Effect of water treatment on the growth potential of *Vibrio cholerae* and *V. parahaemolyticus* in seawater.

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Abstract

In laboratory experiments we added *Vibrio cholerae* and *V. parahaemolyticus* to bottles with seawater previously treated by filtration, UV, chlorine or ozone. The purpose was to investigate the influence of different treatment techniques on the growth potential of these bacteria in simulated ballast water tanks. Residual oxidants were removed before inoculation, and the bottles were incubated at 21 ± 1 °C. The growth potential of the vibrios was investigated in two different experimental setups, i.e. in presence and absence of added natural microorganisms. In general, *V. cholerae* and *V. parahaemolyticus* rapidly lost their culturability after inoculation and storage in untreated seawater, but showed increased survival or growth in the treated water. Highest growth was observed in water previously exposed to high concentrations of ozone. Addition of natural microorganisms reduced the growth of *V. cholerae* and *V. parahaemolyticus*.

Keywords: Ballast water; Bacteria; Water treatment; Ozone; Vibrio cholerae; Vibrio parahaemolyticus

1. Introduction

The introduction of invasive marine species into new environments by ships' ballast water has been identified as one of the greatest threats to the world's oceans. For avoiding such bio invasions, the International Maritime Organization (IMO) Convention on ballast water management has required overseas vessels to limit the number of viable organisms and indicator microbes in ballast water prior to discharge (IMO, 2004). To meet these requirements ships may install ballast water treatment equipment. Technologies for treatment of ballast water often include disinfection (e.g. UV treatment, ozonation or chlorination) for

inactivation of microorganisms. While disinfection of ballast water during uptake substantially reduces the number of viable bacteria, it does not sterilize the water, allowing surviving bacteria or bacteria already present in the ballast tank (seed reservoir) to multiply during storage in the ballast tanks (Waite *et al.* 2003; Perrins *et al.* 2006; Hess-Erga *et al.* 2010).

Ballast water treatment systems generally do not include removal of natural occurring dissolved organic matter (DOM) and other nutrients for bacterial growth. Oxidation-based disinfection processes (e.g. ozonation) are well known to increase the fraction of bio-available DOM in drinking water by oxidizing non biodegradable organic molecules to smaller and more biodegradable molecules, thereby promoting growth of heterotrophic bacteria in the distribution system (Yavich *et al.* 2004; Swietlik *et al*. 2009). Ozonation and UV-treatment are also shown to create easily degradable substrates for bacterial growth in seawater (Hess-Erga *et al.* 2010).

For avoiding regrowth of heterotrophic bacteria in ballast tanks, the importance of obtaining a high level of total residual oxidant (TRO) was reported by Perrins *et al*. (2006). They treated marine ballast water with ozone and showed that when the TRO concentration fell below the bacterial inhibition threshold (below 0.5-1.0 mg/L as Br_2), heterotrophic bacteria grew rapidly, sometimes to higher levels than in the controls.

Additional disinfection at discharge is an approach to reduce the number of heterotrophic bacteria in the discharged water. For technologies which do not include disinfection at discharge, large numbers of viable bacteria, grown up during storage in ballast tanks, can be discharged to surface water by ships' ballasting operations. This does not represent a problem as long as the bacteria are harmless for humans and aquatic life. As a reference, heterotrophic bacteria multiplying in drinking water in general is not considered as a significant health risk for people with normal immune defence (WHO, 2003).

Little is known about the potential health relevance of the regrowth in ballast tanks after different disinfection processes. A previous study indicated that ozonation of seawater, with subsequent removal of TRO by sodium thiosulphate, created conditions that favoured the growth of vibrios at 20 °C, most likely by production of bio-available DOM (Tryland *et al.*, 2010). The vibrio group includes the causative agent of cholera, *V. cholerae* and other human

pathogenic species like *V. vulnificus* and *V. parahaemolyticus*, but also other non-harmful species (Farmer and Hickman-Brenner, 1991). Regrowth of vibrios relative to other heterotrophic bacteria is known to depend on factors such as temperature, salinity, predation by eukaryotes, infection by viruses and bio-available DOM. For example cyanobacterial-derived DOM (homogenized *Nodularia spumigena*) was shown to stimulate growth of *V. cholerae* and *V. vulnificus*, and the contribution of *V. cholerae* to total *Vibrio* spp. abundance and total bacterial counts increased with increasing DOM concentration (Eiler *et al*., 2007).

The purpose of this study was to investigate whether treatment of seawater (filtration, UV-, chlorine- or ozone treatment) altered the growth potential of *V. cholerae* and *V. parahaemolyticus*. Simulation of recontamination in ballast tanks was studied by adding *V. cholerae* and *V. parahaemolyticus* to treated seawater in microcosms/bottles with or without a natural community of microorganisms. TRO was removed by sodium thiosulphate before inoculation, and bacterial survival and growth was studied at 21 ± 1 °C for 5 days.

2. Materials and methods

2.1. Water samples

Seawater samples were collected from the Inner Oslofjord, at the Huk beach the three first sampling days, and at Bjørvika (near the outlet of river Akerselva) the last sampling day. Characteristics of the water samples are shown in Table 1.

2.2. Treatment of water samples

The seawater samples were treated by different techniques in laboratory experiments at room-temperature: 1) No treatment, 2) Sterile filtration using a 0.2 µm pre-washed cellulose nitrate filter, 3) UV-treatment (120 mWs/cm^2) using collimated beam equipment as described by Liltved *et al.* (1995), 4) Chlorination by adding sodium hypochlorite (3 mg/L) (total residual oxidant (TRO) was measured to 1.0-1.9 mg Cl_2/L after 30 minutes contact time using the colorimetric DPD-method (Hach Lange GmbH, Düsseldorf, Germany)), and 5) Ozonation by bubbling ozone-containing oxygen (Liltved *et al.* 1995) to 300-600 ml test waters in glass flasks via a diffusor for 4 sec (low dose) or 30 sec (high dose). Immediately after removal of the diffusor the TRO level was measured to 1.6-1.9 mg Cl_2/L (low dose) and 5-6 mg Cl_2/L (high dose), and the TRO level remained constant for 30 min. Sodium thiosulphate (30 mg/L)

was added to the chlorinated and ozonated water samples after 30 min reaction time for neutralizing the residual oxidants.

2.3. Bacterial strains and inoculum preparation

2.3.1. Bacterial strains

Vibrio parahaemolyticus (CCUG 14474) was used in the three first experiments, i. e. in all experiments dealing with *V. parahaemolyticus*. The strain is pathogenic to man and was isolated from shirasu food poisoning in Japan. Different *V. cholerae* strains were used in the different experiments because a low recovery on selective agar was observed for the strains used in the first experiments. This encouraged the testing of different *V. cholearae* strains: In the first experiment, *V. cholerae* (CCUG 537) was used. This strain was anhaemolytic and is an opportunistic pathogen to man. The strain belongs to serotype O:13 and the cholera toxin gene *ctx*A is not present. In experiment 3, a β-haemolytic strain of *V. cholerae* (NCTC 7254) was used. This strain is pathogenic to man, and belongs to serovar O:1/Subgroup I. The strain was isolated from a cholera epidemic in Egypt. In experiment 4, *V. cholerae* strain 503 (NVH isolate from beach water, non O:1/O:139, with a good recovery on selective agar) was used.

2.3.2. Broth enrichment of test bacteria and preparation of inoculum

V. cholerae and *V. parahaemolyticus*, stored as stock cultures in freezer at -80 ºC, were streaked on blood agar plates (BA) and incubated for 24 hours at 37 °C. One single colony of each strain was transferred to enrichment broth. *V. cholerae* was inoculated in nutrient broth. For *V. parahaemolyticus* nutrient broth with 6 % sodium chloride was used. Broth cultures were incubated at 37 °C for 24 h. Tenfold dilutions were plated on Blood agar and/or thiosulfate-citrate-bile salts-sucrose agar (TCBS) (experiment 4) and incubated for 24 hours at 37 °C to determine the concentration.

The broth cultures of *V. cholerae* and *V. parahaemolyticus* were washed in sterile seawater by a repeated centrifugation and further diluted in sterile seawater for preparation of bacterial inoculum.

2.4. Experimental design

Four experiments were performed:

1. Addition of pure cultures of *V. cholerae (*CCUG 537) or *V. parahaemolyticus* to treated and untreated seawater
2. Addition of *V. parahaemolyticus* and natural microorganisms to treated and untreated seawater
3. Addition of *V. cholerae (*NCTC 7254) or *V. parahaemolyticus* and natural microorganisms to treated and untreated seawater
4. Addition of *V. cholerae* strain 503 and natural microorganisms to untreated and ozonated water, followed by detection with PCR

The following procedure where used for all experiments:
Fifty ml of the different seawater samples (no treatment, sterile filtered, UV-treated, chlorinated, low-ozonated and high-ozonated) were placed in 100 ml brown glass bottles/microcosms (simulating ballast tanks). The bottles were inoculated with *V. parahaemolyticus* (Experiment 1, 2 and 3) or *V. cholerae* (experiment 1, 3 and 4) to a final expected concentration of 10^2-10^3 cfu/ml. All experiments were done with triplicate bottles. For investigating the potential growth in presence of a natural environment (with both competitive heterotrophic bacteria, viruses and predators), native inoculum (0.5 ml untreated seawater from the same locality as the treated) was also added to a set of 3 bottles in experiment 2, 3 and 4. After the addition of inoculum the microcosms were stored at 21±1 °C and the numbers of *V. cholerae*/*V. parahaemolyticus* and HPC were measured after 0, 2 and 5 days storage. Quantification of bacteria on day 0 verified the expected bacterial numbers in the added inoculums.

2.5. Microbiological methods

From appropriate dilutions of the microcosms *V. cholerae* and *V. parahaemolyticus* were recovered by spread plate technique on TCBS (TCBS-agar CM 333, Oxoid Ltd, Basingstoke, Hampshire, UK) agar and blood agar (BA). Colonies were examined based on morphology and counted after 24 h incubation at 37 °C.
TCBS is the standard selective agar medium for human pathogenic vibrios in food (Nordic Committee on Food Analysis,1997, International Organization for Standardization, 2007), and is frequently used for detection of *V. cholerae* in ballast water. BA is a commonly used non-selective medium for isolation of bacteria. For *V. cholerae* strains (CCUG 537) and (NCTC

7254), a much higher recovery on BA compared to TCBS was observed, i. e. the recovery on TCBS was only about 1% compared with the recovery on BA. As a consequence we did our calculations for the numbers of *V. cholerae* strains (CCUG 537) and (NCTC 7254) from the counts on BA. *V. cholerae* (CCUG 537) was counted as anhaemolytic, grey, opaque colonies with 2-3 mm in diameter when no other heterotrophic bacteria were added. In the experiments adding natural microorganisms (1 % seawater) the background growth interfered with *V. cholerae* (CCUG 537), and a satisfactory recognition of anhaemolytic target colonies could not be conducted. Consequently, *V. cholerae* (NCTC 7254) was used for experiment 2 because it produced a clear zone of β-haemolysis on BA and was easy to distinguish from the background growth.

For *V. parahaemolyticus* and *V. cholerae* strain 503 the recovery on TCBS compared to BA was much better, 84 % and 73%, respectively, and TCBS counts were used for calculation of the bacterial numbers.

As an indicator of the natural microbial community the total number of culturable bacteria (heterotrophic plate count, HPC) was examined by spread plate on Difco Marine agar (Marine Agar 2216, Catalogue no. 212185. BD Diagnostics, Maryland, USA). Colonies were counted after 3 days incubation at 22°C.

2.6. PCR method

Experiment 4 was conducted to verify the results in experiment 1 and 3 with a culture independent method. Quantitative real-time PCR (qPCR) targeting the *gro*El gene specific for *V.cholerae* was used to monitor the concentration of *V. cholerae* 503 in experiment 4. Samples (2 x 1ml) were withdrawn on day 0, 2 and 5, and concentrated in PCR grade water (100µl) by centrifugation for 5 min. at 10 000rpm. qPCR was performed on Bio-Rad CFX96 (Bio-Rad) with a PCR reaction mix (20µl reaction volume) consisting of: SooFast^{TM} Probes Supermix (Biorad) (10µl), *gro*El primer forward and reverse (0.25 µM final concentration), *gro*El hydrolysis probe (0.25 µM final concentration) and 2µl template. The primer and probe sequence and specificity has been documented in Fykse *et al*., 2012. The reaction mixture was denatured at 95°C for two min, followed by 40 cycles of 95°C for 10sec and 64°C for 30sec. The experimental setup had tree parallels of each treatment, thus two technical parallels were considered sufficient for each experimental parallel. Thus, duplicate reactions were run for each template and standard concentration. The results were managed in Bio-Rad software

program: CFX Manager Version: 2.1.1022.0523 software. A standard curve with three tenfold dilution of *V. cholerae* 503 was run parallel to the samples for calculation of sample concentrations. The *V. cholerae* 503 culture used for the standard curve was 10 fold diluted and quantified by spread plate technique on TCBS, and the CFX Manager software used this information to calculate probable concentration of the unknown samples.

2.7. Calculation of bacterial numbers

From the three parallels in each experiment arithmetic mean were calculated and presented.

3. Results and discussion

In untreated seawater, the number of culturable *V. cholerae* and *V. parahaemolyticus* decreased significantly (> 1 $\log_{10}$ unit reduction) after 5 days storage (Figure 1-4). Although the temperature during storage (21 ± 1°C) was adequate for growth of *V. cholerae* and *V. parahaemolyticus*, other conditions in the untreated seawater, like presence of competitive bacteria, predators and viruses and low levels of bio-available nutrients, may have prevented the establishment and growth of the added vibrios.

Previous disinfection of the seawater strongly affected the survival and growth of the added *V. cholerae* and *V. parahaemolyticus*. In the first experiment, where no natural microorganisms were added, growth of both *V. cholerae* (Figure 1A) and *V. parahaemolyticus* (Figure 1B) were observed in all bottles containing treated seawater. The strongest growth (close to 10^6 cfu/ml at day 5) was observed in seawater previously exposed to high levels of ozone (Figure 1). This may be explained by lower levels or absence of predators and competitors in the disinfected seawater, as well as higher levels of easy degradable substrates generated by the strong oxidation process. An increase of available nutrients for bacterial growth after disinfection may take place by different mechanisms: Rupture or killing of cells with a concurrent release of cellular matter, which is further degraded by released enzymes into DOM, and by chemical modification (oxidation) of existing DOM by the disinfectants (Swietlik *et al*. 2009; Hess-Erga *et al.* 2010).

The experimental design without added natural microorganisms (Figure 1), may have overestimated the growth of the two added bacteria compared to a realistic ballast water

management situation. Under real conditions, *V. cholerae* and V. *parahaemolyticus* will not be present as the sole or dominating bacteria in the treated ballast water. If some *V. cholerae* or *V. parahaemolyticus* survive the disinfection of influent ballast water, e. g. protected by particles during disinfection (Liltved and Cripps 1999, Hess-Erga *et al*., 2008) or if a contamination occur downstreams (e. g. from sediments or biofilms in the ballast tanks), there will always be other heterotrophic bacteria present, competing for available growth substrates.

A following up study was therefore performed. In these experiments 0.5 ml untreated seawater was inoculated to the microcosms simultaneously with the *V. cholerae* or *V. parahaemolyticus*. The volume of untreated seawater should illustrate a practical situation where an assumed fraction of approximately 1% of the native heterotrophic bacteria, viruses and predators bypass the water treatment. The purpose of this experiment was to identify if any of the water treatment processes produced conditions which gave the potential pathogenic vibrios a competitive advantage relative the other heterotrophic bacteria in a more natural microbial environment.

Growth of *V. cholerae* was only observed in the microcosms with seawater previously exposed to high ozone doses (Figure 2A). A rapid decrease of culturable *V. cholerae* was observed in the untreated and filtered seawater. A slower decrease was observed in the seawater previously treated by UV, chorine and low ozone dose (Figure 2A). The corresponding HPC are shown in Figure 2B. An increase in HPC was observed in all the disinfected water samples, and the results therefore indicate that all the applied disinfection processes will increase the regrowth potential with regard to heterotrophic bacteria. Increased growth of *V. cholerae* was not revealed, except in water treated with high ozone dose. Absence of growth can be explained by outnumbering of *V. cholerae* by the competitive heterotrophic bacteria, e. g. due to available growth factors and/or higher survival from predators and viruses.

Growth of *V. parahaemolyticus* was observed in most of the microcosms containing disinfected seawater, even in presence of added natural microorganisms (Figure 3). Results from two different experiments based on seawater collected in January 2010 and April 2010 are shown in figure 3A and 3B, water characteristics are reported in Table 1. Although the pattern of Figure 3A and 3B are somewhat different, it illustrates how *V. parahaemolyticus* can grow in disinfected seawater, in contrast to the rapid decay observed in untreated

seawater. The disinfected seawater initially contained only 1% of native microorganisms compared with untreated seawater. Lower competition- and predation pressure, as well as higher levels of easy degradable substrates generated by oxidation processes, may explain the different fate of *V. parahaemolyticus* in the disinfected seawater compared with untreated seawater.

In the present study both TCBS and BA were used for the isolation and enumeration of *V. cholerae* and *V. parahaemolyticus.* While *V. parahaemolyticus* and the environmental isolate *V. cholerae* strain 503 showed good recovery on TCBS, the strains *V. cholerae* (CCUG 537) *and V. cholerae* (NCTC 7254) showed only 1% recovery on TCBS compared to BA. *V. cholerea* (CCUG 537) is an opportunistic pathogen to man. The strain belongs to serotype O:13 and the cholera toxin gene *ctx*A is not present. *V. cholerae* (NCTC 7254) is pathogenic to man. It belongs to serovar O:1/Subgroup I. The strain was isolated from a cholera epidemic in Egypt. *V.cholerae* (NVH 503) is a non O:1/O:139 environmental isolate. These results indicate that the different *V. cholerae* strains may behave different after dilution in seawater, i.e. some strains may no longer be culturable on the highly selective TCBS, but still be culturable on BA.

It is well known that species of several bacterial genera, including the vibrios, are able to enter a viable but non-culturable (VBNC) state in seawater (Kogure *et al.* 1979, Eiler *et al*., 2007). This will also apply to ballast water, indicating that specific bacteria can be present in a sample even though growth is absence on a conventional media. It has further been reported that some human pathogens, most notably *Vibrio cholerae*, can maintain their infectious potential even after entering the VBNC state (Grimes *et al*. 1986). A follow up study was performed, including a culture independent method, to investigate whether the plating techniques affected the result. In experiment 4 *V. cholerae* strain 503 was added to untreated seawater and seawater treated with high concentration of ozone with and without competitive native inoculum. The *V. cholerae* strain used in this experiment grew well on both TCBS and BA. The recovery of *V. cholerae* on TCBS was followed on day 0, 2 and 5 together with a quantification by qPCR and plating of HPC. The seawater quality deviated from the earlier experiments (see table 1), but the result of the plating techniques showed a similar development of the cultures to the earlier experiments (Figure 4 A and C): *V. cholerae* had increased growth in ozonated water, and decreased growth in untreated water. The result of the qPCR (Standardcurve Efficiency 94%,

306 Slope – 3.473, $R^{\wedge 2}$: 0.968) confirmed the result of the plate technique: *V. cholerae* multiplied
307 in ozonated seawater, but was repressed by competitive heterotrophic bacteria (Figure 4 B).
308 The decrease in the number of *V. cholerae* in untreated seawater demonstrated by qPCR
309 showed that *V. cholerae* were killed (i.e. by predators or virus), not just in an unculturable
310 state. The qPCR method might have overestimated the concentration of viable bacteria since
311 the DNA in the PCR reaction target can be intact after the cell is dead. However, the possible
312 error in concentration due to dissolved DNA in the culture was reduced by centrifugation of
313 the samples and replacing the seawater medium with PCR grade water.
314
315 Growth of potential pathogenic vibrios relative to other harmless heterotrophic bacteria in
316 ballast tanks is a complex process which depends on factors such as temperature (≥ 20°C),
317 salinity, predation by eukaryotes, infection by viruses, bio-available DOM, as well as initial
318 population of microorganisms. In our experiments *V. parahaemolyticus* and *V. cholerae* were
319 added after the disinfection process and after removal of residual oxidants, thus simulating
320 recontamination. In a realistic situation, initial presence of pathogens is of course required for
321 their growth. In general, disinfection of influent ballast water is expected to reduce the risk of
322 human pathogens in the ballast water since most pathogens seem to be more sensitive to
323 common disinfection processes than several other competitive heterotrophic bacteria (Hijnen
324 *et al.*, 2006; Yasar, 2007). Introduction of pathogens may, however, potentially occur
325 downstream the disinfection, e. g. from sediments or biofilms in the ballast tanks. Presence of
326 a residual oxidant in the ballast tank will also affect the microbial ecology. For the highest
327 ozone dose used in our experiment, without addition of sodium thiosulphate a significant
328 TRO would most probably have been maintained for several days in a real ballast tank and
329 thus prevented bacterial growth (Perrins *et al*., 2006; Wright *et al*., 2010), unless high levels
330 of oxidant-consuming materials were present.
331
332 Time of storage will also affect the microbial ecology in ballast tanks. Immediately after
333 disinfection (if not a significant disinfection residual is present), growth of bacteria with high
334 maximum growth rate will occur. After such an initial phase, competition due to crowding
335 and nutrient limitation will favour bacteria with high substrate affinity (Hess-Erga *et al.*
336 2010). Joachimsthal *et al.* (2003) found a gradual creation of anaerobic conditions in a ballast
337 tank and suggested that this could lead to the accumulation of facultative anaerobic
338 microorganisms, which might represent a potential source of pathogenic species.
339

4. Conclusion

Disinfection and consecutive storage in ballast tanks may alter the bacterial community in the ballast water both due to selective inactivation and due to selective regrowth. In our experiments low levels of bio-available substrates and presence of competitive heterotrophic bacteria and predators seemed to prevent the growth of the added pathogenic vibrios in untreated seawater. The experiments illustrated how disinfection of seawater, most probably by generating more easy degradable substrates and removing predators and competitors, can alter the growth potential with regard to rapid multiplying heterotrophic bacteria, including *V. parahaemolyticus* and *V. cholerae*.

Growth of potential pathogenic bacteria relative to other harmless heterotrophic bacteria in ballast tanks is a complex process which depends on several factors, including possible contamination routes, survival in sediments, different physiological and chemical growth factors and different sensitivity to treatments. More work is required to study this complex mechanism, to be able to give recommendations with regard to the relevance of regrowth of heterotrophic bacteria in disinfected ballast water (both for humans and aquatic life) and the potential requirement for additional disinfection at discharge.

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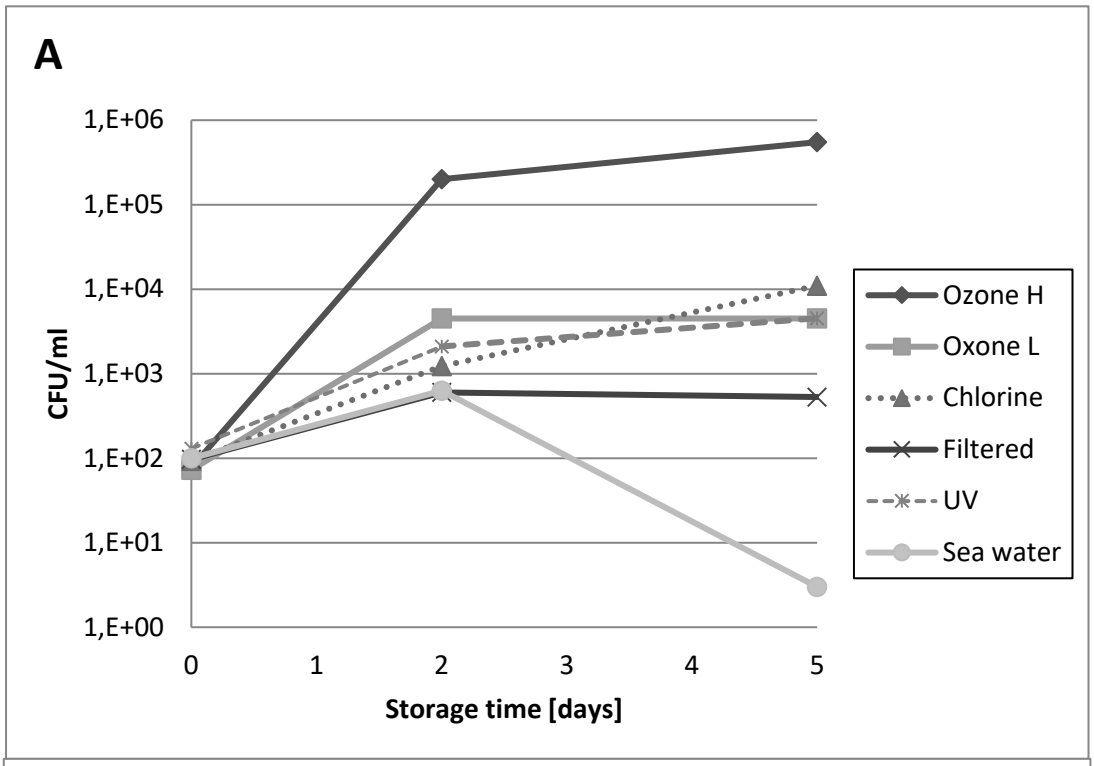
Figure captions

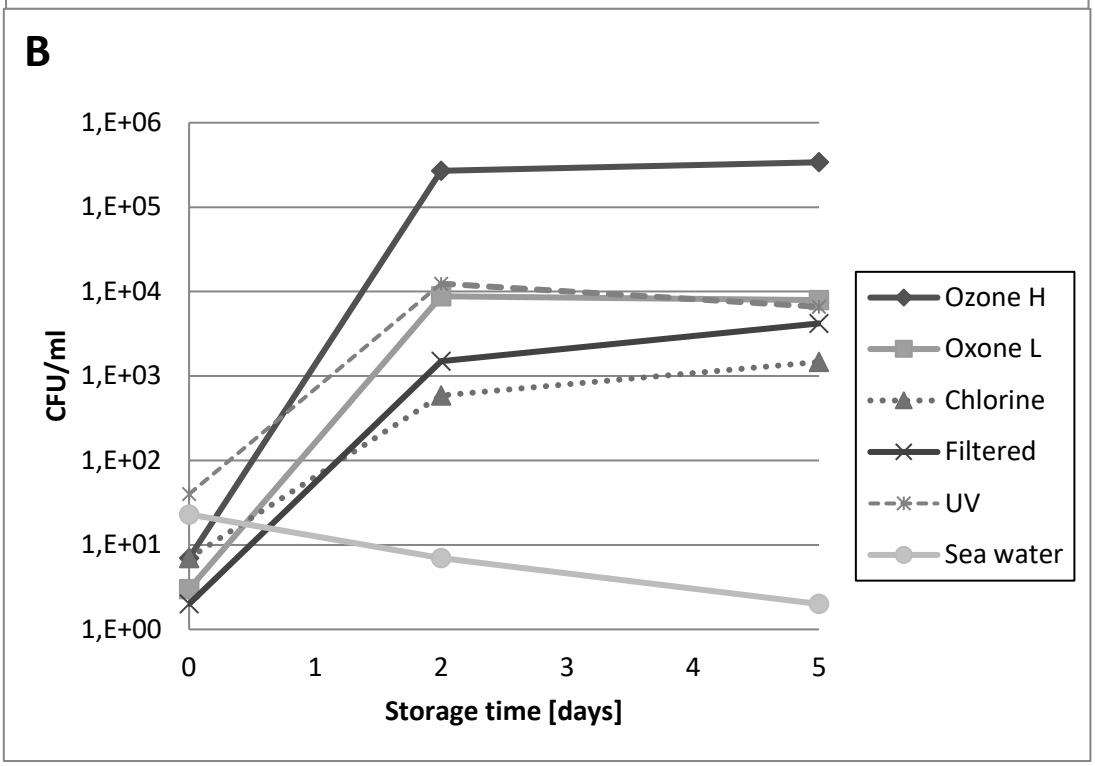
Figure 1. Fate of *V. cholera* (Figure A) and *V. parahaemolyticus* (Figure B) inoculated to seawater previously treated by different disinfection methods, after 0, 2 and 5 days storage at 20 °C. Each data point represents mean value from 3 microcosms.

Figure 2. Fate of *V. cholerae* (Figure A) inoculated to seawater previously treated by different disinfection methods, in presence of added competitive heterotrophic bacteria, after 0, 2 and 5 days storage at 20 °C. Corresponding HPC are shown in Figure B. Each data point represents mean value of 3 microcosms.

Figure 3. Fate of *V. parahaemolyticus* added to seawater previously treated by different disinfection methods, in presence of added competitive heterotrophic bacteria, after 0, 2 and 5 days storage at 20 °C. Results from two different experiments are shown in Figure A and B. Each data point represents mean value of 3 microcosms.

Figure 4. Recovery on TCBS agar of *V. cholerae* (Figure A) inoculated to seawater previously treated by ozonation, after 0, 2 and 5 days storage at 22 °C with and without competitive heterotrophic bacteria. Corresponding qPCR results targeting *V. cholerae* specific *gro*El DNA are shown in Figure B, and corresponding HPC are shown in Figure C. Each data point represents mean value of 3 microcosms with standard deviation presented as error bars.


A
1,E+06
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
CFU/ml
0
1
2
3
4
5
Storage time [days]
Ozone H
Oxone L
Chlorine
Filtered
UV
Sea water


B
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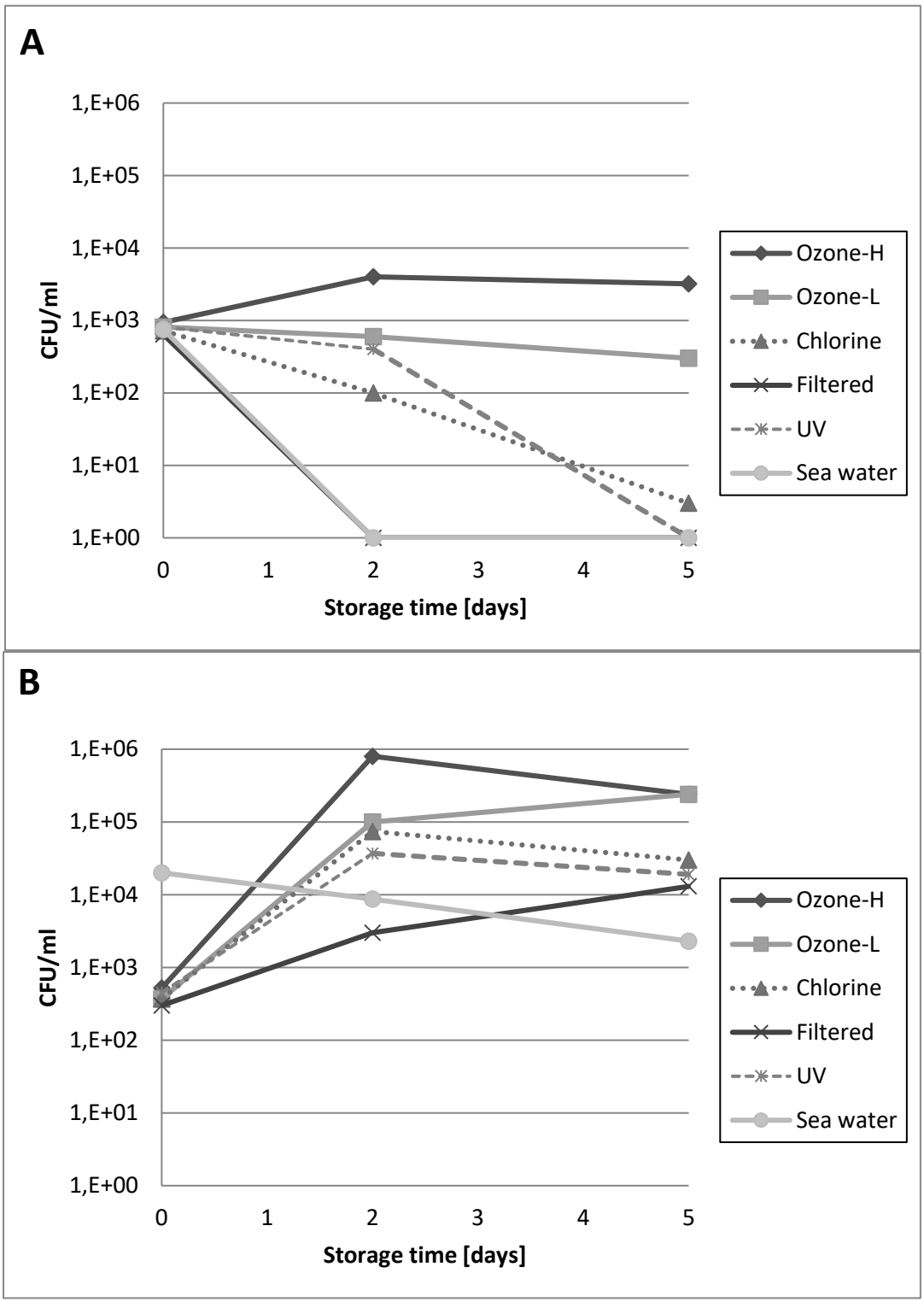
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
CFU/ml
0
1
2
3
4
5
Storage time [days]
Ozone H
Oxone L
Chlorine
Filtered
UV
Sea water


A
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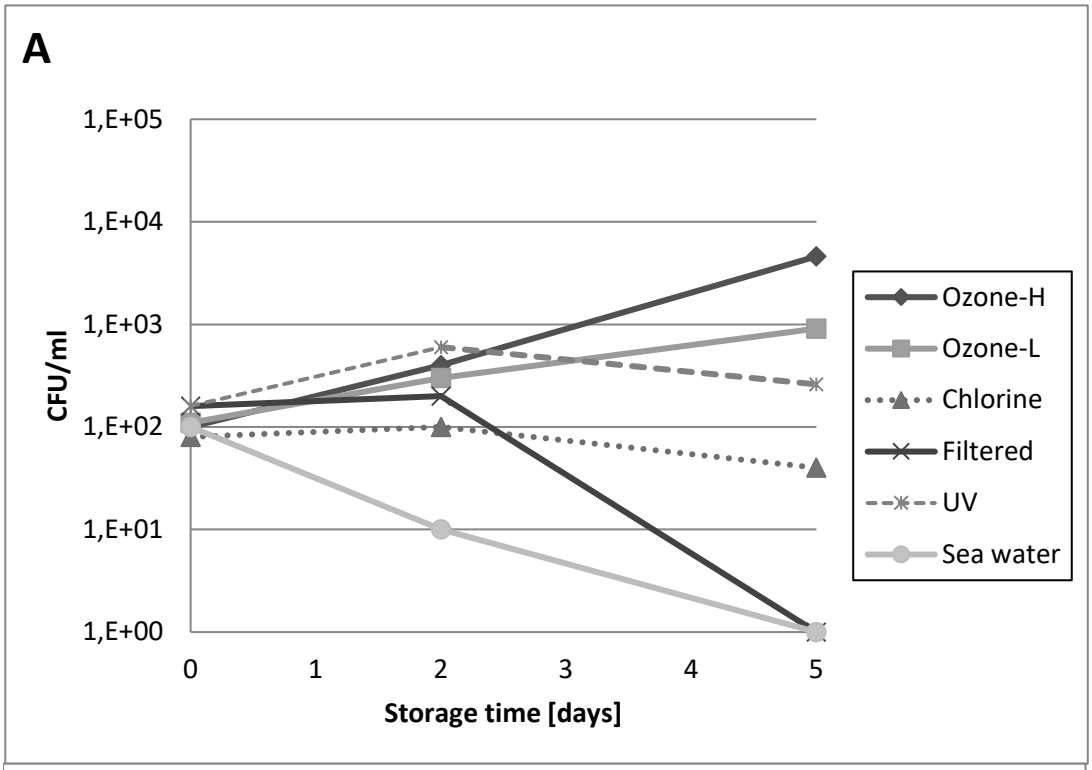
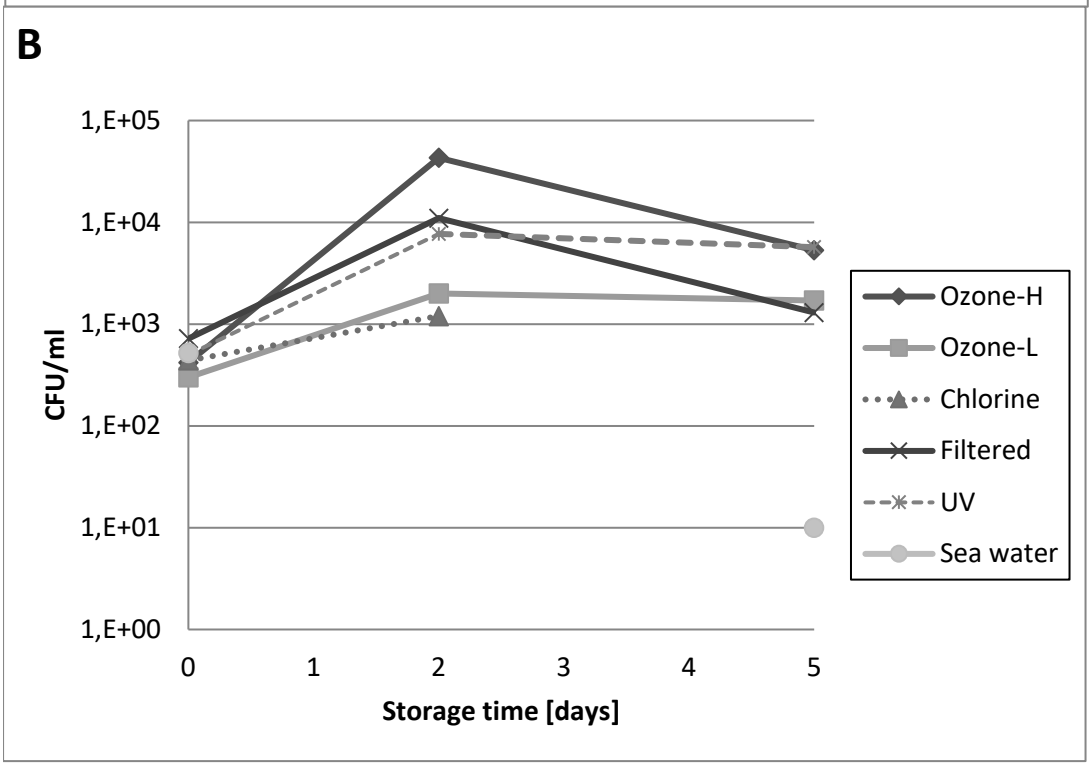
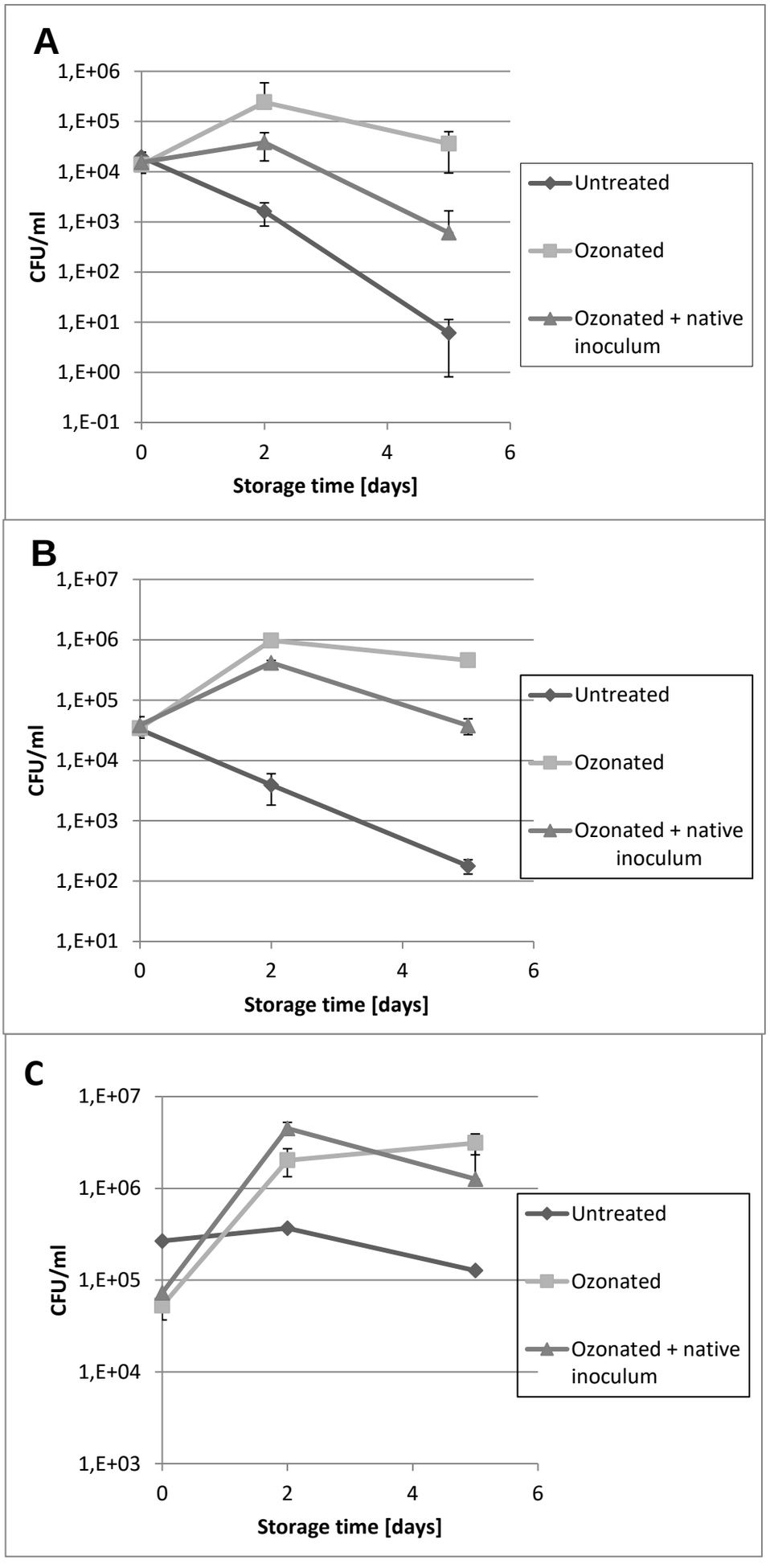
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
CFU/ml
0
1
2
3
4
5
Storage time [days]
Ozone-H
Ozone-L
Chlorine
Filtered
UV
Sea water
B
1,E+06
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
CFU/ml
0
1
2
3
4
5
Storage time [days]
Ozone-H
Ozone-L
Chlorine
Filtered
UV
Sea water


A
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
CFU/ml
0
1
2
3
4
5
Storage time [days]
Ozone-H
Ozone-L
Chlorine
Filtered
UV
Sea water


B
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
CFU/ml
0
1
2
3
4
5
Storage time [days]
Ozone-H
Ozone-L
Chlorine
Filtered
UV
Sea water


A
1,E+06
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1,E-01
CFU/ml
0
2
4
6
Storage time [days]
Untreated
Ozonated
Ozonated + native inoculum
B
1,E+07
1,E+06
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
CFU/ml
0
2
4
6
Storage time [days]
Untreated
Ozonated
Ozonated + native inoculum
C
1,E+07
1,E+06
1,E+05
1,E+04
1,E+03
CFU/ml
0
2
4
6
Storage time [days]
Untreated
Ozonated
Ozonated + native inoculum