed in October 3rd, 2018.

Beller, M. (1992). Hepatitis A outbreak in Anchorage, Alaska, traced to ice slush beverages. *The Western Journal Of Medicine,* 156(6), 624-627.

Bischoff, W. E., Wallis, M. L., Tucker, B. K., Reboussin, B. A., Pfaller, M. A., Hayden, F. G., & Sherertz, R. J. (2006). "Gesundheit!" Sneezing, common colds, allergies, and *Staphylococcus aureus* dispersion. *The Journal of Infectious Diseases*, 194(8), 1119–1126. doi:10.1086/507908

Bisson, J. W., & Cabelli, V., J. (1980). *Clostridium perfringens* as a water pollution indicator. *Journal of Water Pollution Control Federation*, 52(2), 241-248.

Braam, P. (2005a). *Diarrhea caused by Escherichia Coli* in American Public Health Association (Ed.) Control of Communicable Diseases Manual - An Official Report of the American Public Health Association (18th edition, pp 176-187) [pdf]. Retrieved from http://navybmr.com/study%20material/CCDM.pdf. Accessed in December 23rd, 2018.

Braam, P. (2005b). *Salmonellosis* in American Public Health Association (Ed.) Control of Communicable Diseases Manual - An Official Report of the American Public Health Association (18th edition, pp 508-512) [pdf]. Retrieved from http://navybmr.com/study%20material/CCDM.pdf. Accessed in December 23rd, 2018.

Burnett, I. A., Weeks, G. R., & Harris, D. M. (1994) A hospital study of ice-making machines: their bacteriology, design, usage and upkeep. *Journal of Hospital Infection*, 28(1) 305-313.

Cannon, R. O., Poliner, J. R., Hirschhorn, R. B., Rodeheaver, D. C., Silverman, P. R., Brown, E. A., Talbot, G. H., Stine, S. E., Monroe, S. S., Dennis, T. D., & Glass, R. I. (1991). A multistate outbreak of Norwalk virus gastroenteritis associated with consumption of commercial ice. *Journal of Infectious Diseases*, 164(5), 860–863. doi:10.1093/infdis/164.5.860

Carvalho, L., & Oliveira, E. (2018). Avaliação físico-química, microbiológica e macroscópica do gelo comercializado no município de Teresina-PI [Physical-chemical, microbiological and macroscopic evaluation of ice commercialized in Teresina-PI municipality]. *Cadernos Cajuína*, 3(1), 183–194.

Center for Disease Control and Prevention (2018a). Typhoid fever and paratyphoid. Retrieved from https://www.cdc.gov/typhoid-fever/index.html. Accessed in October 25th, 2018.

Center for Disease Control and Prevention (2018b). Salmonellosis. Retrieved from https://www.cdc.gov/salmonella/general/index.html. Accessed in October 25th, 2018.

Center for Disease Control and Prevention (2018c). Shiga toxin-producing E. coli (STEC). Retrieved from https://www.cdc.gov/ecoli/general/index.html. Accessed in October 25th, 2018.

Chaignat, C. (2005). *Typhoid fever* in American Public Health Association (Ed.) Control of Communicable Diseases Manual - An Official Report of the American Public Health Association (18th edition, pp 624-629) [pdf]. Retrieved from http://navybmr.com/study%20material/CCDM.pdf. Accessed in November 13th, 2018.

Chapin, R. C. (1909). *Standard of living among workingmen's families in New York City*. New York, NY: Russell Sage Foundation. Retrieved from: https://archive.org/stream/standardlivinga02chapgoog#page/n6. Accessed in November 30th, 2018.

Chavasit, V., Sirilaksanamanon, K., Phithaksantayothin, P., Norapoompipat, Y., Parinyasiri, T. (2011). Measures for controlling safety of crushed ice and tube ice in developing country. *Food Control*, 22(1), 118-123. doi:10.1016/j.foodcont.2010.05.016

Cheng, H., Hung, M., Chen, W., Lo, Y., Su, Y., Wei, H., Chen, M., Tuan, Y., Lin, H., Lin, H., Liu, T., Wang, Y., & Wu, F. (2017). Ice-associated norovirus outbreak predominantly caused by GII.17 in Taiwan, 2015. *BMC Public Health*, 17(1). doi: 10.1186/s12889-017-4869-4

David, E. (1994). *Harvest of the Cold Months: The Social History of Ice and Ices* [e-book]. London: Faber and Faber. Available at https://books.google.pt/books?id=1elpxy2oCW4C&printsec=frontcover&hl=pt-PT. Accessed in November 17th, 2018.

Dewey-Mattia, D., Manikonda, K., Hall, A.J., Wise, M.E., & Crowe, S. J. (2018). *Surveillance for foodborne disease outbreaks—United States, 2009–2015*. MMWR Surveillance Summaries. 67(10), 1–11. Retrieved from Center for Disease Control and Prevention: https://www.cdc.gov/mmwr/volumes/67/ss/ss6710a1.htm. Accessed in October 13th, 2018.

Dickens, D. L., DuPont, H. L., & Johnson, P. C. (1985). Survival of bacterial enteropathogens in the ice of popular drinks. *Journal of the American Medical Associatio*n, 253(21), 3141-3143. doi: 10.1001/jama.253.21.3141

Durgesh P. M., Ranjana G. K., & Varsha K. V. (2008). Microbiological analysis of street vended fruit juices from Mumbai City, India. *Internet Journal of Food Safety*, 10(1), 31-34.

Eaton, A. D., Clesceri, L. S., Greenberg, A. E., Franson, M. A. H., American Public Health Association., American Water Works Association., & Water Environment Federation. (1998). *Standard methods for the examination of water and wastewater*. Washington, DC: American Public Health Association.

Economou, V., Gousia, P., Kemenetzi, D., Sakkas, H., & Papadopoulou, C. (2016). Microbial quality and histamine producing microflora analysis of the ice used for fish preservation. *Journal of Food Safety*, 37(1). doi:10.1111/jfs.12285

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The gut microbiota of marine fish. *Frontiers in Microbiology*, 9. doi:10.3389/fmicb.2018.00873

Empresa Portuguesa das Águas Livres (n.d). *Ficha informativa – Dureza [Information sheet - Hardness]* [PDF file]. Retrieved from https://www.epal.pt/EPAL/docs/default-source/agua/ficha-dureza.pdf?sfvrsn=2. Accessed in October 20th, 2018.

Empresa Portuguesa das Águas Livres. (2015). *Certificado de Acreditação [Accreditation Certificate]* [PDF file]. Retrieved from https://www.epal.pt/EPAL/menu/%C3%A1gua/controlo-da-qualidade-da-%C3%A1gua/acredita%C3%A7%C3%A3o. Accessed in October 20th, 2018.

Empresa Portuguesa das Águas Livres. (2018). Torneiras na cidade de Lisboa [Water taps in the city of Lisbon]. Retrieved from https://www.epal.pt/EPAL/menu/%C3%A1gua/divulga%C3%A7%C3%A3o-de-dados-da-qualidade-da-%C3%A1gua/controlo-legal/torneiras-na-cidade-de-lisboa. Accessed in October 20th, 2018.

Estados Unidos Mexicanos. Secretaría de Comercio y Fomento Industrial (1994). Norma Oficial Mexicana NOM-006-SCFI-1994 [Official Mexican Norm]. Retrieved from

http://www.colpos.mx/bancodenormas/noficiales/NOM-006-SCFI-1994.PDF. Accessed in December 2nd, 2018.

European Food Safety Authority and European Centre for Disease Prevention and Control (2016). *The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015*. EFSA Journal, 14(12). doi:10.2903/j.efsa.2016.4634

European Parliament and Council (2004). *Regulation (EC) N.º 852/2004 of the European Parliament and Council of April 29th 2004*. Official Journal of the European Union. 139(1), 1-54. Accessed in October 19th, 2018.

Evans, W. E. (1963). *The Chemistry of Death*. Springfield: Thomas.

Falcão, J. P., Falcão, D. P., & Gomes, T. A. T. (2004). Ice as a vehicle for diarrheagenic *Escherichia coli*. *International Journal of Food Microbiology*, 91(1), 99–103. doi:10.1016/s0168-1605(03)00327-1

Feng, P. (2012) *Escherichia coli* (ETEC, EPEC, EHEC, EIEC) in Lampel, K. A., Al.Khaldi, S., & Cahill, S. M. (Ed.) Bad Bug Book - Foodborne Pathogenic Microorganisms and Natural Toxins (2nd edition). Retrieved from Food and Drug Administration https://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf. Accessed in December 23rd, 2018.

Ferreira, C., Marques, T., & Guerra, P. (2015). Fairs and markets in Oporto: old and new formats of economic activity and urban animation. *GOT, Revista de Geografia e Ordenamento do Território*, 8. doi:10.17127/got/2015.8.005.

Fisher, K., & Phillips, C. (2009). The ecology, epidemiology and virulence of Enterococcus. *Microbiology*, 155(6), 1749–1757. doi:10.1099/mic.0.026385-0

Fontes, M. C., Esteves, A., Caldeira, F., Saraiva, C., Vieira-Pinto, M., & Martins, C. (2007). Estado de frescor e qualidade higiênica do pescado vendido numa cidade do interior de Portugal [State of freshness and hygiene quality of fish sold in a city from the interior of Portugal]. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 59(5), 1308–1315. doi:10.1590/s0102-09352007000500031

Food and Environmental Hygiene Department of Hong Kong. (2005). *The microbiological quality of edible ice from ice manufacturing plants and retail businesses in Hong Kong*. Risk Assessment Studies Report No. 22. Retrieved from https://www.cfs.gov.hk/english/programme/programme_rafs/files/edible_ice_ra.pdf. Accessed in November 21st, 2018.

Food Safety Authority of Ireland. (2002). *1st Quarter National Microbiological Survey: Microbiological quality of ice for cooling drinks* (Volume 1, Number 28). Available at https://www.fsai.ie/uploadedFiles/Monitoring_and_Enforcement/Monitoring/Surveillance/ice_cooling_drinks(1).pdf. Accessed in December 17th, 2018.

Fraser, O. P., & Sumar, S. (1998). Compositional changes and spoilage in fish (part II) - Microbiological induced deterioration. *Nutrition & Food Science*, 98(6), 325–329. doi:10.1108/00346659810235242

Freire, A. J., Assunção, G. M., Araújo J. C., & Barin, C. S. (2008). Análise físicoquímica e microbiológica de gelo comercializado em postos de combustível [Physical-chemical analysis of ice commercialized in gas stations]. Presented at *II Congresso Nacional de Extensão Universitári*a, Londrina. UNOPAR

Furtado, T. (1997). Sistema de informação para o património arquitectónico - Real Fábrica de Gelo de Montejunto [Information system for architectural heritage – Royal Ice Factory of Montejunto]. Retrieved from http://www.monumentos.gov.pt/Site/APP_PagesUser/SIPA.aspx?id=6279. Accessed in November 5th, 2018.

Gaglio, R., Francesca, N., Di Gerlando, R., Mahony, J., De Martino, S., Stucchi, C., Moschetti, G., & Settanni, L. (2017). Enteric bacteria of food ice and their survival in alcoholic beverages and soft drinks. *Food Microbiology*, 67(1), 17–22. doi:10.1016/j.fm.2017.04.020

Gebo, K. A., Srinivasan, A., Perl, T. M., Ross, T., Groth, A., & Merz, W. G. (2002). Pseudo-outbreak of *Mycobacterium fortuitum* on a Human Immunodeficiency Virus ward: transient respiratory tract colonization from a contaminated ice machine. *Clinical Infectious Diseases*, 35(1), 32–38. doi:10.1086/340741

Geldreich, E. E., & Clarke, N. A. (1966). Bacterial pollution indicators in the intestinal tract of freshwater fish. *Applied Microbiology*, 14(3), 429–437.

Gerokomou, V., Voidarou, C., Vatopoulos, A., Velonakis, E., Rozos, G., Alexopoulos, A., Plessas, S., Stavropoulou, E., Bezirtzoglou, E., Demertzis, P.G., & Akrida-Demertzi, K. (2011). Physical, chemical and microbiological quality of ice used to cool drinks and foods in Greece and its public health implications. *Anaerobe*, 17(6), 351–353. doi:10.1016/j.anaerobe.2011.06.005

Giampietro, A. & Rezende-Lago, N. C. M. (2009) Qualidade do gelo utilizado na conservação de pescado fresco [Quality of ice used in the conservation of fresh fish]. *Arquivo do Instituto de Biologia*, 76(3), 505-508.

Godfrey, E., & Pond, M. (1940). A hospital epidemic of Flexner dysentery caused by contaminated ice. *Journal of The American Medical Association*, 114(13), 1151-1154. doi: 10.1001/jama.1940.02810130013005

Gomes, K., Gomes, F., Ponte, A., & Silva, A. (2012). Avaliação microbiológica e físico-química do gelo comercializado em lojas de conveniência de postos de combustíveis da cidade de Sobral – Ceará [Microbiological and physical-chemical evaluation of ice commercialized in convenience stores from gas stations in the city of Sobral – Ceará]. Presented at VII CONNEPI - Congresso Norte Nordeste de Pesquisa e Inovação. Tocantins. Retrieved from http://propi.ifto.edu.br/ocs/index.php/connepi/vii/paper/view/1355. Accessed in October 18th, 2018.

Grimes, D.J, Jacobs, D., Swartz, D.G., Brayton, P.R., & Colwell, R.R. (1993) Numerical taxonomy of Gram-negative, oxidase-positive rods from carcharhinid sharks. *International Journal of Systemic Bacteriology*, 43(1), 88-98.

Hait, J. (2012) *Staphylococcus aureus* in Lampel, K. A., Al.Khaldi, S., & Cahill, S. M. (Ed.) Bad Bug Book - Foodborne Pathogenic Microorganisms and Natural Toxins (2nd edition). Retrieved from Food and Drug Administration https://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf . Accessed in November 30th, 2018.

Hamedy, A., Khosravi, A., & Omidy, A. (2004). Contamination of ice and ice water by *Vibrio Cholera*, in different regions of Mashad, Iran. *The Internet Journal of Infectious Diseases*, 3(2).

Hammack, T. (2012) *Salmonella* species in Lampel, K. A., Al.Khaldi, S., & Cahill, S. M. (Ed.) Bad Bug Book - Foodborne Pathogenic Microorganisms and Natural Toxins (2nd edition). Retrieved from Food and Drug Administration

https://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf. Accessed in November 30th, 2018.

Hampikyan, H., Bingol, E.B., Cetin, O., & Colak, H. (2017) Microbiological quality of ice and ice machines used in food establishments. *Journal of Water and Health,* 15(3), 410–417. doi.org/10.2166/wh.2017.159.

Hosseini, B., & Namazian, A. (2012) An overview of Iranian ice repositories, an example of traditional indigenous architecture. *Journal of the Faculty of Architecture METU,* 29(2), 223–234. doi:10.4305/metu.jfa.2012.2.10.

Instituto Nacional de Estatística. (2007). *Classificação Portuguesa das Actividades Económicas – Revisão 3 [Portuguese Classification of Economic Activities – Revision 3]*. Retrieved from https://www.ine.pt/ine_novidades/semin/cae/CAE_REV_3.pdf. Accessed in January 4th, 2019.

Instituto Português da Qualidade. (1966). *Água – Determinação de cloretos [Water – Chloride determination]* (NP423:1966). Retrieved from http://www1.ipq.pt/PT/site/clientes/pages/Norma.aspx?docId=IPQDOC-185-149307. Accessed in November 10th, 2018.

Instituto Português da Qualidade. (1966a). *Água – Determinação das durezas[Water – Hardness determination]* (NP424:1966). Retrieved from http://www1.ipq.pt/PT/site/clientes/pages/Norma.aspx?docId=IPQDOC-185-77973. Accessed in November 10th, 2018.

Instituto Português da Qualidade. (1969). *Água - Determinação da condutividade eléctrica [Water – Electrical conductivity determination]* (NP732:1969). Retrieved from http://www1.ipq.pt/PT/site/clientes/pages/Norma.aspx?docId=IPQDOC-185-77985. Accessed in November 10th, 2018.

Instituto Português da Qualidade. (1998). *Qualidade da água – Técnicas de avaliação e controlo dos meios de cultura utilizados na contagem de colónias de controlo da qualidade da água [Water quality – Evaluation and medium control techniques used in the count of colonies for water quality control]* (NP4354:1998). Retrieved from http://www1.ipq.pt/PT/site/clientes/pages/Norma.aspx?docId=IPQDOC-185-149766. Accessed in November 10th, 2018.

Instituto Português da Qualidade. (1998a). *Qualidade da água – Pesquisa e quantificação de Estafilococos [Water quality – Analysis and quantification of* Staphylococcus*]* (NP4343:1998). Retrieved from http://www1.ipq.pt/PT/site/clientes/pages/Norma.aspx?docId=IPQDOC-185-80282. Accessed in November 10th, 2018.

International Organization for Standardization. (1993). *Water quality -- Determination of permanganate index* (ISO 8467:1993(E)). Retrieved from https://www.iso.org/standard/15669.html. Accessed in November 10th, 2018.

International Organization for Standardization. (1999). *Microbiology of food an animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 1: General rules for the preparation of the initial suspension and decimal dilutions* (ISO 6887-1:1999(E)). Retrieved from https://www.iso.org/standard/26850.html. Accessed in November 10th, 2018.

International Organization for Standardization. (1999a). *Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium* (ISO 6222:1999(E)). Retrieved from https://www.iso.org/standard/28960.html. Accessed in November 10th, 2018.

International Organization for Standardization. (2003). *Water quality – Sampling – Part 3: Guidance on the preservation and handling of water samples* (ISO 5667-3:2012(E)). Retrieved from https://www.iso.org/standard/53569.html. Accessed in November 10th, 2018.

International Organization for Standardization. (2006). *Water quality – Sampling for microbiological analysis* (ISO 19458:2006(E)). Retrieved from https://www.iso.org/standard/33845.html. Accessed in November 10th, 2018.

International Organization for Standardization. (2007). *Microbiology of food an animal feeding stuffs – General requirements and guidance for microbiological examinations* (ISO 7218:2007(E)). Retrieved from https://www.iso.org/standard/36534.html. Accessed in November 10th, 2018.

International Organization for Standardization. (2009). *Microbiology of food an animal feeding stuffs – Guidelines on preparation and production of culture media – Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory.* (ISO/TS 11133:2014(E)). Retrieved from https://www.iso.org/standard/53610.html. Accessed in November 10th, 2018.

International Organization for Standardization. (2010). *Water quality – Detection of Salmonella spp.* (ISO 19250:2010(E)). Retrieved from https://www.iso.org/standard/43448.html. Accessed in November 10th, 2018.

International Organization for Standardization. (2012). *Water quality – Enumeration of Escherichia coli and coliform bacteria – Part 2: Most probable number method* (ISO 9308-2:2012(E)). Retrieved from https://www.iso.org/standard/52246.html. Accessed in November 10th, 2018.

International Packaged Ice Association. (2017). IPIA Today. Retrieved from http://packagedice.com/ipia-today/. Accessed in October 2nd, 2018.

International Packaged Ice Association. (2018) Ice Manufacturers Worldwide. Retrieved from http://packagedice.com/ipia-ice-manufacturer-locations-worldwide/. Accessed in October 2nd, 2018.

Istituto Nazionale Ghiaccio Alimentare. (2015). *Manuale di corretta prassi operativa per la produzione di ghiaccio alimentare*. Retrieved from http://www.ghiaccioalimentare.it/download/manuale.pdf. Accessed in October 2nd, 2018.

Khan, A. S., Moe, C. L., Glass, R. I., Monroe, S. S., Estes, M. K., Chapman, L. E., Jiang, X., Humphrey, C., Pon, E., & Iskander J. (1994). Norwalk virus-associated gastroenteritis traced to ice consumption aboard a cruise ship in Hawaii: comparison and application of molecular method-based assays. *Journal of Clinical Microbiology*, 32(2), 318-322.

Laussucq, S., Baltch, A., Smith, R., Smithwick, R., Davis, B., Desjardin, E. K., Silcox, V. A., Spellacy, A. B., Zeimis, R. T., Gruft, H. M., Good, R. C., & Cohen, M. L. (1988). Nosocomial *Mycobacterium fortuitum* colonization from a contaminated ice machine. *American Review of Respiratory Disease*, 138(4), 891-894. doi: 10.1164/ajrccm/138.4.891

Lee, K.H., Ab Samad, L.S., Lwin, P.M., Riedel, S.F., Magin, A., Bashir, Vaishampayan, P. A., & Lin, W.J. (2017) On the rocks: microbiological quality and microbial diversity of packaged ice in Southern California. *Journal of Food Protection*, 80(6), 1041–1049. doi:10.4315/ 0362-028X.JFP-16-295

Lee, P. S. (2008). Quantitation of Microorganisms. in Goldman, E., & Green, L. H. (Ed.) *Practical Handbook of Microbiology* (2nd edition, pp 11-22) [e-book]. Boca Raton, FL: CRC Press. Retrieved from https://books.google.pt/books?id=qVyPr57Q2lUC&printsec=frontcover&hl=pt-PT&source=gbs_ge_summary_r&cad=0#v=onepage&q&f=false. Accessed in December 22nd, 2018.

Mako, S. L., Harrison, M. A., Sharma, V., & Kong, F. (2014). Microbiological quality of packaged ice from various sources in Georgia. *Journal of Food Protection*, 77(9), 1546-1553. doi:10.4315/0362-028x.jfp-13-398

Maruyama, H. (Producer), & Yasuhiro, I. (Director). (2009). *Hagane no Renkinjutsushi: Furumetaru Arukemisuto [Fullmetal Alchemist: Brotherhood]* [Television series]. Japan: Bones Studio.

Mendes, A. L. S. (2009). *Qualidade microbiológica do gelo para consumo em bebidas - Um estudo nos estabelecimentos das zonas balneares do Porto [Microbiological quality of ice used for beverages – A study in beach side estabelishments in Porto]* (Master's thesis). Retrieved from Repositório Aberto UPorto: https://repositorio-aberto.up.pt/handle/10216/22456. Accessed in October10th, 2018.

Moita, P. A. P. (2016). *Estudo da estabilidade química, nutricional e microbiológica durante o processo tradicional de preparação do bacalhau salgado seco para congelação* [Study of the chemical, nutritional and microbiological stability of dried, salted codfish during its traditional preparation process for freezing] (Master's thesis). Retrieved from Repositório IPSantarém: http://repositorio.ipsantarem.pt/bitstream/10400.15/1728/1/MoitaPat__teseMestradoESAS_2016.pdf. Accessed in December 6th, 2018.

Moore E.W., Brown E.W., & Hall E.M. (1953). Sanitation of crushed ice for iced drinks. *American Journal of Public Health*. 43(10), 1265-1269. doi: 10.2105/ajph.43.10.1265

Moyer, M. P., Breuer, G. M., Kempf, J. L., Friell, L. A., Roland, G. W., & Hausler, W. J. (1993). Quality of packed ice purchases at retail establishments in Iowa. *Journal of Food Protection*, 56(5), 426–431.

Neto, A. A. R., Vasconcelos, U., Almeida, F. R., & Calazans, G. M. T. (2009). Avaliação bacteriológica do gelo em pacote comercializado em Recife, PE [Bacteriological evaluation of packaged ice commercialized in Recife, PE]. *Revista de Higiene Alimentar*, 23(168-169), 172-175.

Nichols, G., Gillespie, I., & De Louvois, J. (1999). The microbiological quality of ice used to cool drinks and ready-to-eat food from retail and catering premises in the United Kingdom. *Journal of Food Protection*, 63(1), 78-82. doi: 10.4315/0362-028x-63.1.78

Noor Izani, N.J., Zulaikha, A.R., Mohamad Noor, M.R., Amri, M.A., & Mahat, N.A. (2012). Contamination of faecal coliforms in ice cubes sampled from food outlets in Kubang Kerian, Kelantan. *Tropical Biomedicine*. 29(1),71-76

Northcutt, J. K., & Smith, D. (2009). Microbiological and chemical analyses of ice collected from a commercial poultry processing establishment. *Poultry Science*, 89(1), 145–149. doi:10.3382/ps.2009-00205

Novotny L., Dvorshka, L., Lorencova, A., Beran, V., & Pavlik, I. (2004). Fish: a potential source of bacterial pathogens for human beings. *Veterinary Medicine*. 49(9),343-358

Pal, D., & Gupta, C. D. (1992). Microbial pollution in water and its effect on fish. *Journal of Aquatic Animal Health*, 4(1), 32–39. doi:10.1577/1548-8667(1992)004<0032:mpiwai>2.3.co;2

Pedro, S., Costa, M.P., Silva, H.A., & Nunes, M.L (2010). Qualidade microbiológica de pescado pronto a consumir comercializado em Portugal [Microbiological quality of ready-to-eat fish commercialized in Portugal] [Abstract]. Presented at 3rd Annual PORTFIR Meeting. Retrieved from http://www2.insa.pt/sites/INSA/Portugues/ComInf/Noticias/Documents/2011/Dezembro/P15.pdf. Accessed in December 7th, 2018.

Pitzer, K. S. (1991). *Activity coefficients in electrolyte solutions* (2nd ed.). Boca Raton: CRC Press.

Quick, R., Paugh, K., Addiss, D., Kobayashi, J., & Baron, R. (1992). Restaurant-associated outbreak of Giardiasis. *Journal of Infectious Diseases*, 166(3), 673–676. doi:10.1093/infdis/166.3.673

Rosenberg, F. A. (2003). The microbiology of bottled water. *Clinical Microbiology Newsletter*, 25(6), 41–44. doi:10.1016/s0196-4399(03)80019-3

Schmidt, R. H., & Rodrick, G. E. (1999). Microbial, physical, and chemical quality of packaged ice in Florida. *Journal of Food Protection*, 62(5), 526–531. doi:10.4315/0362-028x-62.5.526

Scotch Whisky Association. (2009). *The Scotch Whisky Regulations*. Retrieved from http://www.scotch-whisky.org.uk/. Accessed in October 14th, 2018.

Settanni, L., Gaglio, R., Stucchi, C., De Martino, S., Francesca, N., & Moschetti, G. (2017). Presence of pathogenic bacteria in ice cubes and evaluation of their survival in different systems. *Annals of Microbiology*, 67(12), 827–835. doi:10.1007/s13213-017-1311-1

Shamsuddeen, U., Bukar, A., Usman, A., Kabir, M., & Abdulmalik, S. (2010). Bacteriological quality of water used for ice making in some parts of Kano metropolis, Nigeria. *Bayero Journal of Pure and Applied Sciences*, 3(1). doi:10.4314/bajopas.v3i1.58785

Shawyer, M., & Pizzali, A. F. M. (2003). *The use of ice on small fishing vessels* (FAO Fisheries Technical Paper 436). Retrieved from Food and Agriculture Organization of the United Nations, http://www.fao.org/docrep/006/Y5013E/y5013e00.htm#Contents. Accessed in November 15th, 2018.

Sheikhi, R., Alimohammadi, M., Askari, M., & Moghaddasian, M. (2014). Decay of free residual chlorine in drinking water at the point of use. *Iranian Journal of Public Health*, 43(4), 535-536.

SICAE. (n.d.). *Consultas de CAE [CAE consultation]*. Retrieved from http://www.sicae.pt/Consulta.aspx. Accessed in January 23rd, 2019. Accessed in January 16th, 2019.

Soares, M., & Neves, A. (2017). Microbiological evaluation of dried salted codfish shredded cooled in retail trade. *Revista da Unidade de Investigação do Instituto Politécnico de Santarém*, 5(2), 53-55. ISBN: 2182-9608

Toranzo, A.A, Novoa, B., Romalde, J.L., Nunez, S., Devesa, S., Marino, E., Silva R., Martinez, E., Figueras, A., & Barja, J.L. (1993) Microflora associated with healthy and diseased turbot (*Scophthalmus maximu*s) from 3 farms in Northwest Spain. *Aquaculture*, 114(1), 189-202.

Toyofuku, H. (2005). *Foodborne Intoxications* in American Public Health Association (Ed.) Control of Communicable Diseases Manual - An Official Report of the American Public Health Association (18th edition, pp 233-235) [pdf]. Retrieved from http://navybmr.com/study%20material/CCDM.pdf. Accessed in November 19th, 2018

U.S. Food & Drug Administration (2017). *FDA Regulates the Safety of Packaged Ice*. Retrieved from https://www.fda.gov/food/resourcesforyou/consumers/ucm197586.htm. Accessed in October 14th, 2018.

Ukwo, S.P., Ndaeyo, N.U., & Udoh, E.J. (2011). Microbiological quality and safety evaluation of fresh juices and edible ice sold in Uyo metropolis, South-South, Nigeria. *Internet Journal of Food Safety*, 13(1), 374-378.

van de Mortel, T. F. (2008) Faking it: social desirability response bias in self-report research. *The Australian Journal of Advanced Nursing*, 25(4), 40-48.

Vieira, R. H. F., Souza, O. V., & Patel, T. R. (1997) Bacteriological quality of ice used in Mucuripe Market. Fortaleza, Brazil. *Food Control*, 8(2) , 83-85. doi:10.1016/s0956-7135(96)00076-x

Vieira, R. H. S. F., Tavares, L. A., Gambar, R. C., & Pereira, M. L. (1998). *S. aureus* em camarão fresco e superfícies de bancada da feira livre de pescado do Mucuripe, Fortaleza, Ce. Registro de pontos críticos e medidas de controle [*S. aureus* in fresh shrimp and stall surfaces in the fish market of Mucuripe, Fortaleza, Ce. Record of critical points and control measures]. *Revista Higiene Alimentar*.12(55), 47-50.

Wilson, I. G., Hogg, G. M., & Barr, J. G. (1997). Microbiological quality of ice in hospital and community. *Journal of Hospital Infection*, 36(3), 171–180. doi:10.1016/s0195-6701(97)90192-4

World Health Organization. (2011). *Guidelines for drinking-water quality, 4th edition*. Malta: World Health Organization. Retrieved from http://www.who.int/water_sanitation_health/publications/2011/dwq_guidelines/en/. Accessed in October 20th, 2018.

Zhang, L. J., Wang, X. J., Bai, J. M., Fang, G., Liu, L. G., Zhang, Y., & Fontaine, R. E. (2009). An outbreak of hepatitis A in recently vaccinated students from ice snacks made from contaminated well water. *Epidemiology and Infection*, 137(03), 428. doi:10.1017/s0950268808001337

Zhang, G. (2012) *Escherichia coli* (ETEC, EPEC, EHEC, EIEC) in Lampel, K. A., Al.Khaldi, S., & Cahill, S. M. (Ed.) Bad Bug Book - Foodborne Pathogenic Microorganisms and Natural Toxins (2nd edition). Retrieved from Food and Drug Administration https://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf. Accessed in November 23rd, 2018.

Annexes

Annex 1a – Microbiology parameters: production stage.

				Staphylococcus		**Total microbial count**			
Sample	**Coliforms**	***E. coli***	***Enterococcus***	**Total**	**Coagulase +**	**37ºC**	**22ºC**	**5ºC**	***Salmonella***
104	0	0	0	**NP**	**NP**	0	1	**NP**	ND
128	0	0	0	**NP**	**NP**	0	0	**NP**	ND
193	0	0	0	**NP**	**NP**	1	0	**NP**	ND
240	0	0	0	**NP**	**NP**	1	0	0	ND
273	0	0	0	**NP**	**NP**	2	20	0	ND
289	0	0	0	**NP**	**NP**	0	0	0	ND
419	0	0	**NP**	**NP**	**NP**	0	0	0	ND
423	0	0	**NP**	**NP**	**NP**	0	0	0	ND
447	0	0	0	**NP**	**NP**	0	0	0	ND
466	0	0	0	**NP**	**NP**	2	19	0	ND
520	0	0	0	**NP**	**NP**	0	1	0	ND
542	0	0	0	**NP**	**NP**	1	0	0	ND
633	0	0	0	**NP**	**NP**	0	0	0	ND
673	0	0	0	**NP**	**NP**	0	0	0	ND
684	0	0	0	**NP**	**NP**	0	0	0	ND
687	0	0	0	**NP**	**NP**	1	1	0	ND
827	0	0	0	0	0	0	0	1	ND
830	0	0	0	0	0	0	0	1	ND
842	0	0	0	0	0	0	4	0	ND
865	0	0	0	0	0	0	0	1	ND
871	0	0	0	0	0	0	0	0	ND
874	0	0	0	0	0	0	0	0	ND
900	0	0	0	0	0	0	0	1	ND
903	0	0	0	0	0	1	0	0	ND
923	0	0	0	0	0	0	0	0	ND
926	0	0	0	0	0	0	0	0	ND
944	0	0	0	0	0	2	0	1	ND
946	0	0	0	0	0	0	0	0	ND
949	0	0	0	3	0	0	1	0	ND

NP – not performed; ND – not detected.

Green cells indicate that results are within acceptable range for parametric or recommended values. Yellow cells indicate detection of microorganisms in parameters with no legal requirement for water analysis.

Annex 1b – Physical-chemical parameters: production stage.

Sample	Ammonia	Conductivity	Chlorides	FRC	Nitrate	Nitrite	pH	OMC	Hardness
104	0,10	462	**NP**	**NP**	0,68	<0,02	8,3	0,86	**NP**
128	0,17	261	65	**NP**	1,16	<0,02	8,2	1,77	36
193	<0.06	474	132	**NP**	1,57	<0,02	7,7	0,98	42
240	0,09	496	NP	**NP**	1,63	<0,02	7,7	0,96	44
273	0,10	438	99	**NP**	1,59	<0,02	8,8	2,22	52
289	0,09	541	124	**NP**	1,25	<0,02	7,9	1,58	52
419	0,09	473	91	0,30	4,45	<0,02	8	0,89	68
423	0,11	451	123	0,30	5,94	<0,02	8,2	0,85	40
447	<0.06	424	112	0,40	5,48	<0,02	8,1	0,63	32
466	0,07	10900	203	**NP**	2,23	<0,02	8,1	10,24	50
520	0,10	845	127	0,20	1,64	<0,02	8	1,63	32
542	0,07	519	119	0,04	1,74	<0,02	7,7	1,84	32
633	<0,06	600	181	0,10	1,68	<0,02	7,8	1,30	32
673	0,07	697	210	0,10	1,89	<0,02	7,8	2,03	34
684	0,09	873	175	0,09	2,13	<0,02	7,9	1,66	32
687	0,08	424	71	0,10	1,79	<0,02	8,1	0,97	22
827	<0,06	1124	334	0,30	1,95	<0,02	7,9	1,52	28
830	<0,06	315	71	0,30	2,21	<0,02	8,1	1,30	32
842	<0,06	430	75	0,04	1,53	<0,02	7,9	2,40	60
865	0,20	372	82	0,09	2,12	<0,02	8,4	**Damaged**	44
871	0,19	1561	522	0,05	2,17	<0,02	7,5	**Damaged**	80
874	0,21	546	147	0,10	1,73	<0,02	8,2	**Damaged**	62
900	0,06	617	175	0,10	1,77	<0,02	7,5	4,44	60
903	0,07	2556	875	0,07	1,36	<0,02	7,7	6,30	60
923	0,09	419	86	0,10	1,76	<0,02	7,8	1,44	80
926	0,10	152	111	0,10	0,96	<0,02	7,9	36,00	58
944	0,10	5240	2237	0,20	1,99	<0,02	8	6,37	71
946	0,09	741	199	0,06	2,04	<0,02	7,9	2,04	63
949	0,10	510	136	0,10	2,08	<0,02	8,1	2,63	62

FRC: Free Residual Chlorine; OMC: Organic Matter Content; NP: not performed.

Green cells indicate that results are within acceptable range; red cells indicate results are outside of parametric or recommended values.

Annex 2a – Microbiology parameters: storage stage.

				Staphylococcus		**Total microbial count**			
Sample	**Coliforms**	***E. coli***	***Enterococcus***	**Total**	**Coagulase +**	**37ºC**	**22ºC**	**5ºC**	***Salmonella***
103	0	0	0	**NP**	**NP**	0	2	**NP**	ND
129	0	0	0	**NP**	**NP**	0	3	**NP**	ND
194	15	7	4	**NP**	**NP**	86	>300	**NP**	ND
241	0	0	0	**NP**	**NP**	0	0	1	ND
274	0	0	0	**NP**	**NP**	0	1	0	ND
290	0	0	0	**NP**	**NP**	0	0	0	ND
420	0	0	0	**NP**	**NP**	1	0	0	ND
424	0	0	0	**NP**	**NP**	0	1	0	ND
448	0	0	0	**NP**	**NP**	0	1	0	ND
467	0	0	0	**NP**	**NP**	1	0	0	ND
521	48	0	1	**NP**	**NP**	94	>300	127	ND
543	108	0	6	**NP**	**NP**	>300	>300	>300	ND
631	0	0	0	**NP**	**NP**	0	2	0	ND
634	0	0	0	**NP**	**NP**	1	0	0	ND
674	0	0	0	**NP**	**NP**	0	0	0	ND
685	0	0	0	**NP**	**NP**	0	0	0	ND
688	0	0	0	**NP**	**NP**	0	0	0	ND
828	0	0	0	0	0	0	22	0	ND
831	0	0	0	3	2	1	65	0	ND
843	0	0	0	0	0	0	1	0	ND
866	0	0	0	0	0	0	0	1	ND
872	2	1	0	0	0	0	2	72	ND
875	0	0	0	0	0	0	5	0	ND
901	0	0	0	0	0	0	0	1	ND
904	0	0	0	0	0	0	1	1	ND
924	0	0	0	5	0	1	107	38	ND
927	0	0	0	2	0	4	174	6	ND
945	0	0	0	0	0	1	1	0	ND
947	0	0	0	0	0	>300	>300	100	ND
950	0	0	0	0	0	0	0	0	ND

NP – not performed; ND – not detected.

Green cells indicate that results are within acceptable range for parametric or recommended values; red cells indicate results outside the acceptable range. Yellow cells indicate detection of microorganisms in parameters with no legal requirement for water analysis.

Annex 2b – Physical-chemical parameters: storage stage.

Sample	Ammonia	Conductivity	Chlorides	FRC	Nitrate	Nitrite	pH	OMC	Hardness
103	0,12	474	**NP**	**NP**	0,93	<0,02	8,4	0,91	**NP**
129	0,15	206	48	**NP**	1,09	0,03	8	0,89	36
194	<0,06	379	99	**NP**	1,60	<0,02	9,2	1,14	42
241	0,09	469	**NP**	**NP**	1,88	<0,02	9,3	0,88	42
274	0,10	427	84	**NP**	1,69	<0,02	7,6	2,38	50
290	0,09	609	138	**NP**	1,51	<0,02	8,2	1,98	56
420	0,09	470	105	0,60	4,25	<0,02	9,1	0,65	56
424	0,11	416	108	0,20	6,08	<0,02	9,1	0,73	38
448	<0,06	368	90	0,10	5,03	<0,02	9,5	0,69	32
467	<0,06	10970	206	**NP**	2,24	<0,02	8	9,76	60
521	0,11	376	88	0,10	1,02	<0,02	7,9	1,14	64
543	0,09	326	72	0,20	1,56	<0,02	8,8	2,00	28
631	0,08	468	105	0,08	1,25	<0,02	9,4	0,87	30
634	0,06	679	196	0,08	1,71	<0,02	7,9	1,71	36
674	0,07	707	202	0,20	1,89	<0,02	7,8	1,58	36
685	0,07	660	187	0,07	1,68	<0,02	9,6	1,31	40
688	0,07	382	68	0,05	0,84	<0,02	9	1,94	32
828	0,14	535	146	0,08	2,12	<0,02	8,8	**Damaged**	24
831	<0,06	423	99	0,20	2,08	<0,02	9,4	1,19	28
843	0,07	596	135	0,03	2,28	<0,02	9,8	3,49	60
866	0,23	478	108	0,07	2,04	<0,02	9,1	**Damaged**	52
872	0,14	338	82	0,08	1,77	<0,02	9,3	**Damaged**	48
875	0,12	835	247	0,10	2,04	<0,02	8,9	**Damaged**	52
901	<0,06	474	127	0,20	1,68	<0,02	7,8	1,53	**Damaged**
904	0,06	1402	443	0,10	1,23	<0,02	9,6	1,70	64
924	0,09	342	97	0,06	1,26	<0,02	7,9	1,30	68
927	0,10	459	95	0,10	1,48	<0,02	9,9	1,44	36
945	0,09	8000	3195	0,08	2,43	<0,02	8,6	6,89	73
947	0,10	573	167	0,08	2,52	<0,02	9,6	1,61	68
950	0,11	624	199	0,10	2,79	<0,02	8,3	2,13	70

FRC: Free Residual Chlorine; OMC: Organic Matter Content; NP: not performed.

Green cells indicate that results are within acceptable range; red cells indicate results are outside of parametric or recommended values.

Annex 3a – Microbiology parameters: product-contact stage.

				Staphylococcus		**Total microbial count**			
Sample	**Coliforms**	***E. coli***	***Enterococcus***	**Total**	**Coagulase +**	**37ºC**	**22ºC**	**5ºC**	***Salmonella***
195	122	7	21	**NP**	**NP**	>300	>300	**NP**	ND
242	613	0	1	**NP**	**NP**	>300	>300	>300	ND
275	2420	0	3	**NP**	**NP**	>300	>300	>300	ND
291	6	0	0	**NP**	**NP**	115	>300	>300	ND
421	1554	7	2420	**NP**	**NP**	>300	>300	>300	ND
425	829	8	388	**NP**	**NP**	>300	>300	>300	ND
449	2420	57	2420	**NP**	**NP**	>300	>300	>300	ND
468	2420	1	866	**NP**	**NP**	>300	>300	>300	ND
522	387	0	3	**NP**	**NP**	>300	>300	>300	ND
523	2420	0	5	**NP**	**NP**	>300	>300	>300	ND
544	2420	16	2420	**NP**	**NP**	>300	>300	>300	ND
564	548	1	93	**NP**	**NP**	>300	>300	>300	ND
632	44	23	67	**NP**	**NP**	232	>300	>300	ND
635	12	0	4	**NP**	**NP**	45	170	29	ND
675	276	1	23	**NP**	**NP**	>300	>300	>300	ND
686	2420	0	2420	**NP**	**NP**	>300	>300	>300	ND
689	138	0	12	**NP**	**NP**	>300	>300	>300	ND
829	1400	0	29	**CG**	**CG**	>300	>300	**D**	ND
832	770	3	55	**CG**	**CG**	>300	>300	**D**	ND
844	152	1	5	380	0	101	>300	>300	ND
867	2420	4	1120	10	0	>300	>300	>300	ND
873	866	3	10	267	0	>300	>300	>300	ND
876	58	0	1	30	0	>300	>300	>300	ND
902	15	0	61	534	0	>300	>300	>300	ND
905	3	0	4	37	2	135	>300	>300	ND
925	411	2	2420	401	0	>300	>300	>300	ND
928	435	0	93	201	0	>300	>300	>300	ND
948	2420	13	84	**CG**	**CG**	>300	>300	>300	ND
951	387	0	7	271	0	>300	>300	>300	ND

NP – not performed; ND – not detected; D: damaged; CG: confluent growth.

Green cells indicate that results are within acceptable range for parametric or recommended values; red cells indicate results outside the acceptable range. Yellow cells indicate detection of microorganisms in parameters with no legal requirement for water analysis.

Annex 3b – Physical-chemical parameters: product-contact stage.

Sample	Ammonia	Conductivity	Chlorides	FRC	Nitrate	Nitrite	pH	OMC	Hardness
195	0,14	457	102	**NP**	1,99	<0,02	9,1	3,09	46
242	0,25	1432	**NP**	**NP**	1,64	<0,02	9,2	4,88	34
275	0,19	305	77	**NP**	1,43	<0,02	9	2,69	36
291	0,12	388	78	**NP**	1,58	<0,02	9,7	1,90	46
421	1,98	457	94	0,10	25,45	<0,02	9,5	10,53	62
425	7,44	521	140	0,20	56,83	0,54	7,6	23,09	44
449	3,73	229	44	0,20	16,50	<0,02	6,9	7,79	32
468	1,27	7430	266	0,30	0,16	<0,02	7,1	26,47	40
522	2,25	417	107	0,03	0,88	0,05	7,7	5,63	20
523	6,20	649	161	0,07	0,88	0,07	7,1	8,82	26
544	0,48	87	42	<0,03	0,88	0,09	8	7,60	12
564	0,33	600	1821	0,06	0,89	<0,02	7,5	8,86	32
632	0,10	194	54	0,08	0,89	<0,02	9,3	0,96	18
635	0,07	100	36	0,07	0,89	<0,02	6,8	7,21	14
675	0,35	508	141	0,04	0,89	<0,02	9	7,16	34
686	0,53	518	126	0,04	0,89	0,07	8,7	6,47	34
689	0,26	262	73	0,10	0,89	<0,02	9,2	7,00	30
829	9,59	572	133	0,06	1,02	0,13	8,5	15,30	32
832	0,39	88	13	0,07	0,75	<0,02	9,1	12,77	20
844	0,30	620	144	0,03	2,08	<0,02	9,5	20,00	72
867	1,33	291	62	0,10	0,84	0,09	7,5	**Damaged**	32
873	0,87	471	110	0,10	2,21	<0,02	9,1	**Damaged**	72
876	0,19	782	234	0,06	1,65	<0,02	8,8	**Damaged**	52
902	0,43	713	191	0,10	1,90	<0,02	8,7	30,40	72
905	0,08	394	102	0,05	1,24	<0,02	9,4	2,60	56
925	1,40	285	85	0,10	0,44	<0,02	7,7	26,90	48
928	0,75	542	194	0,06	1,73	<0,02	9,3	28,60	74
948	4,56	348	105	0,20	1,90	<0,02	9	66,90	79
951	2,17	567	129	0,06	2,04	<0,02	9,1	14,10	72

FRC: Free Residual Chlorine; OMC: Organic Matter Content; NP: not performed.

Green cells indicate that results are within acceptable range; red cells indicate results are outside of parametric or recommended values.

Annex 2 – Sample questionnaire

Market: _____ Date: _____

Worker

Work station: _____

Education: _____

Work clothes/hair: _____

Stand

Cleaning

How: _____

When: _____

Location within the market: _____

Who gets the ice: _____

Exposure (>1h/day)

Water: _____

Ice: _____

Fresh and/or dried fish: _____

Arconditioning system: _____

Health

Skin infection: _____

When: _____

Nail infection: _____

When: _____

Pulmonary infection: _____

When: _____

Gastrointestinal disorder: _____

When: _____

Allergies: _____

Other: _____

Observations