

Evaluation of the effectiveness of ozone as a sanitizer for fish experimentally contaminated with *Salmonella sp.*

Avaliação da eficácia de ozônio como sanitizante para peixes contaminados experimentalmente por Salmonella sp.

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Abstract

Salmonellosis is a major public health problem related to food contamination and ensuing food poisoning. Brazilian resolution RDC n° 12/2001 of the Brazilian National Health Surveillance Agency (ANVISA) established the absence of *Salmonella* in 25 g of fish for consumption. However, the significant increase in the occurrence of fish contamination by *Salmonella* and other pathogenic bacteria shows that the currently applied strategies are not sufficient and that, in addition to the implementation of good health practices, the application of new sanitizer technologies in the fish industry is also necessary. In this context, the present study evaluated the effectiveness of ozone in an aqueous medium as a sanitizer for *Salmonella* contaminated fish. The experiment was carried out using a completely randomized design with eight treatments and five replicates, giving a total of 40 experimental units. Each sample consisted of three fishes, totalizing 120 fishes. The treatments consisted of different combinations of temperature and water-dissolved ozone (O₃) concentrations (21 °C × 0.35 ppm; 20 °C × 0.45 ppm; 21 °C × 0.60 ppm; 20 °C × 0.80 ppm; 19 °C × 1.7 ppm; 6 × 5.1 ppm; 4 °C × 7.2 ppm; and 2 °C × 9.1 ppm). *Colossoma macropomum* (Tambaqui) samples were experimentally infected with *Salmonella typhimurium* (ATCC 14028) and immersed in water with the different treatments. After three minutes, the fish samples were collected and subjected to qualitative *Salmonella* analyses. The ozone tests were not efficient in eradicating *Salmonella* under the experimental conditions presented here, indicating the need for the identification of effective sanitizers in order to meet the determinations of Brazilian law.

Keywords: Microbiology; Tambaqui; *Colossoma macropomum*; Ozonation.

Resumo

A contaminação de alimentos com *Salmonella* e, conseqüentemente, a toxinfecção alimentar, é um importante problema de saúde pública. No Brasil, a Resolução da ANVISA RDC n° 12/2001 para pescado preconiza que a *Salmonella* deve estar ausente em 25 gramas do produto a ser consumido. Porém, o aumento significativo na ocorrência de contaminação de pescado por *Salmonella* e por outras bactérias patogênicas demonstra que as estratégias atualmente utilizadas não têm sido suficientes e que, além da implementação de boas práticas sanitárias, é necessária, também, a aplicação de novas tecnologias sanitizantes na indústria de pescado. Neste contexto, o presente estudo avaliou a eficácia de ozônio em meio aquoso como agente sanitizante de pescado contaminado. O experimento foi desenvolvido em delineamento inteiramente casualizado com oito tratamentos, que consistiram em diferentes combinações de temperatura e concentração de ozônio



Evaluation of the effectiveness of ozone as a sanitizer for fish experimentally contaminated with *Salmonella* sp.

Luiz, D. B. et al.

(21 °C × 0,35 ppm; 20 °C × 0,45 ppm; 21 °C × 0,60 ppm; 20 °C × 0,80 ppm; 19 °C × 1,7 ppm; 6 °C × 5,1 ppm; 4 °C × 7,2 ppm; 2 °C × 9,1 ppm), com cinco repetições por tratamento, totalizando 40 amostras compostas (três peixes por amostra). Para tanto, 120 peixes da espécie *Colossoma macropomum* (Tambaqui) foram contaminados experimentalmente com *Salmonella typhimurium* (ATCC 14028) e, em seguida, imersos em recipientes contendo os tratamentos testados. Após um período de três minutos, as amostras de peixes foram coletadas e submetidas à análise qualitativa de *Salmonella*. Os testes realizados com ozônio não foram eficientes para erradicar *Salmonella* nas condições experimentais apresentadas, indicando a necessidade de identificação de agentes sanitizantes eficientes, a fim de atender à determinação da legislação brasileira.

Palavras-chave: *Microbiologia*; *Tambaqui*; *Colossoma macropomum*; *Ozonização*.

1 Introduction

The size of the contamination and the kind of microorganisms found in fish and fishery products depend on several aspects, such as location and method of capture or farming, season, handling, processing and storage, amongst others (GONÇALVES; OLIVEIRA, 2016). Foodborne diseases are usually caused by the ingestion of contaminated products. In addition, the microbiological quality of these products is also related to post-harvesting management, in other words, good handling and manufacturing practices are essential for food security in the transport, storage, handling and processing stages (JAKABI et al., 1999).

Amongst the main food contaminants, *Salmonella* is one of the main causes of foodborne illnesses in many parts of the world. The ubiquitous nature of this microorganism and the numerous serotypes present in various foodstuffs are a major challenge for researchers who strive for its elimination. Thus, in the last decade, many innovative technologies have emerged with great potential to inactivate *Salmonella* and other foodborne pathogens, particularly with regard to fresh fish (CARRASCO et al., 2012; MUKHOPADHYAY; RAMASWAMY, 2012; SCALLAN et al., 2015; SILVA, 2015; GONÇALVES; OLIVEIRA, 2016; BAKA et al., 2017).

Sodium hypochlorite is the most widely used compound in food sanitation. However, chlorine is not a friendly chemical, as it can react with organic material, forming organochlorines such as trihalomethanes, considered to be potential carcinogens (NASCIMENTO; SILVA, 2010; AL-OMARI et al., 2014). Thus studies are now being carried out to evaluate alternative sanitizers that show lesser impact on receiving water bodies, that do not form toxic byproducts and that can easily eliminate their excess concentrations (RAMOS et al., 2013; ACHILLEOS et al., 2005; EPA, 2004).

Amongst these emerging technologies, the use of ozone, which, due to its powerful oxidant effect, is characterized as one of the most powerful disinfectants, is noteworthy (LANITA; SILVA, 2008; GONÇALVES, 2016), causing cell inactivation by direct membrane and cell wall damage, disruption of enzymatic reactions and DNA damage (EPA, 2004; VON GUNTEN, 2003; GONÇALVES; OLIVEIRA, 2016). In addition, several studies have shown that its use presents low potential with regard to changes

in the sensory characteristics of food, which expands the possibilities for its use (USEPA, 1999; WHANG et al., 2004; ALEXANDRE et al., 2011; MUKHOPADHYAY; RAMASWAMY, 2012).

It is not enough to just select a powerful sanitizing agent; the concentrations required and the efficiency of the disinfection process are of critical importance with respect to the prevention of pathogen survival, ensuring that it is safe to use the final product. In addition, maintaining an adequate level of sanitizer in the washing water can be a challenge to the industry, due to the high organic load of their washing systems (ZHOU et al., 2015).

This aspect becomes more relevant in the fish processing scenario in Brazil, since the RDC Resolution No. 12 of January 2, 2001, of the *Agência Nacional de Vigilância Sanitária* (ANVISA) of the Brazilian Ministry of Health (MS), states that *Salmonella* must be absent in 25 g of the product to be consumed (BRASIL, 2001). In addition, in Brazil, chlorine is currently the only sanitizer permitted by law by the national inspection agency (Ministério da Agricultura, Pecuária e Abastecimento, MAPA) for fish cleaning processes.

The efficiency of using ozone in reducing microbial counts depends on many factors, e.g. ozone concentration and time of exposure, type of produce, temperature, microbial populations and packaging material (GLOWACZ; REES, 2016; HORVITZ; CANTALEJO, 2014; GONÇALVES; OLIVEIRA, 2016).

Considering these factors and the lack of studies on the application of ozone as a sanitizer in the initial washing process of whole fish in fish warehouses, the present study aimed to evaluate the effect of using different combinations of temperature and water-dissolved ozone concentrations for the elimination of *Salmonella* during fish cleaning processes in fish warehouses, considering the microbiological standards set forth by Brazilian law.

2 Material and methods

2.1 Fish samples

Tambaqui (*Colossoma macropomum*) specimens, a species representative of commercial interest in northern Brazil, were purchased slaughtered after fasting for 24 hours,

Evaluation of the effectiveness of ozone as a sanitizer for fish experimentally contaminated with *Salmonella* sp.

Luiz, D. B. et al.

originating from farms located in Palmas, TO, Brazil. The specimens had live weights of from 400 g to 750 g.

2.2 *Salmonella* strains

Salmonella typhimurium strains (ATCC 14028) were used, acquired from the reference microorganism collection at the Oswaldo Cruz Foundation (FIOCRUZ, Brazil). Freeze-dried bacterial strains were re-suspended and homogenized in 0.5 mL Brain Heart Infusion Broth (BHI), according to the culture rehydration instructions provided by FIOCRUZ. After resuspension, the strains were inoculated into the same broth and incubated in a bacteriological incubator at 37 °C for 24 hours. The strains were then maintained in BHI broth, under refrigeration, and either sub-cultured periodically or inoculated into Selective Nutrient Broth for *Salmonella* (3M™, Brazil) and incubated for 24 hours at 41 °C to carry out the experimental fish contamination.

2.3 Contaminant suspension preparation

The *Salmonella typhimurium* concentrations in the contaminant suspension were estimated by the turbidimetric method. After 24 hours in Selective Nutrient Broth for *Salmonella* (3M™, Brazil), a 2 mL aliquot of the bacterial suspension was removed for spectrophotometric measurements using *Salmonella*-free Selective Nutrient Broth as the blank. The contaminant suspension was adjusted to an optical density range of 0.1 to 0.5 (10^4 to 10^8 CFU mL⁻¹) at 540 nm and kept refrigerated until analyzed (HANNA et al., 2011).

2.4 Experimental fish contamination

Fish contamination was carried out in a previously sanitized and disinfected container. Using a sterile spray, the contaminating *Salmonella* suspension was sprayed twice on each side of the fish, using about 0.5 mL of the bacterial suspension. After 5 to 10 minutes, the contaminated samples were homogenized for the ozone treatments.

2.5 Treatments

2.5.1 Experimental design

The experiment was carried out using a completely randomized design with eight treatments and five replicates, giving a total of 40 experimental units. Each sample consisted of three fishes, totalizing 120 fishes. The treatments consisted of different combinations of temperature and water-dissolved ozone (O₃) concentrations (21 °C x 0.35 ppm; 20 °C x 0.45 ppm; 21 °C x 0.60 ppm; 20 °C x 0.80 ppm; 19 °C x 1.7 ppm; 6 °C x 5.1 ppm; 4 °C x 7.2 ppm; and 2 °C x 9.1 ppm). Each experimental unit was placed in a previously sterilized 20 L capacity plastic container, which was attached to a 500 L-capacity tank.

2.5.2 Ozone treatments

The experimental units were supplied with the ozonated water solution in a continuous flow with an average flow of 7 L min⁻¹. Three fish previously infected with *Salmonella typhimurium* were immersed for three minutes in each experimental unit. The fish were subsequently removed with sterile tongs and sent for qualitative analysis regarding *Salmonella*. The three fish formed a composite sample. Thus, for each treatment, five samples were analyzed, giving a total of 15 fish. The three-minute period was previously determined to be the maximum time for the first fish washing procedure before starting sample processing (in cylinders or on washing mats) in fish processing industries.

In addition to the five repetitions, parallel tests were also carried out to verify if the fish were free from prior *Salmonella* infection and if contamination by spraying was effective. These tests were termed "positive and negative controls." Thus, three fish from each batch used in the treatments were analyzed for the presence of *Salmonella* (negative control), while three others were contaminated by spraying, according to the methodology employed herein, and analyzed for the presence of *Salmonella* (positive control).

2.5.3 Water ozonation

For the experimental tests designed to resemble the initial industrial washing of whole fish, a high capacity ozonation piece of equipment was used. The assembled system allowed for simultaneous repetitions and ozonation, water flow and system temperature control. These features contributed to the configuration of the system as a "pilot-scale", which facilitates the projection of the treatment on an industrial scale.

An ozonizer (Philozon, O₃R) with an oxygen concentrator at 92% (10 L min⁻¹), maximum ozone generation of 15 g of O₃ at 8 min⁻¹ L of O₂ with a static mixer injection system/diffuser, bypass and one-inch venturi injector, was used. This equipment stored the ozonized water in a 500 L tank with a predetermined constant concentration displayed on the digital oxi-reduction (redox) potential (ORP) controller.

In addition to confirmation by the redox potential, the quantification of the dissolved ozone was also carried out using spectrophotometry (HACH DR 5000 spectrophotometer) with a 1.0 cm optical path quartz cell, considering a molar extinction coefficient of $\epsilon = 2950 \text{ M}^{-1} \text{ cm}^{-1}$ at 258 nm (APHA, 2012).

2.5.4 Sample analyses

The three fish in each experimental unit were placed in sterile bags with 125 mL of peptone water, which was gently agitated for three minutes. A 25 mL aliquot of the peptone water was then removed and diluted with 225 mL

Evaluation of the effectiveness of ozone as a sanitizer for fish experimentally contaminated with *Salmonella* sp.

Luiz, D. B. et al.

of enrichment medium (3M™) and placed in an incubator for 24 hours at 41 ± 0.5 °C. For the qualitative *Salmonella* analysis (presence/absence), a rapid *Salmonella* detection system was used, according to the manufacturer's instructions (Plate Petrifilm™ *Salmonella* Express System, 3M™).

The same procedure was carried out for each fish used in the positive and negative controls, with the difference that, in this case, each fish represented an experimental sample.

3 Results and discussion

The present study evaluated the efficacy of eight temperature and water-dissolved ozone concentration combinations as potential sanitizers for fish experimentally contaminated with *S. typhimurium*. When the treatments had dissolved ozone concentrations below or equal to 1.7 ppm (0.35 ppm, 0.45 ppm, 0.6 ppm, 0.8 ppm and 1.7 ppm) they were carried out at room temperature (19 °C to 21 °C). These concentrations were unable to eliminate the presence of *Salmonella* after three minutes of treatment (Table 1).

To test the effect of higher dissolved ozone concentrations the water used to dissolve the ozone had to be cooled, so as to expand the saturation capacity of the solution (USEPA, 1999). Thus the effectiveness of the treatments with 5.1 ppm, 7.2 ppm and 9.1 ppm were tested at temperatures of 6 °C, 4 °C and 2 °C respectively. All treatments showed the presence of *Salmonella* after 3 minutes of fish immersion (Table 1).

Different studies have reported the application of ozone in food sanitization, using different microorganisms as indicators. Koidiset al. (2000) evaluated the sanitizing effect of two aqueous ozone concentrations (1.4 ppm at 22 °C and 3.0 ppm at 4 °C) in shelled eggs inoculated with *Salmonella enteritidis* (7 log). The contaminated eggs were immersed for 30, 60 and 90 seconds in ozone solutions at the aforementioned concentrations.

Decreases of 1.02, 1.19 and 1.37 log units, respectively, were observed at 1.4 ppm and at 3.0 ppm, decreases of 2.12, 2.34 and 2.47 log units, respectively, were observed. However, the microorganism evaluated was not eliminated from any of the samples.

In another study, Ölmez and Akbas (2009) evaluated the use of aqueous ozone as a sanitizer for lettuce leaves contaminated with *Listeria monocytogenes* (8 log). The authors immersed lettuce leaves in contaminated solutions with concentrations ranging from 0.5 to 4.5 ppm with exposure times from 0.5 to 3.5 minutes. Under these conditions, they found decreases ranging between 1.5 and 2.4 log *Listeria* CFU. It is noteworthy that the maximum decreases were observed above 3 ppm, with higher concentrations showing no significant changes in the microbial counts. Furthermore, the highest microbial decreases were observed between two and three minutes of exposure, with no further significant decreases identified after two minutes.

Alexandre et al. (2013) tested the sanitizing effect of sodium hypochlorite, hydrogen peroxide, water-dissolved ozone, UV radiation and ultrasound to assess their applicability as pretreatments for freezing peppers contaminated with *Listeria* (7.27 log). Regarding ozone, the peppers were dipped into a 0.3 ppm solution for two minutes, and a decrease of 2.25 log *Listeria* CFU was noted. For this experiment the authors concluded that, despite hydrogen peroxide (1% and 5%) showing greater decreases in *Listeria* CFU (2.54 and 3.68 log, respectively), its application resulted in negative impacts regarding pepper color and firmness, impacts which were not observed with the use of ozone. Thus, of the treatments evaluated, ozone was elected the most suitable prior to pepper freezing.

Singh et al. (2003) tested different concentrations of ozonated water (4.60 ppm, 9.27 ppm and 14.3 ppm) and different immersion times (3, 5 and 10 minutes) to eliminate the presence of *Escherichia coli* O157:H7 in

Table 1. Analysis of the presence of *Salmonella* after the application of different ozone concentrations to experimentally contaminated tambaqui (*Colossoma macropomum*) samples.

O ₃ concentrations (ppm)	Temperature (°C)	<i>Salmonella</i> analyses (Number of replicates/Number of replicates with presence of <i>Salmonella</i>) ¹
0.35	21	5/5
0.45	20	5/5
0.6	21	5/5
0.8	20	5/5
1.7	19	5/5
5.1	6	5/5
7.2	4	5/5
9.1	2	5/5

¹ = 05 replicates were carried out for each treatment.

Evaluation of the effectiveness of ozone as a sanitizer for fish experimentally contaminated with *Salmonella* sp.

Luiz, D. B. et al.

alfalfa seeds. The presence of the bacterium was still observed in the seeds after all the treatments. Recently, Karaca and Velioglu (2014) tested the use of ozonated water (12 ppm) on the inactivation of *Escherichia coli* and *Listeria innocua* inoculated onto spinach, lettuce and parsley by dipping. The authors observed a reduction of 2.0 ± 0.3 log for *E. coli* and 2.2 ± 0.1 log for *L. innocua* and the use of ozone in an aqueous medium caused no changes in the characteristics of the vegetables analyzed.

Sengun (2014) tested the efficacy of ozonated water in reducing the counts of *Salmonella Typhimurium*. The treatment of carrots with ozonated water (0.5 ppm, 1.0 ppm and 1.5 ppm) for 3 min, 5 min and 10 min caused reductions in the number of *Salmonella Typhimurium* ranging between 0.37 and 2.72 log units, depending on the ozone concentration and exposure time used. However none of the treatments resulted in the complete inactivation of *Salmonella*.

Al-haddad et al. (2005) evaluated the effectiveness of *Salmonella* inactivation on chicken skin using ozone in a gaseous medium. According to this study, the authors inoculated 0.1 mL of a saline *Salmonella infantis* suspension (1.5×10^4 CFU mL⁻¹) onto 10 cm² of chicken skin, resulting in 150 CFU cm⁻². After inoculation, the samples were exposed to different treatments: (a) exposure to gaseous ozone near 3.000 (> 2.000) ppm for from 0 to 30 minutes; and (b) exposure to gaseous ozone concentrations near 3.000 (> 2.000) ppm for 15 minutes, followed by storage at 7 °C using a controlled atmosphere (70% CO₂:30% N₂) for nine days. None of the treatments resulted in complete *Salmonella* inactivation, with maximum values of 97%, the best condition for treatment being O₃ gas for 15 minutes followed by nine days of storage in a controlled gaseous environment. The authors indicated that, although a bactericidal effect was observed, the *Salmonella* was not completely eradicated from the natural chicken substrate. Moreover, the authors attributed this result to the fact that the organic matter naturally present in the chicken skin absorbs and reduces the active ozone concentration. Consequently, the living *Salmonella* cells tend to be incorporated into the folds and skin pores, protecting the microorganisms from contact with the ozone molecules.

In a review on the use of emerging technologies in the control of *Salmonella* in foods, Mukhopadhyay and Ramaswamy (2012) indicated that the effectiveness of ozone treatments in both gaseous and liquid media was limited, when applied to substrates that show the presence of organic matter, such as foods (meat and vegetables) and environmental substrates (effluent and air). Furthermore, the type of organic matter may also interfere with the inactivation rate of the bacterium. The authors also observed that "it is not the total ozone applied, but indeed the residual ozone that has the best effect on *Salmonella* cells." Thus, long periods of storage may be required after

applying ozone, whereas temperature is the main factor regarding the stability of residual ozone and the inactivation effect on the microorganisms. The authors noted that ozone treatments tend to be long in order to increase the efficiency of *Salmonella* inactivation. However, treatment times exceeding 10 minutes are not feasible for industrial food processing. Therefore, to reduce the treatment time and increase the inactivation rates of *Salmonella*, combinations of treatments such as UV + O₃, increased temperature + O₃, amongst others, are indicated (MUKHOPADHYAY; RAMASWAMY, 2012; XUE et al., 2016).

The complete elimination of the microorganisms analyzed was not observed in any of these studies, corroborating the data presented in the present study. However, Resolution ANVISA RDC No. 12/2001 for fish states that *Salmonella* must be absent in 25 grams of the product to be consumed. Thus considering this norm and the fact that the initial *Salmonella* contamination in the tambaqui samples was of 10⁴ to 10⁸ CFU, the ozone treatment was not effective in the eradication of *Salmonella* under the experimental conditions evaluated.

Regarding the inadequacy of ozone in eliminating *Salmonella* from the tambaqui samples under the conditions evaluated, some assumptions can be postulated, which corroborate those raised by Al-haddad et al. (2005) and cited previously:

- 1) The fish evaluated in the present study have scales and release a characteristic mucus, both of which probably act as physical barriers, hindering the action of the sanitizers in removing the microorganisms;
- 2) Scales, pores and the operculum located over the gills constitute a physical barrier to diffusion of the water containing the disinfecting agent, and make it difficult to complete the disinfection process;
- 3) The characteristic mucus and other impurities naturally present on fish surfaces are organic substances which react with the ozone molecules, i.e. are organic compounds that act as ozone molecule sequestering agents (ozone oxidizes organic matter), decreasing the availability (concentration) of this oxidant in the medium and consequently reducing the efficient inactivation of *Salmonella* on the surface of the fish.

4 Conclusions

Although studies that show the efficiency of ozone as a bactericide are available, reports of the complete inactivation of *Salmonella* in foods were not found. The present study demonstrated the inefficiency of ozone in the eradication of *Salmonella* under experimental conditions. These results suggest that (i) *Salmonella* can impregnate the pores present in the fish scales, hindering the effects of the ozone; (ii) the presence of a high organic load on the surface of the fish or diluted in the aqueous medium

Evaluation of the effectiveness of ozone as a sanitizer for fish experimentally contaminated with *Salmonella* sp.

Luiz, D. B. et al.

decreases the ozone concentration and the efficiency of *Salmonella* inactivation.

However, although studies indicate the possibility of greater treatment efficiency by exposing the food to low ozone concentrations for longer periods of time, the search for emerging and safe bactericidal agents should consider the feasibility, both technical and economic, of the efficient eradication of *Salmonella*, also evaluating the sensory aspects and food safety.

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Evaluation of the effectiveness of ozone as a sanitizer for fish experimentally contaminated with *Salmonella* sp.

Luiz, D. B. et al.

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