

# Zooplankton and aggregates as refuge for aquatic bacteria: protection from UV, heat and ozone stresses used for water treatment

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## Summary

**Aggregates and zooplankton may provide refuge for aquatic bacteria against external hazards. The ability of attached bacteria to survive and recover from stressors commonly used for water treatment was tested in the laboratory. Without zooplankton or aggregates, both UV and ozone significantly reduced abundance of free-living bacteria in both freshwater and marine medium. The presence of zooplankton carcasses and aggregates, however, allowed some of the attached bacteria to survive and recover quickly within 3 days. Heat exposure was the least effective as both free-living and attached bacteria were able to recover quickly. Selective survival of bacterial phylogenotypes led to large changes in bacterial community composition after stress exposures, and some of the bacteria that recovered belonged to groups with known pathogens. This study demonstrates that zooplankton and aggregates protected various aquatic bacteria from external stressors, and organic remains generated from zooplankton and aggregates after stress exposure even enabled the surviving bacteria to quickly regrow and subsequently be released into the surrounding water. Hence, water disinfection treatments that overlooked the potential persistence of bacteria associated with organisms and aggregates may not be effective in preventing the spread of undesirable bacteria.**

## Introduction

Within the water column bacteria may exist as free-living cells or be attached to various surfaces, e.g. aggregates

and living organisms such as zooplankton. Bacterial abundances associated with aggregates and zooplankton can be highly variable; nevertheless, calculations based on literature data suggest that bacteria attached to aggregates and zooplankton are orders of magnitude more concentrated than ambient bacteria, and can constitute a significant portion of total water column bacteria (Simon *et al.*, 2002; Tang *et al.*, 2010). The physiology and ecology of these attached bacteria are inherently dependent on the micro-environment in which they are present, and thus cannot be accurately characterized by traditional bulk water measurements. For example, cell–cell interactions on aggregate surfaces can modulate the development of the attached bacterial community (e.g. Grossart *et al.*, 2003a), and the bacterial community structure associated with a zooplankton is influenced by the host's feeding history (Grossart *et al.*, 2009; Tang *et al.*, 2009a). Direct association of bacteria with aggregates and zooplankton enhances bacterial dispersal rate and distance (Grossart *et al.*, 2010), and may also drive biogeochemical reactions at rates higher than in the ambient water (Smith *et al.*, 1992; Tang *et al.*, 2001; Grossart *et al.*, 2003b).

An obvious advantage for bacteria to attach to aggregates and/or zooplankton is direct access to rich organic substrates within these micro-environments, which allow the bacteria to attain much higher growth rates than their free-living counterpart (Tang, 2005; Grossart *et al.*, 2007). Another potential benefit is protection from external hazards, similar to other well-studied biofilms (Mah and O'Toole, 2001). Physical and chemical stressors are commonly used in commercial water disinfection (Shannon *et al.*, 2008). By residing in interstitial spaces within an aggregate or inside the body of a zooplankton, attached bacteria will significantly benefit from protection against these external stressors. For example, bacteria inside protozoans can survive disinfectants that normally kill free-living bacteria (Barker and Brown, 1994; Bichai *et al.*, 2008). In addition, bacteria and bacterial spores in nematode's gut can also survive UV radiation during drinking water processing and thus pose potential threats to public health (Bichai *et al.*, 2009). Inside a zooplankton's gut microbes face challenges such as digestive enzymes and low oxygen concentration, but this micro-environment is

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more predictable and stable than the ambient water, and arguably would allow bacteria to adapt more readily (Harris, 1993). Indeed, the observed high numbers of enteric bacteria inside zooplankton suggest that many bacteria do survive and thrive in this micro-environment (Nagasawa *et al.*, 1985; Nagasawa and Nemoto, 1988). The association of pathogenic bacteria, such as *Vibrio* spp., with copepods and aggregates has been well documented (Huq *et al.*, 1983; Cottingham *et al.*, 2003; Lyons *et al.*, 2005; 2010). Therefore, increased dispersal of zooplankton and aggregates and their attached microflora because of anthropogenic activities such as ballast water shipping can have severe consequences for human and ecosystem health if these pathogens can survive mandatory ballast water treatment. The potential ability of the attached bacteria to survive several stressors is an important consideration not only for understanding their ecology but also for formulating a more effective public health management (Vezzulli *et al.*, 2010).

In this study, we tested the hypothesis that, in contrast to free-living bacteria, those attached to aggregates and zooplankton can better survive external stressors and are subsequently released into the surrounding water. We tested three stressors commonly applied in water treatment in both freshwater and marine medium: UV, heat and ozone. UV can have multiple negative effects on bacteria, such as direct damage of DNA (e.g. Sinha and Häder, 2002), whereas excessive heat mainly causes enzyme deactivation and protein denaturing (e.g. Nguyen *et al.*, 1989). Ozone is a strong oxidizing agent that causes multiple structural damages to bacterial cells (Komanapalli and Lau, 1996; Thanomsub *et al.*, 2002). Because of their strong negative effects on cell physiology, all three stressors are commonly used for treatments of drinking and ballast waters. Thus, the ability of bacteria

including potential pathogens to survive these stressors by attaching to aggregates and zooplankton has important ecological and environmental implications.

Survival of both free-living and attached bacteria was determined by their ability to regrow after exposure to the aforementioned stressors. To test whether different stressors select for the growth of specific bacteria and lead to changes in bacterial community composition (BCC), we performed denaturing gradient gel electrophoresis (DGGE) and DNA clone library analysis. Our results indicate that attached bacteria not only had a higher ability to survive than their free-living counterpart, they even benefited from the organic remains of the aggregates and zooplankton after stress exposures and attained rapid regrowth. This resulted in a significant change of community composition of the attached as well as free-living bacteria over time.

## Results

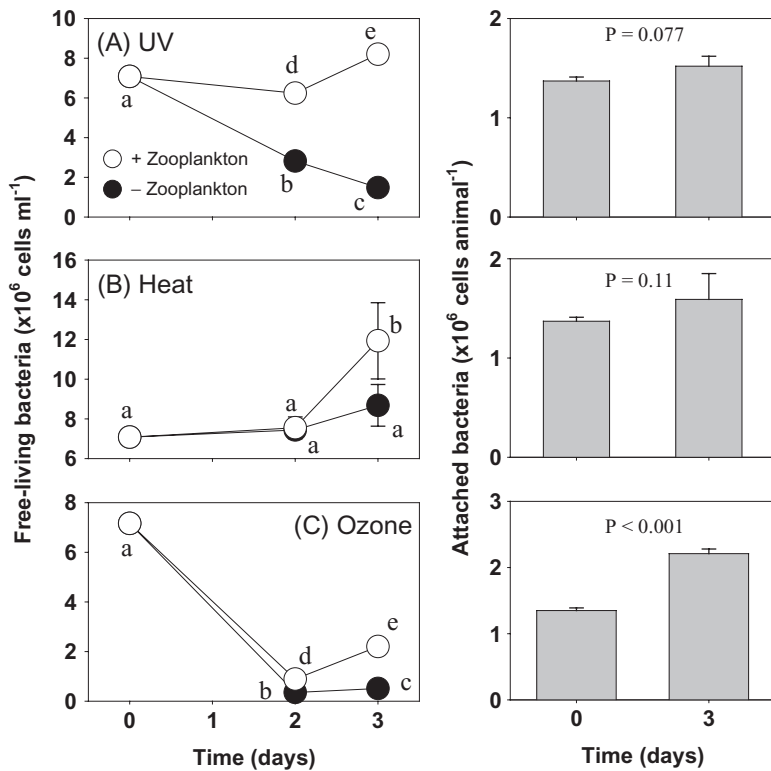
### *Bacterial recovery after UV, heat and ozone exposures*

**Freshwater zooplankton.** UV radiation in the presence of the copepod *Eudiaptomus gracilis* led to an initial decrease in the abundance of free-living bacteria, which thereafter increased significantly (Table 1). In contrast, without copepods (control) it decreased by 80% within 3 days after UV radiation (Fig. 1A). There was no significant change in bacterial abundance attached to the copepod (Table 1), which remained at a high level of  $1.4\text{--}1.5 \times 10^6$  cells per animal (Fig. 1A). In the presence of the daphnid *Ceriodaphnia* sp., the abundance of free-living bacteria increased fivefold in 3 days (Fig. 2); at the same time, numbers of attached bacteria also significantly increased after UV radiation (Table 1). Without daphnids, however,

**Table 1.** Summary of *P*-statistics for bacterial abundance data.

	Free-living bacteria			Attached bacteria ( <i>t</i> -test)
	(two-way RM ANOVA)			
	Treatment	Time	Interaction	
UV experiments				
<i>Eudiaptomus gracilis</i>	< 0.001	< 0.001	< 0.001	0.077
<i>Ceriodaphnia</i> sp.	< 0.001	< 0.001	< 0.001	0.013
<i>Acartia tonsa</i>	< 0.001	< 0.001	< 0.001	0.043
Aggregates <sup>a</sup>	< 0.001	< 0.001	< 0.001	
Heat experiments				
<i>Eudiaptomus gracilis</i>	0.033	0.001	0.038	0.011
<i>Acartia tonsa</i>	0.125	< 0.001	0.193	0.006
Aggregates <sup>a</sup>	< 0.001	< 0.001	< 0.001	
Ozone experiments				
<i>Eudiaptomus gracilis</i>	< 0.001	< 0.001	< 0.001	< 0.001
<i>Acartia tonsa</i>	< 0.001	< 0.001	< 0.001	0.088
Aggregates <sup>a</sup>	< 0.001	< 0.001	< 0.001	

a. Statistics for aggregate treatments are for total bacteria.



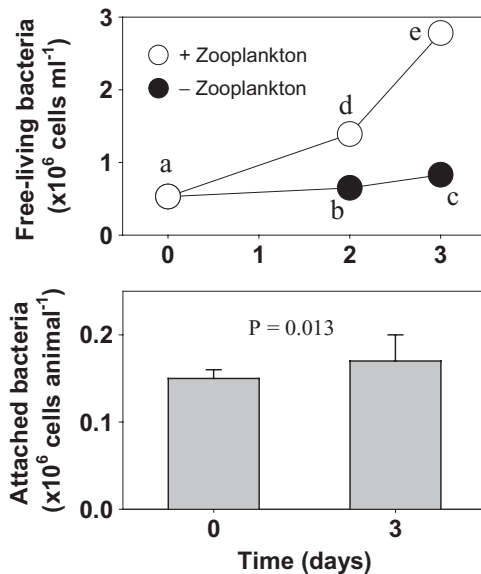
**Fig. 1.** *Eudiatomus gracilis*. For UV (A) and heat (B) experiments, free-living bacterial abundances during regrowth incubations are presented on the left column and attached bacterial abundances on the right column. For ozone experiment (C), regrowth incubations were done with bacteria recovered from the < 5 µm and > 5 µm size fractions, and bacteria in the > 5 µm fraction were further separated into free-living (left column) and attached bacteria (right column). Data are shown as mean ± SD (n = 3). Some of the error bars are within the symbols. Different letters next to symbols indicate significant difference at P < 0.05 according to Tukey's *post hoc* comparisons. See Table 1 for additional statistics.

growth of free-living bacteria remained much lower after exposure to UV radiation (Fig. 2).

In the heat experiment, abundance of free-living bacteria in both the control and the *E. gracilis* treatment increased (Fig. 1B), but the presence of copepods led to

a significantly higher number of free-living bacteria on day 3 after heat exposure (Table 1). Concurrently, attached bacterial abundance also increased significantly (Fig. 1B; Table 1).

For the ozone experiment, to remove residual ozone in the water after ozonation, bacteria were first recovered on filters for the < 5 µm (without zooplankton carcasses) and > 5 µm (with carcasses) size fractions prior to 'regrowth incubations'. Hence, the < 5 µm fraction was analogous to the controls in the UV and heat experiments. The > 5 µm fraction of the regrowth incubation was further separated into 'free-living' and 'attached' bacteria. Counts of free-living bacteria were normalized to the original jar volume (82 ml) for comparison. In the treatment with *E. gracilis*, numbers of free-living bacteria sharply decreased in both < 5 µm and > 5 µm fractions during the first 2 days, but rebounded more quickly in the > 5 µm fraction (Fig. 1C). In contrast, the number of bacteria attached to the copepod significantly increased on day 3 after exposure to ozone (Fig. 1C; Table 1). Our results indicate that the tested freshwater zooplankton species acted as an effective bacterial refuge against external stressors, in particular UV radiation and ozone.



**Fig. 2.** *Ceriodaphnia* sp. Only UV experiment was conducted. Free-living bacterial abundances are presented in the upper panel and attached bacterial abundances in the lower panel. See Fig. 1 caption for further explanation.

*Marine zooplankton.* UV radiation reduced free-living bacterial abundance by > 90% in the control (Fig. 3A). In contrast, until 3 days after the radiation it significantly increased by 47% in the presence of the copepod *Acartia*

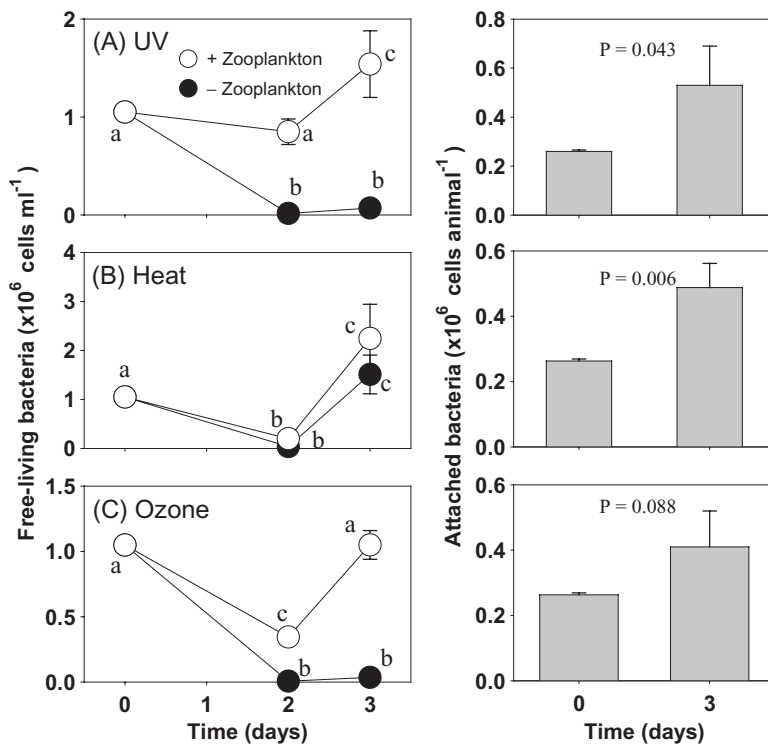


Fig. 3. *Acartia tonsa*. See Fig. 1 caption for explanation.

*tonsa* (Table 1); attached bacterial abundance even doubled during that time (Fig. 3A; Table 1).

In the heat experiment, there was no overall significant difference between abundance of free-living bacteria in the control and in the presence of copepods (Fig. 3B; Table 1). On day 3 after the heat exposure, free-living bacteria were even able to recover at almost the same rate with or without copepods (Fig. 3B). At the same time, attached bacterial abundance increased significantly by nearly 100% (Table 1).

Ozone exposure resulted in an initial decrease in abundance of free-living bacteria without or with the copepod *A. tonsa* (Fig. 3C). However, the recovery was significantly faster in the presence of copepod carcasses (Table 1), suggesting release of bacteria from the carcasses into the surrounding water. Abundance of bacteria attached to the copepod carcasses also increased during the 3 day incubation (Fig. 3C). However, this increase was statistically insignificant (Table 1), possibly because of detachment of bacteria from the copepod carcasses. These findings point to the fact that the tested marine zooplankton species also had the potential to effectively protect bacteria from external stresses and hence allowed for rapid regrowth and release of bacteria into the surrounding water.

**Freshwater aggregates.** We did not distinguish between bacteria attached to the aggregates and those in the surrounding water; hence, for UV and heat experiments,

free and attached bacteria in the aggregate treatment were combined and reported as total bacterial abundance. For the ozone experiment, bacterial counts are given as total bacteria recovered from either the  $< 5 \mu\text{m}$  (absence of aggregates) or the  $> 5 \mu\text{m}$  (presence of aggregates) size fractions.

Initial bacterial abundance in the UV experiment was three times higher in the aggregate treatment than in the control because of additional bacteria from the aggregates (Fig. 4A). While total bacterial abundance in the control remained less than  $2 \times 10^7$  cells per millilitre after UV radiation, it significantly increased in the aggregate treatment (Table 1) and reached up to  $1.7 \times 10^8$  cells per millilitre on day 3 (Fig. 4A).

Heat exposure did not greatly reduce total bacterial number without or with aggregates. In the presence of aggregates, bacterial abundance significantly increased throughout the experiment (Table 1) and reached a very high number on day 3 ( $2.0 \times 10^8$  cells per millilitre) whereas it remained much lower ( $0.48 \times 10^8$  cells per millilitre) without aggregates (Fig. 4B).

For the ozone experiment a similar pattern was observed with highly increased bacterial abundances on day 3, in particular when aggregates were present (Fig. 4C; Table 1). These results indicate that, similarly to zooplankton, aggregates effectively protected bacteria from external stresses and allowed for high survival rates and regrowth once the stressor had been removed.

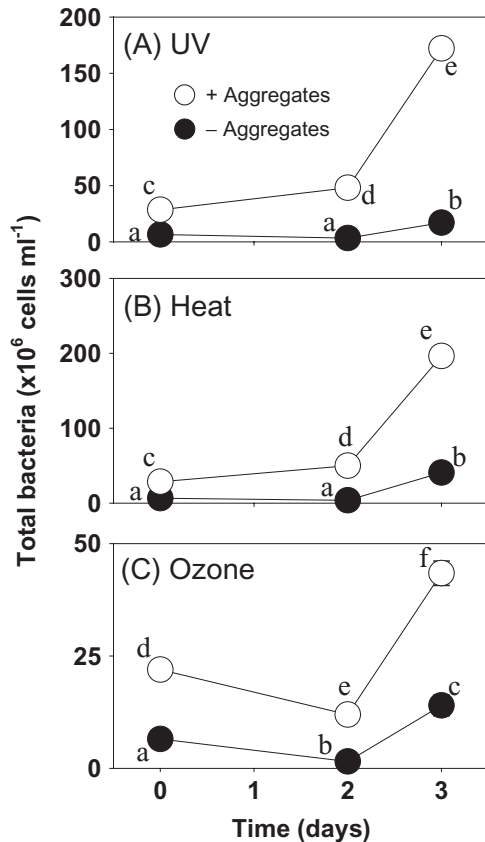


Fig. 4. Freshwater aggregates. Only total bacteria are shown. See Fig. 1 caption for further explanation.

#### BCCs following UV, heat and ozone exposures

**Freshwater zooplankton.** Cluster analysis of the DGGE banding pattern shows that similarity in BCC between water and zooplankton was  $\leq 50\%$ . After stress exposures the BCC diverged greatly such that there was  $\leq 20\%$  similarity among bacteria attached to the zooplankton, bacteria in the water around the animals and bacteria in the control, but there was no grouping for the water with zooplankton and the zooplankton itself (Fig. 5A). Nevertheless, in the presence of zooplankton free-living bacteria contained more sequences similar to those attached to zooplankton than free-living bacteria of the control (Fig. 6). Furthermore, UV and ozone exposures resulted in more pronounced changes in BCC than heat when compared with the BCC of the initial water samples (Fig. 5A).

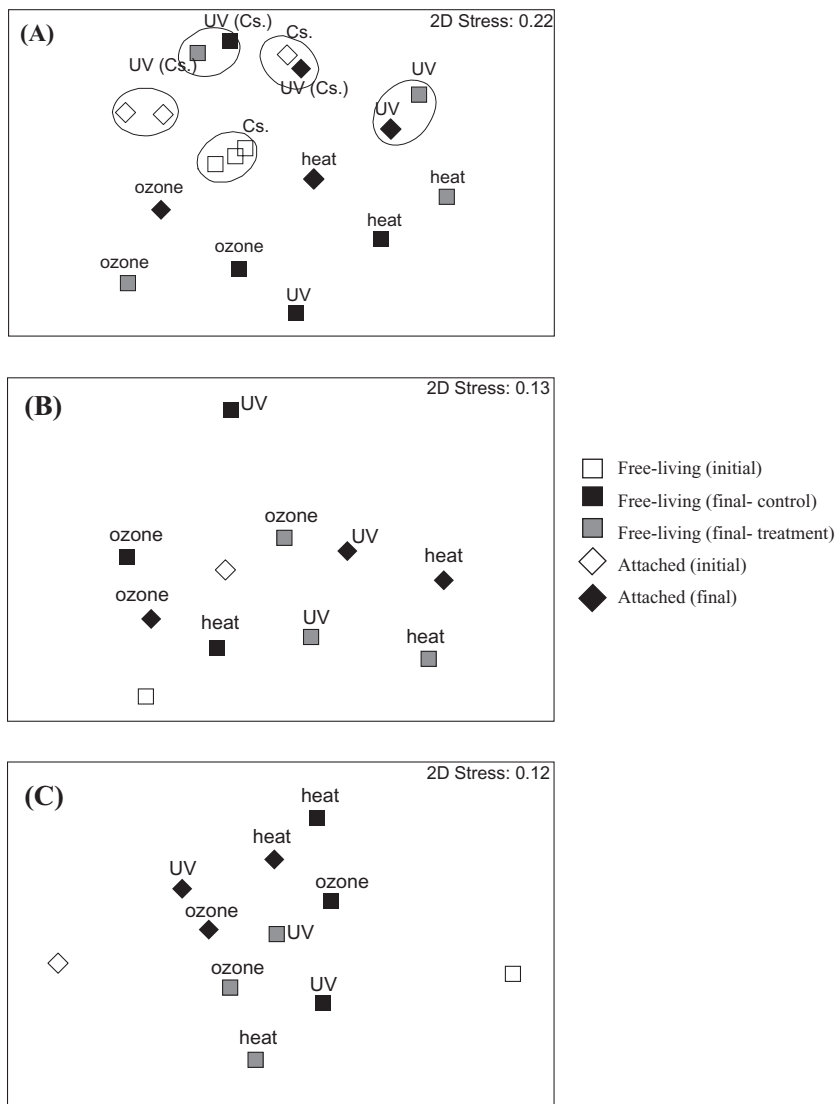
**Marine zooplankton.** Cluster analysis of the DGGE banding pattern reveals that the similarity of BCC (based on DNA) between initial samples from water and zooplankton was very low (40%), indicating that the zooplankton naturally carried a BCC different from that in ambient water. This difference was even more pronounced when

only active bacteria (based on cDNA) were considered (Fig. 5B vs. C), suggesting that a different subset of bacteria was active in the respective samples. BCC based on both DNA and cDNA showed a clear divergence of BCC between the control and the free-living and attached bacteria in the zooplankton treatments after exposure to each stressor (Fig. 5B and C). This indicates that the presence of zooplankton differentially affected the survival rate of specific bacterial phylotypes and hence their regrowth and subsequent release into the surrounding water.

**Phylogenetic analysis.** Phylogenetic analysis of clones obtained from free-living bacteria on day 3 after stress exposures shows that these clones were more often found on the zooplankton than in the control (Fig. 6). Interestingly, exposure to UV strongly selected for members of the genera *Variovorax* and *Alteromonas*, whereas ozone strongly selected for members of the genera *Herbaspirillum* and *Pseudoalteromonas*, for freshwater and marine zooplankton respectively (Table 2). These genera do not contain known pathogens. In contrast, many sequences retrieved from freshwater zooplankton samples after the heat exposure are related to potential pathogens (Fig. 6, Table 2), indicating that the zooplankton carcasses acted as a source of potential pathogens. Sequence analysis of bacteria associated with the marine copepod *A. tonsa* revealed a much lower number of clones closely related to potential pathogens (Table 2). This suggests that the studied freshwater and marine zooplankton species may differ in their role as carriers of potential pathogenic bacteria.

#### Discussion

In a recent review paper, Bichai and colleagues (2008) cautioned that higher organisms may protect pathogens from drinking water treatments, posing a hidden threat to public health. A subsequent study by Bichai and colleagues (2009) confirmed that bacteria and bacterial spores inside nematode's gut survived UV radiation. Unlike Bichai and colleagues (2009), who inoculated the nematode with selected microbes (*Escherichia coli* and *Bacillus subtilis* spores), we followed the survival of bacterial populations naturally occurring on aggregates and zooplankton. Consistent with their observations, our results also showed that UV and ozone, while effectively reduced free-living bacteria, did not eliminate bacteria associated with zooplankton and aggregates that are frequently present in both freshwater and marine environments. By residing inside an aggregate or a zooplankton, bacteria will be shielded from UV. The rich organic matter provided by aggregates and zooplankton carcasses might also effectively lower the total residual oxidant dose in the ozone treatment (Westerhoff *et al.*, 1999), allowing for



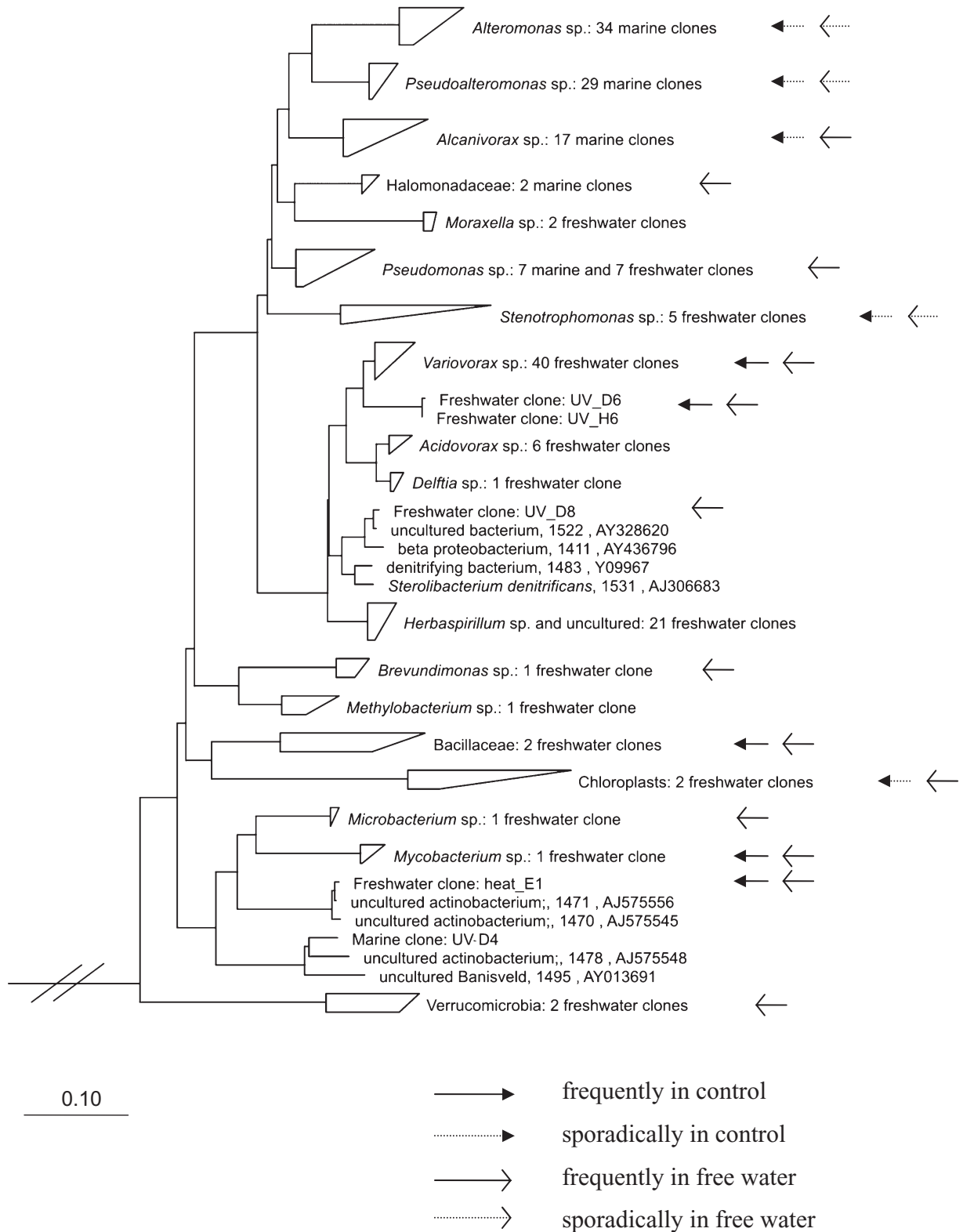
**Fig. 5.** Multidimensional scaling results for zooplankton experiments based on DGGE similarity obtained by cluster analysis with Dice coefficient (presence/absence of bands). Distances between dots represent the similarities between samples. For freshwater samples (A), 'Cs.' refers to samples from the *Ceriodaphnia* sp. experiments whereas all other samples were from the *Eudiaptomus gracilis* experiments. To cross-check the validity of the multidimensional scaling, the clusters were superimposed (black lines, similarity of 60%). Marine samples from the *Acartia tonsa* experiments were analysed based on DNA (B), or cDNA from rRNA representative of active bacteria (C).

higher survival and recovery of attached bacteria. In comparison, heating (up to 50°C) was not effective in removing either free-living or attached bacteria as both were able to rapidly recover in 3 days.

Timescale is also of critical consideration when evaluating a water treatment protocol. In many of our experiments abundance of free-living bacteria initially decreased and recovery was not obvious until day 3. These observations indicate that while the majority of the bacteria succumbed to the stressors, a small fraction nevertheless persisted and recovered over time, leading to an overall shift in BCC. This is also supported by our DGGE data based on DNA (and also cDNA for *A. tonsa*) as well as by our clone libraries. More importantly, some of the clones recovering from stress exposures, in particular those found in the presence of freshwater zooplankton after heat exposure, belonged to groups with known human pathogens. This suggests that zooplankton and

presumably aggregates potentially act as reservoirs for pathogenic bacteria (Lyons *et al.*, 2005; Vezzulli *et al.*, 2010), which will be well protected from external stresses, and their subsequent growth can be even stimulated because of increased availability of organic matter and nutrient upon death of the zooplankton and other organisms. Hence, a water treatment protocol may be mischaracterized as effective based on the initial response of the bacterial community, but a hidden threat is likely to remain in the water.

In an effort to stem the spread of invasive species via ballast water discharge, the International Maritime Organization (IMO) recommends that ballast water is to be treated to certain standards before discharge (Gollasch *et al.*, 2007). UV, heat and ozone are among the disinfecting agents commonly considered for that purpose (e.g. Rigby *et al.*, 1999; Waite *et al.*, 2003; Herwig *et al.*, 2006). Although our experimental set-up was of very different



**Fig. 6.** Maximum likelihood phylogenetic tree with number of clones obtained from samples with zooplankton. Arrows indicate if respective bands were found in DGGE analysis in the control and in the ambient water (free-living) of the zooplankton treatments. Clones of several bacterial groups differed enough to yield different DGGE bands. Dotted arrows indicate that at least one but relatively few of DGGE bands were found in the respective sample (sporadically); solid arrows indicate a common occurrence of DGGE bands in the respective sample (frequently).

**Table 2.** Affiliation of bacterial clones from zooplankton to bacterial genera with and without known pathogens.

Phylum	Genus	Comprise known pathogens	Freshwater			Marine		
			UV	Heat	Ozone	UV	Heat	Ozone
Gammaproteobacteria	Alteromonas	No				24		10
	Pseudoalteromonas	No					1	28
	Alcanivorax	No					16	1
	Halomonadaceae	No						2
	Moraxella	Yes		2				
	Pseudomonas	Yes	1		6		7	
	Stenotrophomonas	Yes		1	4			
Betaproteobacteria	Variovorax	No	39	1				
	Acidovorax	Yes	4	2				
	Delftia	Yes	1					
	Herbaspirillum & uncultured	No						21
Alphaproteobacteria	Brevundimonas	Yes						1
	Methylobacterium	Yes		1				
Firmicutes	Bacillaceae	Yes		2				
	Chloroplasts	No	2					
Actinobacteria	Microbacterium	Yes		1				
	Mycobacterium	Yes		1				
Verrucomicrobia	Verrucomicrobia	Yes		2				

Clone libraries were conducted with DNA from zooplankton samples (day 3 after stress exposures).

scale than commercial water treatment systems, our results will help regulators evaluate the effectiveness of various ballast water treatment technologies.

Present IMO regulations require that the treatment kills zooplankton and larger organisms in the ballast water prior to discharge, but not necessarily the microbes that are associated with them. For microorganisms the IMO regulations focus only on a few known human pathogens, notably *Vibrio*, *Enterococci* and *E. coli*. Based on the results of this study, there is reason to be concerned about the current IMO standards and their implementation: In our experiments all stressors resulted in 100% mortality of the zooplankton, and some of the carcasses showed visible signs of decomposition after 3 days, comparable with earlier observations of zooplankton carcass decomposition (Tang *et al.*, 2006; 2009b). Our results confirmed that UV, heat and ozone, when applied properly, are effective in killing zooplankton, but not necessarily the bacteria associated with the zooplankton and aggregates. Hence, while a treatment that kills zooplankton may be sufficient to meet the IMO standards, the treated water may still pose potential environmental threat if discharged. First of all, bacteria associated with zooplankton and aggregates can survive in high numbers and second, the remains of zooplankton and aggregates provide high concentrations of organic substrates for further proliferation of specific bacteria. Indeed, our study showed that zooplankton and aggregates carry a very diverse community of bacteria, many of those recovered from the zooplankton treatments are potential pathogens to human and wildlife but are not currently regulated by IMO. It is therefore very likely that ships unknowingly transport and enrich a large amount of

these bacteria, which are subsequently discharged into coastal waters and ports.

The huge amount of ballast water transported around the globe every day (more than  $57.6 \times 10^6$  gallons per day in the USA alone; <http://www.serconline.org/ballast/faq.html>) allows for increased dispersal of specific bacterial species (Drake *et al.*, 2001; 2002; Duggan *et al.*, 2005). According to the metacommunity concept, continuous supply of non-native species may allow for establishment of stable populations even in less suitable habitats. Venail and colleagues (2009) could demonstrate that dispersal rate plays a key role in determining community structure and function (for example, productivity) over both ecological and evolutionary timescales. However, high dispersal rates (e.g. via ballast water) may also lead to homogenization through mass effects (Leibold and Norberg, 2004), and the evolutionary as well as functional consequences for natural ecosystems remain largely unknown.

Besides drinking water and ballast water treatments, our results also have implications for general microbial ecology. Aquatic and marine bacteria are naturally exposed to environmental stressors such as UV, temperature fluctuation and oxidative chemicals. UV in particular has been shown to inhibit growth of free-living bacteria in polar as well as coastal waters (Thomson *et al.*, 1980; Davidson and van der Heijden, 2000), and is expected to play an increasingly important role in restructuring the Antarctic food web because of ozone depletion over the Southern Ocean (Davidson and Belbin, 2002). Furthermore, UV radiation in combination with high humic acid concentrations in lakes has the potential to generate a

variety of reactive oxygen species (ROS, Zepp *et al.*, 1977) with different half-lives and several partly contrasting effects. In a recent study, Glaeser and colleagues (2010) have shown that ROS have the potential to greatly affect BCC in a lake rich in humic matter. Zooplankton, however, can minimize direct and indirect (via ROS) UV effects by behavioural (vertical migration) or physiological (pigmentation) adaptation (Hansson *et al.*, 2007). As such, bacteria attached to zooplankton, especially those that are inside the zooplankton body, including some *Vibrio* species (Cottingham *et al.*, 2003), may be less vulnerable to ambient UV or even ozone than their free-living counterpart.

Our experiments confirmed that, similar to biofilms (e.g. Pozos *et al.*, 2004), aggregates and zooplankton provided protection to attached bacteria from UV, heat and ozone exposures, allowing them to survive and recover more quickly than free-living bacteria. It is also important to note that aggregates and zooplankton not only provided a refuge for bacteria, they also provided organic substrates to support subsequent growth of the surviving bacteria such that aggregates and zooplankton carcasses act as a source of bacteria to the surrounding water (Tang *et al.*, 2009b; this study). As such, aggregates and zooplankton, whether dead or alive, may function as important reservoirs to replenish the free-living bacterial populations after their decimation by predation or other environmental factors (Vezzulli *et al.*, 2010). The present results, like other related studies (reviewed in Simon *et al.*, 2002; Grossart, 2010; Tang *et al.*, 2010), reaffirms the fact that 'free-living' and 'attached' bacteria are not strictly separated in nature, and therefore the ecology of 'free-living' bacteria cannot be fully understood in isolation from the ecology of microbial hot spots represented by aggregates and higher organisms in the water column (Grossart and Tang, 2010).

## Experimental procedures

### Collection of zooplankton and aggregates

Freshwater zooplankton and water were collected from Lake Grosse Fuchskuhle (53°10'N, 13°02'E), northeastern Germany. The lake was experimentally divided into four sections with different amounts of humic matter input (see Hütalle-Schmelzer and Grossart, 2009). For this study zooplankton and water were collected from the eutrophic NE basin, which has the lowest humic matter content of all basins and an almost neutral pH of 6.8. Upon return to the laboratory the water was passed through a 44 µm screen to remove large organisms. Two of the most numerous zooplankton species were chosen for experiments: *E. gracilis* (Copepoda; female, ca. 1.5 mm body length) and *Ceriodaphnia* sp. (Cladocera; ca. 0.8 mm body length).

The marine calanoid copepod *A. tonsa* (CV-female; ca. 1 mm) was obtained from a culture collection at the Danish

Technical University, Denmark. Seawater was collected on 20 March 2009 in the Northern German Bight close to the island of Helgoland and immediately passed through a 44 µm screen to remove large particles and organisms.

Algal aggregates were formed by placing Lake Grosse Fuchskuhle surface water in 1 l Schott-bottles on a roller table over night. Aggregates were individually collected with a wide mouth pipette and diluted for the experiments with 44 µm screened fresh surface water (0.5 m) from Lake Stechlin.

### UV exposure experiments

For experiment with freshwater zooplankton, 20 individuals of *E. gracilis* or 50 individuals of *Ceriodaphnia* sp. were incubated in 50 ml of lake water in 47 mm sterile Petri dishes (in triplicate). For the control only lake water was added to the Petri dishes (in triplicate). For initial samples, 5 individuals of *E. gracilis* or *Ceriodaphnia* sp. were ground in triplicates for attached bacterial abundance (see below). Ten animals in triplicates of each species were washed in sterile-filtered water and transferred to sterile Eppendorf vials for DGGE (see below). Triplicates of 1 and 10 ml aliquots of the incubation water were also collected for bacterial abundance and DGGE respectively. For experiment with marine zooplankton, 20 individuals of *A. tonsa* were incubated in 20 ml of seawater in 47 mm sterile Petri dishes; seawater without zooplankton was used as control (in triplicates). Initial samples for bacterial abundance, DGGE and clone libraries were collected as described above. For experiment with aggregates, 2 ml of aggregate aliquots was added to 20 ml Lake Stechlin water (see above) in 47 mm sterile Petri dishes. Lake water without aggregates was used as control (in triplicates). Triplicates of 1 ml aggregate aliquots and control water were collected for initial bacterial abundances (see below).

All Petri dishes, with lids opened, were exposed to continuous UV for 2 h on a sterile bench. UV light was produced by two 15 W bulbs (Kendro, UVC 30) placed ca. 15 cm above the Petri dishes. During radiation, the Petri dishes were cooled with ice underneath to prevent over-heating because of UV irradiation. UV dosage ( $D$ ; µWs cm<sup>-2</sup>) was calculated as:

$$D = I \times T$$

where  $I$  is UV intensity and  $T$  is exposure time (7200 s). Average UV intensity according to the manufacturer is 1430 µW cm<sup>-2</sup> at the level of the Petri dishes, which yielded a UV dosage of  $1.0 \times 10^7$  µWs cm<sup>-2</sup>. A UV dosage of  $2.2 \times 10^4$  µWs cm<sup>-2</sup> is considered sufficient to eliminate pathogens in drinking water (US Environmental Protection Agency, 2006).

After 2 h the zooplankton was visually confirmed to be dead and the Petri dishes were covered with lids, sealed with parafilm, and kept in a culture room (19 ± 1°C; dark) for bacterial regrowth. After 2 days, 1 ml aliquots of water were collected from all Petri dishes with or without zooplankton for free-living bacterial abundances. The Petri dishes were opened and closed on a sterile bench to avoid contamination. After 3 days, water was sampled from the Petri dishes in the same manner for free-living bacterial abundances. Afterward, five of the zooplankton carcasses were collected for attached bacterial abundances, and the remaining carcasses for DGGE and clone libraries.

For the aggregate treatment we did not separate the aggregates from the surrounding water; instead, 1 ml aliquots of the aggregate/water mixture were taken from the treatment on day 2 and day 3 for total bacteria counts (free-living + attached). Aliquots (1 ml) were also taken from the aggregate-free control for comparison. No DGGE was done for the aggregates.

#### Heat exposure experiments

Heat exposure experiments were done with *E. gracilis*, *A. tonsa* and aggregates. The set-up was basically the same as in the UV experiments except that sterile test tubes were used instead of Petri dishes. All test tubes were capped and placed in a 50°C water bath for 2 h. Thereafter, the zooplankton was visually confirmed to be dead, and the test tubes were placed in the culture room for bacterial regrowth. On day 2 samples were taken for free-living bacterial abundances. On day 3 samples were taken for free-living and attached bacterial abundances, as well as DGGE and clone libraries (except for aggregates). For the aggregate treatment only total bacteria were quantified.

#### Ozone exposure experiments

Ozone exposure experiments were done with *E. gracilis*, *A. tonsa* and aggregates. The experimental set-up was different from UV and heat experiments. Twenty individuals of *E. gracilis* or *A. tonsa* or 2 ml of aggregate/water aliquots were added in triplicates to sterilized glass jars (size = 82 ml). The jars were then filled with the appropriate incubation water (Lake Grosse Fuchskuhle water for *E. gracilis*; seawater for *A. tonsa*; Lake Stechlin water for aggregates). The cap closure of the glass jars had a 25 × 25 mm<sup>2</sup>, 44 µm nylon screen. Each set of glass jars was put in a larger container with 2 l of the same water type. Ozone was pumped into the large containers by an ozonator (Airmaster TC300B, max. ozone output 3.33 mg min<sup>-1</sup>) for 5 h such that the ozonated water continuously mixed into the glass jar while the screened closures protected the animals and aggregates from mechanical damage by the bubbling.

After 5 h the zooplankton was visually confirmed dead, and the jars' contents were filtered through 5 µm polycarbonate membranes to collect bacteria associated with the zooplankton or aggregates; the filtrates (< 5 µm) were then filtered through 0.2 µm PC membranes to collect the free-living bacteria. The membrane filters with materials collected on them were briefly rinsed with sterile water and then transferred to another set of sterile test tubes each with 25 ml of sterile water (fresh or marine water depending on the experiment). The rinsing was to ensure that the stressor (dissolved ozone) was removed prior to regrowth incubation. The test tubes were vortexed rigorously to resuspend bacteria from the filters, and placed in the culture room (19 ± 1°C; dark) for bacterial regrowth. On day 2 samples were taken for free-living bacterial abundances. On day 3 samples were taken for free-living and attached bacterial abundances, as well as DGGE and clone libraries (except for aggregates). Free-living bacterial abundances in the regrowth incubation were normalized to the jar volume (82 ml). For the regrowth incubation

with aggregates we did not separate bacteria attached to aggregate surfaces from those in the surrounding water; hence only total bacteria were reported.

To estimate the ozone dosage, we ozonated 2 l of non-buffered deionized water in the same manner, and measured hourly the aqueous ozone concentration (*C*) as:

$$C = \frac{\epsilon_{260} \times I}{A_{260}}$$

where  $\epsilon_{260}$  is molar absorptivity, which is taken as 3300 M<sup>-1</sup> cm<sup>-1</sup> (Hart *et al.*, 1983); *I* is absorption path at 1 cm, and  $A_{260}$  is absorbance at 260 nm. Absorbance was measured on Hitachi U-2900 spectrophotometer against distilled water blank. The ozone concentration saturated after 1 h at 0.10 mg l<sup>-1</sup>, giving a dosage of ca. 24.4 mg min<sup>-1</sup> over the duration of our experiment. According to the World Health Organization (2004), an ozone dosage of 2.0 mg min<sup>-1</sup> is sufficient to inactivate even encysted bacteria.

#### Bacterial enumeration

To quantify free-living bacteria, water samples were filtered onto 0.2 µm black polycarbonate membrane filters, and SYBR Gold (10 µl stock in 141 µl Moviol including 1 µl ascorbic acid) was applied directly onto the filters (Lunau *et al.*, 2005). For zooplankton-associated bacteria, the zooplankton carcasses were homogenized in 1 ml of 0.2 µm filtered sterile incubation water with a Teflon pestle, and the homogenate was filtered onto 0.2 µm black polycarbonate filters. The pestle and the grinder were rinsed with pre-filtered sterile water onto the same filters, and SYBR Gold was applied directly onto the filters. Bacteria were counted under an epifluorescence microscope. For aggregate-associated bacteria, aggregates within a known volume of water (2 ml) were filtered directly onto the membrane filters. Because we did not disrupt the aggregates prior to counting (e.g. Grossart *et al.*, 2003b), counts for aggregate-associated bacteria should be considered as conservative estimates.

#### Molecular analyses

Animals were transferred to sterile 2 ml Eppendorf vials and stored at -20°C and at -80°C (for freshwater and marine samples, respectively) until DNA extraction. For free-living bacteria the water samples were filtered onto 0.2 µm polycarbonate membrane filters and stored in the same manner until extraction. Procedures for bacterial DNA extraction and DGGE are described in Tang and colleagues (2009b). Briefly, we used the MOBIO Power Soil DNA-extraction kit for extracting DNA and a phenol-chloroform-extraction for rRNA (Zhou *et al.*, 1996). The rRNA extracted from *A. tonsa* was reverse transcribed into cDNA with the TaqMan RT-kit (Applied Biosystems). Thereafter, DNA and cDNA were amplified with the eubacterial primers 341f-gc and 907r (Muyzer and Ramsing, 1995) for DGGE and 341f and 907r for DNA clone libraries. DGGE was done with the Ingeny system and a gradient of acrylamide and urea of 40–70%, the gel was stained with SybrGold and photographed with an Alphamager. The clone libraries were conducted with the pGEM-T-Easy-kit (Promega) after manufacturer's protocol.

Later the plasmids were amplified with the primers SP6 and T7, cleaned with PEG and sequenced commercially (Macrogen).

### Statistics

For free-living bacterial abundance data in the zooplankton experiments, two-way Repeated Measures ANOVA was used to test for significant effect because of time and treatment. Tukey's test was used for *post hoc* comparisons. For zooplankton-associated bacterial abundance data, two-sample *t*-test was used to compare initial and final measurements. For experiments with aggregates, two-way Repeated Measures ANOVA and Tukey's *post hoc* comparison were used to test for differences between treatment and control in total bacterial abundances. Significance level was set at  $P = 0.05$ .

DGGE banding pattern was analysed with the GelComparII software using the Dice coefficient of present/absent bands. The resulting matrix was used in Primer6 for non-metric multidimensional scaling. This method uses rank orders and the distances shown in the plot stand for the similarity of the samples. Sequences from DNA-based clones were phylogenetically analysed with the software ARB (<http://www.arb-home.de>) (Ludwig *et al.*, 2004). Retrieved sequences of all clones are deposited in GenBank and given the following accession numbers: HM363184-HM363368.

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