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Efficacy of Sodium Tripolyphosphate and Non-Phosphate Additives on the survival of *Vibrio parahaemolyticus* on Prawns *(Fenneropenaeus indicus)* (H. Milne-Edwards, 1837) during Frozen Storage

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Abstract

Use of sodium tripolyphosphate (STPP) and non-phosphate additives (NP1) in combination with sodium chloride is practised in soaking to prevent drip loss while thawing the frozen shrimp. In the present study, we have determined the efficacy of these additives on the survival of *Vibrio parahaemolyticus* on prawns (*Fenneropenaeus indicus)*, during block freezing and individual quick freezing conditions. Freshly harvested prawns were beheaded and treated with a blend solution containing STPP/NP1 at 3 g 100 ml^{-1} in combination with sodium chloride at 2g 100 ml^{-1}, in two separate experiments. At appropriate time intervals, whole prawns were sampled and dilutions plated onto TCBS agar medium to determine the number of *V. parahaemolyticus*. Experiments were performed in triplicate. Results indicated that STPP treated samples exhibited significant ($p<0.05$) reduction in the number of *V. parahaemolyticus* at any given time in both frozen conditions over the course of storage period as compared with non- phosphate treated samples. On the other hand, control samples (water dip) showed a gradual reduction in the number of organism, which is attributed to freezing. Therefore, sodium tripolyphosphate can be used as a promising drip loss preventer as well as an antimicrobial, to enhance the safety of frozen seafood.

Keywords: *Vibrio parahaemolyticus*, food safety, seafood, sodium tripolyphosphate, non-phosphate additive

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Introduction

Cochin is a major fish landing centre along the South West coast of India and supports thriving seafood industry which accounts for more than 90% of the state wide exports. Frozen shrimp continued to be the major item of export contributing for a share of 39.53% in quantity and 66.06% of the total export of marine products from India (MPEDA, 2015). Shrimp, due to its intrinsic features, is considered as a suitable medium for the growth of many microorganisms, including pathogenic vibrios. Among vibrios, *V. parahaemolyticus* is the most important species having aquatic habitat as natural niche and associated with seafood borne gastroenteritis cases (Tanaka et al., 2014). Consumption of raw or undercooked seafood have been implicated as major cause of *V. parahaemolyticus* infection in humans (Newton et al., 2012) and cause over half of all food poisoning outbreaks of bacterial origin in Taiwan, Japan and other South East Asian countries (Yu et al., 2013). In previous studies considerable prevalence of *V. parahaemolyticus* has been encountered in fish and shellfish (Sudha et al., 2012; 2014) from retail markets of Cochin. Many of these isolates were also multidrug resistant.

'Thaw drip loss' is one of the major problems in frozen shrimp industry (Mathen, 1968). Seafood processing companies have great concern in retaining this water, first for economic reasons and secondly for the quality of the final product (Masniyom, 2011). Processing of frozen shrimp products encompasses a wide array of pre-and post-treatments to create value addition (Fig. 1). Phosphates are generally recognized as safe (GRAS), and legally permitted additives along with salt to prevent thaw drip loss and thereby improve the quality of meat, fish and seafood products (Campden

BRI Report, 2012). Sodium Tripolyphosphate (STPP) is the most popular choice of polyphosphates used in seafood industry (Ünal et al., 2006). Though the phosphates have a wide application in seafood industry as a quality improving agent, import authorities in some European Union countries banned the imports of salted cod processed with polyphosphates (Pórarinsdóttir et al., 2010). Non phosphate additives such as organic acids, fatty acid esters, dimethyl dicarbonate, nitrites, sodium chloride, sulfites, etc. are common natural preservatives which are classified as "generally regarded as safe" (GRAS) by the USFDA when used in accordance with good manufacturing practices, in the acid form and sodium salt. It could be used directly during processing to control microbial contamination and shows no phosphate (P_2O_5) residue and was reported to be effective in improving the seafood quality (Sallam, 2007). NP1 from commercial source

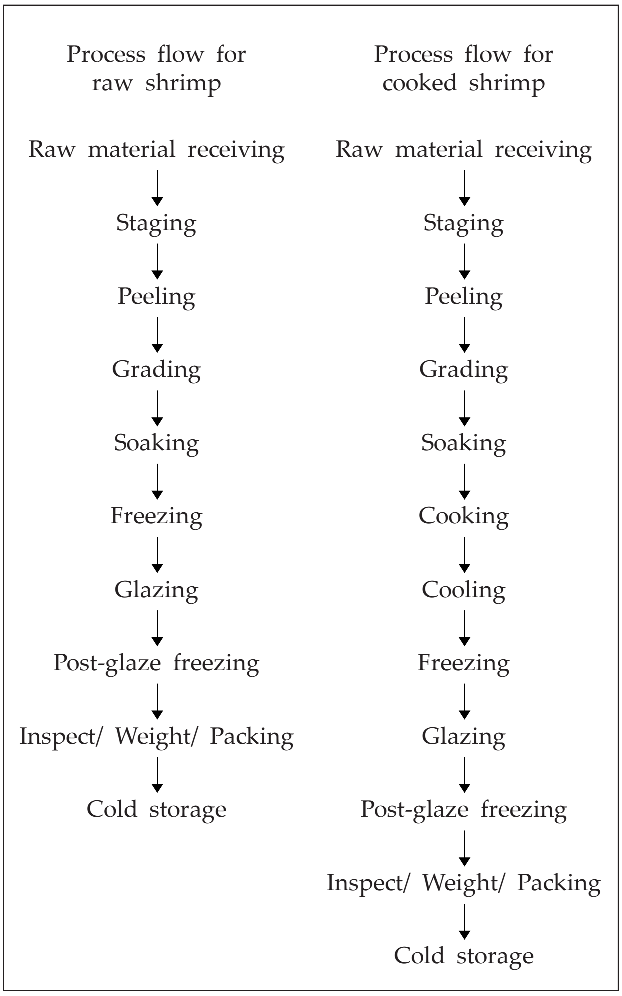

Fig. 1. Process flow for the raw and cooked shrimp (Hatha et al., 2003)

which contain sodium citrate and traces of citric acid is currently used instead of phosphate in some factories in Kerala, India that do freezing of shrimp.

From the literature review, there are indications that polyphosphates may be useful as antimicrobial agents in foods, although at present these compounds are not agreed as approved antimicrobial agents especially against Gram-negative bacteria. Similarly, organic acids and their salts have benefits in shelf-life extension of refrigerated meat, poultry and fish products by inhibiting spoilage, microbial growths and pathogens. Despite these results, use of a mixture of organic acid and organic salts is reported to be more efficient in inhibiting the growth of pathogen and extending product shelf-life (Sallam & Samejima, 2004).

Although STPP and Non-phosphates are primarily used to prevent drip loss while thawing, antimicrobial activity of these substances, if any, is not available in literature except a few reports by Rajkowski et al. (1994) and Lee et al. (2002). There is no published literature on the effect of STPP and non-phosphate additives on *V. parahaemolyticus*. Hence, the study has been taken up with the objectives to assess the efficacy of STPP: NaCl and non-phosphate: NaCl treatment, at levels currently used to control thaw drip loss, on the survival of *V. parahaemolyticus* (a predominant seafood borne pathogen) in block frozen and individually quick frozen *Fenneropenaeus indicus* under storage.

Materials and Methods

A strain of *V. parahaemolyticus* (NCMB 1902) was obtained from National Institute of Cholera and Enteric Disease (NICED), Kolkata, India. The test organism was found to amplify *tlh* gene by PCR. The culture was prepared according to method described by Boonyawantang et al. (2012) with a slight modification. Briefly, a loopful of this culture was revived in 10 ml of sterile nutrient broth (HiMedia, Mumbai) supplemented with 3% NaCl (w/v) and incubated at 37°C for 18-24 h. The culture was then centrifuged at 5000 rpm for 10 min at 5°C. The cell pellet thus obtained was resuspended in 10 ml sterile phosphate buffered saline solution to prepare the suspension of *V. parahaemolyticus*. The cell density was adjusted by spectrophotometer to 0.5 at 600 nm, corresponding to ~ 10^8 cfu ml^{-1}. The actual viable count of the cell suspension was confirmed by serial dilution (in 1.5% saline) and spread plating.

Freshly harvested Indian white shrimps (*Fenneropenaeus indicus*) were purchased directly from local fishermen, selected by size and immediately cooled on ice and transported to the laboratory. This size selection was conducted to minimize possible effects of frozen shrimp shape and thickness (Huan et al. 2003). The samples were rinsed with sterile cold water (about 5ºC), and subsequently with chlorinated water (100 ppm). The shrimps were then beheaded, peeled and deveined. Aseptic techniques were followed throughout the processing of the shrimps.

After processing, these samples were aseptically divided into two equal groups (each contains 26 beheaded, peeled and deveined shrimp). One group was soaked in one liter solution of STPP: NaCl (3:2) in distilled water for one hour at 4-6ºC with moderate agitation according to procedures of Gonçalves & Ribiero (2008) with some modifications. After soaking, the samples were drained well for 10min. The samples kept as control were soaked in sterile cold water only. Another set of sample was treated with sodium citrate containing traces of citric acid in combination with sodium chloride (3:2) by following similar procedure as that of STPP treatment.

Half of the prawns from STPP: NaCl, non-phosphate: NaCl treated and control samples were immersed in 500 ml of *V. parahaemolyticus* suspension (10^8 cfu ml^{-1}) for 1 min followed by a further 10 min draining step. After inoculation, half of them from treated/untreated and inoculated/ uninoculated groups were processed by block freezing and remaining half were processed as individually quick frozen. Block freezing is done in trays of size 2 ft (L) x 1ft (W) X 3 inch (H). Shrimps are arranged in the above trays and water is poured over so that they are clearly immersed in the water. These blocks are then frozen in contact plate freezer at -30ºC. Individual quick freezing is done in deep freezers maintained at -40ºC. All the experiments were done in triplicates.

Both the block frozen and individually frozen samples treated with STPP: NaCl and NP1: NaCl were analysed for the recovery of *V. parahaemolyticus,* soon after inoculation (day 0) and at intervals of 1, 3, 7, 14, 30 and 60 days. From each experimental and control set up, 10 gram samples were drawn and homogenized in 90 ml alkaline peptone water (APW) in a sterile stomacher bag using a stomacher blender (IUL instruments, Barcelona, Spain) for 2 min. A 0.1 ml aliquot of diluted homogenate was spread plated onto Thiosulfate-citrate-bile salts-sucrose agar (TCBS agar) plates without salt as the requirement of salt in these media are sufficient to support the growth of *V. parahaemolyticus* and incubated at 37°C for 24 h. The green coloured colonies were further reconfirmed as *V. parahaemolyticus* by using HiCrome Vibrio agar (HiMedia, Mumbai). The colonies were counted and the number was expressed as log cfu g^{-1} (Moawad et al. 2013). The entire experiment is schematically represented in Fig. 2

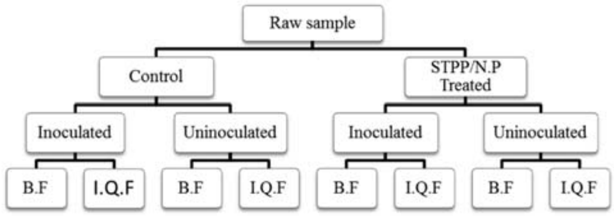

Fig. 2. Schematic flow of the entire experiment

Vibrio parahaemolyticus counts on shrimp samples were converted into logarithms of the number of colony forming units per gram (log_{10} cfu g^{-1}) for the statistical analysis. Statistical analysis was performed with SPSS 20. Analysis of variance (ANOVA) was performed following Duncan multiple range test ($p< 0.05$) to calculate the differences among the mean values with respect to storage period in the treated and untreated samples.

Results and Discussion

The survival of the *V. parahaemolyticus* during storage both in the presence and absence of STPP/ NP1 and at different freezing procedures such as block frozen and IQF differ significantly ($p< 0.05$) from each other. In the block frozen storage experiment (Fig. 3), shrimp samples dipped in STPP: NaCl solution, showed a remarkable reduction of the *V. parahaemolyticus* count as compared to the control. There was a significant reduction ($p<0.05$) in the microbial count by 1 log cfu g^{-1} after 3-4 h of inoculation (i.e. at the initial day of storage), whereas, no such reduction could be observed in the untreated samples. Extensive reduction of *V. parahaemolyticus* was found on the subsequent days of storage in the samples treated with STPP: NaCl solution. However, no such reduction in the population of *V. parahaemolyticus* was observed in the untreated samples. About 3.6 log unit reduction of *V. parahaemolyticus* was apparent within the first 7 days of storage in the treated samples, whereas, only 1 log unit reduction was seen in the untreated samples. Complete elimination of the pathogen had occurred in the treated samples by 14th day, but the untreated samples continued to carry *V. parahaemolyticus* (i.e., about 2.28 log cfu g^{-1}) till the end of the storage period of 60 days.

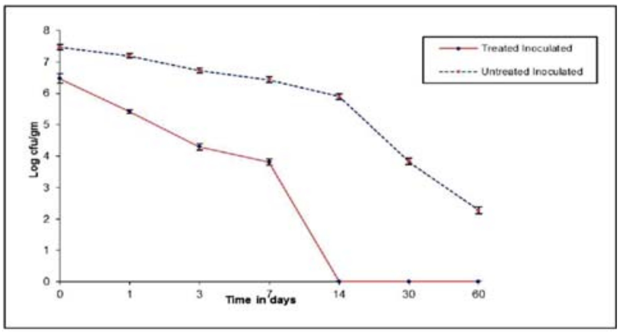

Fig. 3. Effect of STPP: NaCl treatment on the survival of *V. parahaemolyticus* in block frozen shrimps

The effect of sodium tripolyphosphate treatment on the survival of *V. parahaemolyticus* in peeled and deveined *F. indicus* stored under individually quick frozen condition is depicted in Fig. 4. In IQF shrimps, the survival of *V. parahaemolyticus* differ significantly ($p<0.05$) in the presence and absence of STPP/ NaCl treatment during the course of storage. In the presence of STPP/ NaCl, there was a significant decrease ($p<0.05$) of *V. parahaemolyticus* over the course of storage period as compared to untreated samples. About 4.7 log reduction of the pathogen was apparent within the first 14 days of storage in the treated sample, whereas, a significantly lower ($p<0.05$) count of 1.1 log reduction were only occurred in untreated shrimps . On 30th day, pathogen was seldom detected in the treated samples, but the untreated samples continued to carry *V. parahaemolyticus* (3.17 log cfu g^{-1}) till the end of the experiment. While the greater reduction in the treated samples could be attributed to the presence of STPP and freezing, the reduction in untreated sample is very likely due to the low temperature as this pathogen is sensitive to freezing and gradually inactivated at low temperature (Lin et al., 2004).

Our findings agree with the findings of Kilinc et al. (2009) wherein it was shown that that sodium

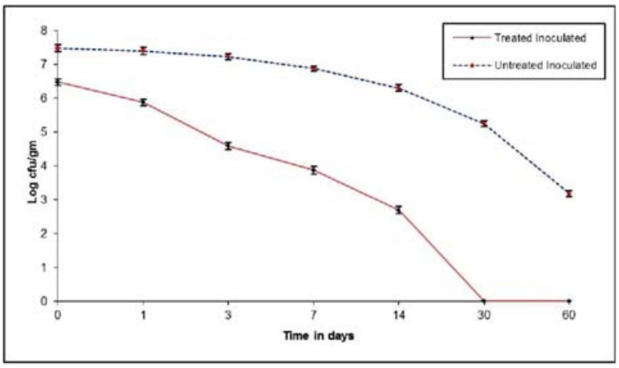

Fig. 4. Effect of STPP: NaCl treatment on the survival of *V. parahaemolyticus* in IQF shrimps

tripolyphosphate could be used as an antimicrobial surface treatment to decrease the populations of pathogens, prevent the growth of spoilage microorganism, extend shelf life of fish and to improve the functionality, especially to increase the water holding capacity. In literatures, it was stated that Gram-positive bacteria are more sensitive to polyphosphates than Gram-negative bacteria (Maier et al., 1999). However, in the present study, it was evident that STPP is having a strong bactericidal effect against the Gram negative pathogen, *V. parahaemolyticus* on shrimps under frozen storage. A significant reduction in the bacterial load from the initial load demonstrates STPP to be effective permeabilizers of the outer membrane (OM), the principle barrier structure in Gram-negative bacteria.

Phosphates often work in conjunction with salt to improve the quality of the product. Salt, specifically sodium chloride (NaCl), has been proven to improve the penetration and effects of the phosphate solutions. Fernández-López et al. (2004) reported that the activity of STPP is synergistically enhanced by the presence of NaCl. Rajkowski et al. (1994) reported that the addition of polyphosphate did not significantly inhibit the growth of *L. monocytogenes*, but combination of NaCl with polyphosphate significantly inhibited their growth. In the present study, addition of 2.0% NaCl also increased the inhibitory effect of STPP. As *V. parahaemolyticus* is a halophilic organism, it requires a minimum of 3% NaCl for its growth. Hence we presume that, NaCl concentration given in the treatment (2%) is not affecting the growth of *V. parahaemolyticus*.

The effect Citric acid: Sodium citrate and NaCl (NP: NaCl) treatment on the survival of *V. parahaemolyticus* in block frozen shrimp under storage was analysed (Fig. 5). In this case, survival of *V. parahaemolyticus* was significantly low ($p<0.05$) in both treated and untreated samples, though the effect was well pronounced in the case of treated samples. About 3.43 log reduction of *V. parahaemolyticus* from the initial bacterial load was observed on the 14th day in treated samples whereas, significantly lower ($p<0.05$) reduction of 1.58 could only observed in the untreated samples. On 30th day onwards, complete elimination of the pathogen was recorded in the treated sample but the untreated sample continued to carry the organisms.

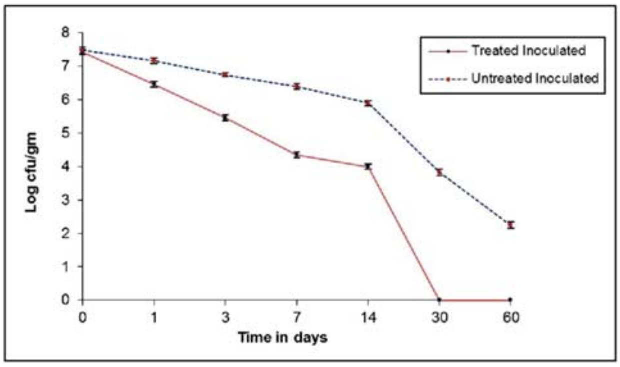

Fig. 5. Effect of Non phosphate: NaCl treatment on the survival of *V. parahaemolyticus* in block frozen shrimps

The effect of Citric acid: Sodium citrate and NaCl treatment on the survival of *V. parahaemolyticus* in individually quick frozen (IQF) shrimps under storage is showed in the Fig. 6. The survival of *V. parahaemolyticus*, both in the presence and absence of Citric acid: Sodium citrate and NaCl treatment differ significantly ($p<0.05$) throughout the storage period. A significant decrease of 3.9 log cfu g^{-1} of the pathogen was visible in treated sample by 30th day, whereas only 2.26 log reduction could be visible in the absence of treatment. On the 60th day, total elimination of the pathogen was apparent in the treated samples, whereas, the untreated sample continued to carry the pathogen until the end of storage period. The reduction of bacterial load in the untreated samples as observed in the current study may be due to the effect of freezing and it was in correlation with the findings of Vasudevan et al. (2002) and Filip et al. (2010). Nevertheless, many microorganisms may be killed after the initial period of freezing, but when they are present in a sufficient number before freezing, they may survive even a long period of storage. Vasudevan et al. (2002) also reported that the freezing cannot be relied upon as a method to reduce *V. parahaemolyticus* in fish, since the time and magnitude of reduction depends on the initial load of the pathogen and the storage temperature. Therefore, freezing as a preservation method may not be appropriate if there is a high initial load of microorganisms before the freezing process which is evident from the present study.

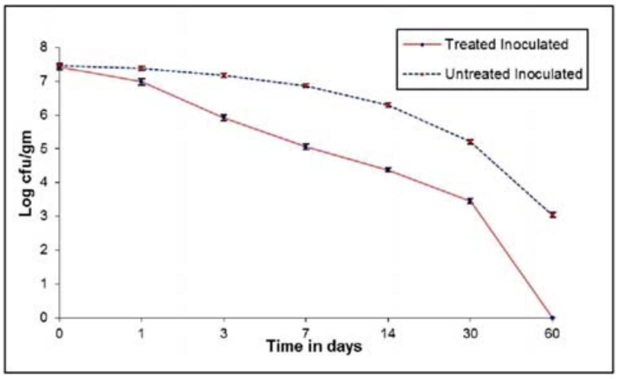

Fig. 6. Effect of Non phosphate: NaCl treatment on the survival of *V. parahaemolyticus* in IQF shrimps

The effects of organic acids and their salt as antibacterial agents in reducing the bacterial colonies during storage in food industries, especially in meat industries have been largely studied. Adding organic acids and their salts to seafood products has been suggested to extend shelf-life, limit pathogen proliferation, and maintain a synthetic preservative free marketing status. Data indicated that, there was a significant reduction of the bacterial load in non-phosphate treated samples compared to the untreated control samples. Similar observations were reported by Mahmoud (2013), wherein it was shown that organic acids such as citric acid was able to reduce growth of spoilage organisms in freshly shucked oyster sample. The results of this study also indicated the antibacterial effect of organic acids against *V. parahaemolyticus.* Citric acid is widely used in the food industries especially for its preservative action due to pH lowering and its synergetic effect for antioxidants due to its metal-chelating action.

The varying bacteriostatic and bactericidal effects of organic acids have been demonstrated in both culture media and food system. The treatments with 2 or 3% of citric acid reduced *E. coli* to undetectable levels (Oulkheir, 2015). Though specific report on antibacterial activity of non-phosphates on

V. parahaemolyticus, which is a gram negative pathogen, is not available, Lee et al. (2002) reported that sodium citrate has strong antimicrobial effect against gram-positive strains. Zhou et al. (2007) demonstrated the efficacy of citric acid and its salts in pathogen control in both fresh and processed meat and poultry. Citric acid and sodium citrate inhibits bacterial cells through metal chelation (Alakomi et al., 2007). Utility of sodium citrate in organic preservation of fish has been highlighted by Sallam, (2007) who reported that this salt has antibacterial activity against the proliferation of various spoilage microorganisms such as psychrotrophic populations, *Pseudomonas* spp. and Enterobacteriaceae. Mani- López et al. (2012) reported that citric acid has been used in food to eliminate pathogenic bacteria including *Salmonella.*

The current study found that dipping of shrimps in an aqueous solution containing organic acid and sodium salts of organic acids; namely citric acid and sodium citrate was efficient against *V. parahaemolyticus* as it reduced the bacterial load during storage; therefore this non phosphate mixture can be utilized as safe organic preservatives for fish under storage. However, our results showed that treatment with STPP is found to be more effective in reducing the *V. parahaemolyticus* load in shrimp as compared to the treatment with non-phosphate additive. Naturally occurring orthophosphates in meats and sodium tripolyphosphates in solutions in which meats were treated resulted in a counter-current diffusion. The initial diffusion rate of orthophosphate from the muscle to solution occurs quickly, but the rate reduces once the protective phosphate barrier is formed around the product (Ünal et al. 2004). A film of STPP and protein on the surface of the shrimp flesh promote the diffusion of phosphate molecules through the surface (Unal et al., 2006) might be the factor responsible for a greater inhibition on the survival of *V. parahaemolyticus* than non-phosphate additive.

In the present study, the effect of STTP: NaCl treatment was more predominant in the block frozen shrimps as the *V. parahaemolyticus* cells were completely eliminated within 14 days during storage whereas in individually frozen samples, *V. parahaemolyticus* showed relatively better survival. It might be due to the better availability of the STPP: NaCl solution to *V. parahaemolyticus* on shrimps that are block frozen, when compared to individually frozen. Ünal et al. (2006) reported that the diffusion of STPP is strongly temperature dependent. Since in the block frozen, shrimp is trapped in the block of ice, we assume that there is a possibility of the STPP: NaCl solution on the surface of the shrimp could be better act against *V. parahaemolyticus.* However, this is just a suggestion, not proven by experiments. No literatures are available with respect to the treatment efficacy of an additive under block frozen and individually quick frozen condition. The results revealed that from bactericidal point of view, non-phosphate treatments are not as effective as STPP: NaCl treatments.

Though the primary objective of phosphate treatments is prevention of drip loss, microbicidal activity would be an added advantage. This is especially important among the seafood processors in India, where microbial problems related to time/temperature abuse is common. *V. parahaemolyticus* has a rapid generation time and therefore even low numbers of the pathogen in raw seafood could outgrow to high levels, representing a potential public health hazard if abuse in storage temperature takes place (Twedt, 1989). Johnson et al. (1971) reported the recovery of *V. parahaemolyticus* from a variety of frozen seafoods, emphasizing the need for adequate control of this organism. In the light of present findings, we recommend continued usage of STPP in a judicious manner as it can better minimize the risk of *V. parahaemolyticus*, infection associated with seafood consumption.

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