Title: *In situ* grazing resistance of *Vibrio cholerae* in the marine environment

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ABSTRACT

Previous laboratory experiments revealed *Vibrio cholerae* A1552 biofilms secrete an antiprotozoal factor that prevents *Rhynchomonas nasuta* from growing and thus prevents grazing losses. The antiprotozoal factor is regulated by the quorum sensing response regulator, HapR. Here we investigate whether the antiprotozoal activity is ecologically relevant. Experiments were conducted in the field as well as under field-like conditions in the laboratory to assess grazing resistance of *V. cholerae* A1552 and N16961 (natural frameshift mutation in *hapR*) biofilms to *R. nasuta* and *Cafeteria roenbergensis*. In laboratory experiments exposing the predators to *V. cholerae* grown in seawater containing high and low glucose concentrations, we determined that *V. cholerae* biofilms showed increased resistance towards grazing by both predators as glucose levels decreased. The relative resistance of the *V. cholerae* strains to the grazers under semi-field conditions was similar to that observed *in situ*. Therefore, the antipredator defense is environmentally relevant and not lost when biofilms are grown in an open system in the marine environment. The *hapR* mutant still exhibited some resistance to both predators and this suggests that *V. cholerae* may co-ordinate antipredator defenses by a combination of density dependent regulation and environmental sensing to protect itself from predators in its natural habitat.

INTRODUCTION

In the last 20 years, cholera has occurred in areas that have been free from outbreaks for almost a century (for review see Tauxe*, et al.*, 1994). The recent increases in occurrences may be due to the fact the *Vibrio cholerae* El Tor biotype (cause of the seventh and current pandemics), may be more environmentally fit than the Classical biotype (etiological agent for the first six pandemics), and thus has replaced the Classical biotype in the environment. This highlights the need to better understand what factors affect the occurrence and survival of *V. cholerae* in the environment. Researchers have begun to use remote sensing to determine if they can identify correlations between cholera outbreaks and ocean parameters (e. g. phytoplankton and zooplankton blooms, seawater temperature, nutrient concentration) in an attempt to predict outbreaks (Lobitz, et al., 2000). Many of the studies monitoring *V. cholerae* in the marine environment have focused on the effect of nutrient availability (Singleton*, et al.*, 1982) and on interactions of *V. cholerae* with copepods (Huq*, et al.*, 1983, Pruzzo*, et al.*, 2008). Several studies have shown that *V. cholerae* attaches preferentially to biotic surfaces such as copepods in the marine environment (Heidelberg*, et al.*, 2002, Mueller*, et al.*, 2007), while others have demonstrated a preference for planktonic growth of *V. cholerae* in the water column (Worden, 2006) in which case the bacterial cells experienced heavy grazing pressure by protozoa.

The interactions of bacteria and protozoa are considered to be one of the oldest predator-prey interactions in nature (Cavalier-Smith, 2002). Grazing by phagotrophic protists is one of the main mortality factors of bacteria in marine and freshwater systems (Azam*, et al.*, 1983, Hahn & Höfle, 2001, Matz & Jürgens, 2001) and a major selective force for evolution of bacterial defense strategies (Matz & Kjelleberg, 2005). Predation can alter bacterial morphology and community structure through direct

(predation (Hahn & Höfle, 1999, Jürgens*, et al.*, 1999)) and indirect (nutrient recycling (Sherr*, et al.*, 1982, Pernthaler*, et al.*, 1997)) interactions. Bacteria have evolved different defense strategies including general avoidance (e.g. motility) and direct consumer effects (e.g. digestional resistance, toxin production) (Matz & Kjelleberg, 2005).

The majority of microbes in natural habitats occur as surface-attached communities called biofilms (Davey & O'Toole, 2000), which function to protect cells in the community from a variety of stresses. The biofilm architecture and bacterial-produced extracellular polymeric substances (EPS) offer important protection against various stresses such as antimicrobial agents (Gilbert*, et al.*, 1997) and grazing (Parry, 2004, Weitere*, et al.*, 2005). Biofilm formation as well as toxin production are controlled by density dependent bacterial gene regulation, or quorum sensing (QS) in many bacterial species (Hammer & Bassler, 2003, Turovskiy*, et al.*, 2007). For example, in the pathogens *Pseudomonas aeruginosa* and *V. cholerae*, QS regulates the production of toxins that have been shown to kill predators resulting in grazing resistance (Matz*, et al.*, 2004, Matz*, et al.*, 2008). While several studies have assessed the interactions of protozoa and *V. cholerae* in the suspended state and planktonically in mesocosms (Macek*, et al.*, 1997, Worden, 2006), surprisingly little is known about the impact of protozoa on the occurrence of attached *V. cholerae.*

In laboratory studies, we have shown that microcolony formation in biofilms of *V. cholerae* (Matz*, et al.*, 2005) and *P. aeruginosa* (Matz*, et al.*, 2004) protected against grazing losses. Further, it has been demonstrated that biofilms have antiprotozoal activity (Matz*, et al.*, 2004, Weitere*, et al.*, 2005). Matz *et al.* (2005) demonstrated that biofilms of *V. cholerae* A1552 wild type strain could prevent the benthic grazer

Rhynchomonas nasuta from growing, while biofilms of a QS mutant (lacking the response regulator, HapR) were grazed. In this study, we investigate the efficacy of the grazing resistance of biofilms observed in laboratory experiments, in an ecologically relevant context. The survival of *V. cholerae* biofilms under grazing pressure was tested *in situ* in environmental diffusion chambers (McFeters & Stuart, 1972), where massive dilution effects occur from the surrounding seawater. The grazing assays were performed in the marine environment over a period of 10 days and survival and persistence in the presence of two marine flagellates, *R. nasuta* and *Cafeteria roenbergensis* was assessed.

Material and Methods

Strains and culture conditions

V. cholerae A1552 wild type, *V. cholerae* N16961 (natural *hapR* frameshift mutant), *V. cholerae hapR* (isogenic genetically modified organism (GMO) lacking the *hapR* gene encoding the QS response regulator) and *E. coli* B were routinely cultured on Luria Bertani agar containing 2 % NaCl (LB20) or grown in LB20 broth overnight at 37°C with shaking (200 rpm). The benthic grazer, *R. nasuta,* was isolated from the field site at the Sydney Institute for Marine Science (SIMS), treated with an antibiotic cocktail (streptomycin, spectomycin, gentamycin, tobramycin, ampicillin and kanamycin at 150 μg mL^{-1}) and serially diluted for many generations to remove the natural contaminating bacterial community. *R. nasuta* and the predominately planktonic flagellate, *C. roenbergensis* (Bicosoecida, Baltic sea, isolated by A. P. Mylnikov), were maintained axenically in 0.5 × nine salts solution (NSS, Väätänen, 1976) supplemented with heat-killed *P. aeruginosa* PAO1 as prey at room temperature, and transferred to fresh medium every two weeks.

121 **Environmental chamber set-up**

122 Four replicate experiments were performed during the period of January 2008 to May
123 2009. Environmental diffusion chambers (McFeters & Stuart, 1972) were suspended
124 in the marine environment at SIMS (see Table 1 for details). The chambers (volume
125 28.3 mL) were sealed with membranes (Supor® -100 membrane filters, 0.1 μm pore
126 size, 90 mm, PALL Life Science) which were permeable to seawater but retained
127 bacteria and protozoans inside the chambers. These were further suspended in a mesh-
128 lined crate to prevent puncture from larger marine animals. The chambers were
129 modified to hold a glass slide as a substratum for biofilm formation. Cover slips were
130 attached to the slide with silicone glue and one cover slip was removed for protozoan
131 counting and one for staining and imaging by confocal laser scanning microscopy
132 (CLSM; Leica DMRB, Leica, Switzerland). For each treatment, at least three, in one
133 case four autoclaved chambers, were inoculated with 28.5 mL of bacteria- (overnight
134 cultures diluted to 10^7 cells mL^{-1}) and protozoa-suspension (10^4 cells mL^{-1}) in 0.5 ×
135 NSS and incubated in the marine environment for 10 days (see Table 1 for details).
136 Following incubation in the marine environment, chambers were collected and
137 immediately transferred to the laboratory in seawater. Although a thin biofilm was
138 sometimes detectable on the outside of the chambers, diffusion of seawater was not
139 impeded as evidenced by the rapid exchange of seawater observed when the chambers
140 were removed. Protozoan numbers inside chambers were determined by microscopy
141 (Leica DMLB, Leica, Switzerland) and suspended bacterial numbers determined by
142 dilution drop plates (Hoben & Somasegaran, 1982). The abundance of *V. cholerae*
143 and absence of contamination was verified by plating on selective CPC agar (Massad
144 & Oliver, 1987) as well as LB20 agar. Microscopy was used to verify absence of
145 protozoan contamination at the end of experiments. Glass slides with the cover slips

were stored in 2% glutaraldehyde at 4°C until staining and imaging. For staining, the cover slips were detached from the glass slides, washed 3 times in sterile PBS and stained with propidium iodide (100 μg mL^{-1}). Stained biofilms were incubated for 10 min in the dark followed by washing with PBS (3 times). The cover slips were inverted on a drop of PBS and "clay feet" on a glass slide and the sides were sealed with liquid candle wax. CLSM images were analysed with Image J (http://rsbweb.nih.gov/ij/).

Grazing assays performed under field-like conditions in the laboratory

To quantify protozoan dynamics on the *V.cholerae* biofilms in a non-destructive environment under conditions similar to those in the field, we performed experiments in the laboratory where the diffusion chambers were place in a circuit with traditional biofilm flow cells (Christensen*, et al.*, 1999) connected with silicon tubing. A peristaltic pump circulated water from the chambers into the flow cells in which protozoan succession on the *V.cholerae* biofilms could be quantified, and back into the chambers. The chambers were held in large plastic containers (25 L) in fresh seawater which was changed twice daily. Four chambers for each experiment were inoculated with 28.5 mL of a suspension of 10^7 cells mL^{-1} of an overnight culture of *V. cholerae* strains in 0.5 × NSS and 10^5 cells mL^{-1} of *R. nasuta* or *C. roenbergensis*. Protozoan abundance was determined by microscopic observation of flow cells over 5 days.

Effect of glucose concentration on persistence of *V. cholerae* under grazing pressure

Due to seasonal differences in the results of our experiments we investigated the influence of different carbon concentrations on the persistence of *V. cholerae* biofilms under grazing pressure, as nutrient levels would be expected to differ between these

seasons. Overnight cultures of *V. cholerae* strains were inoculated (10^5 cells mL^{-1}) in triplicate into 24-well microtiter plates (Sarstedt, Newton, USA) in sterile filtered seawater containing 0.1% or 0.001 % sterile filtered glucose as a carbon source. *R. nasuta* (10^5 cells mL^{-1}) was inoculated 2 hours later to give the bacteria time to attach. As a control flagellates were inoculated into the same medium but with heat-killed *P. aeruginosa* as a food source. Experiments were run for 3 days at room temperature with shaking (60 rpm). Protozoan numbers were determined by microscopy and the biofilm biomass measured as previously described (O'Toole*, et al.*, 1999). Briefly, biofilms were washed 3 × with PBS, stained with 0.3 % crystal violet for 10 minutes and washed 3 times with PBS. The biofilm was destained with 96% ethanol and absorbance read at 490 nm (Wallac 1420 Multilabel Counter, Perkin Elmer Life Sciences).

Statistical analyses

Statistical analyses were performed using SPSS 17.0 software. Pair-wise comparisons were performed using t-tests. Multiple comparisons were done using one- or two-factorial ANOVAs. Tukey-HSD-test was used as post hoc test after significant group effects were detected by ANOVA. In the case of non-homogenous variances, data were log(x+1) transformed prior the analyses. If the variances were still non-homogenous after transformation, non-parametric Kruscal-Wallis H-test for comparing multiple groups or Mann-Whitney U-test for pair-wise comparisons was applied.

RESULTS

Protozoan abundance on *V. cholerae* and *E. coli* biofilms in the marine environment

195 After 10 days in the field, protozoa could be detected on all biofilms grown in the
196 environmental chambers, with the abundance being dependent on the time of year
197 (generally abundance of protozoa was higher in autumn than in summer).
198 Surprisingly, in midsummer (Fig. 1A) the number of the surface-feeder, *R. nasuta,*
199 was significantly higher on the biofilms of the A1552 wild type strain than on the
200 biofilms of the N16961 natural *hapR* mutant strain (t-test $p < 0.01$). Since this strain
201 carries a frameshift mutation in the QS response regulatory gene, *hapR*, it was
202 expected that this strain would support a higher abundance of flagellates than the wild
203 type A1552 strain, as has been shown with the isogenic A1552 *hapR* mutant in
204 laboratory studies (Matz*, et al.*, 2005) even though these two strains are not isogenic.
205 Counts of suspended *V. cholerae* A1552 and N16961 were similar to each other (Fig.
206 1B), but were significantly higher ($p < 0.001$) in the grazed chambers when compared
207 to the grazer free controls.

208 Figure 2A shows the abundance of the suspension feeder, *C. roenbergensis,* on *V.*
209 *cholerae* A1552 and N16961 biofilms after 10 days exposure in the field (experiments
210 were performed middle to end of summer). The abundance of *C. roenbergensis* was
211 10 times higher on the *V. cholerae* N16961 biofilms, in contrast to *R. nasuta*
212 abundances which were 20-fold higher on the A1552 wild type biofilms (Fig. 1A).
213 The abundance of suspended *V. cholerae* A1552 in the chambers was higher in the
214 grazed chamber than in the non-grazed control (Fig. 2B), while the opposite was
215 observed for N16961 where the number of suspended cells was higher in the non-
216 grazed chamber (Fig. 2B). The differences in planktonic cell numbers were significant
217 ($p < 0.05$). These results are similar to those observed in laboratory experiments
218 where the isogenic *hapR* mutant strain supported strong growth of the flagellate
219 resulting in reduced bacterial cell numbers, while the opposite was true for the wild

220 type strain. Biofilm biomass did not differ significantly between the grazed and
221 ungrazed treatments or between strains (data not shown).

222 Experiments with *R. nasuta* or *C. roenbergensis* inoculated in diffusion chambers in
223 the marine environment with either *V. cholerae* A1552 or N16961 biofilms were also
224 performed at the end of autumn (Fig. 3). In the chambers containing *C. roenbergensis,*
225 the trend is the same as for the experiments performed at the end of summer (Fig. 2)
226 with a higher abundance of grazers on the N16961 biofilms. For chambers containing
227 *R. nasuta*, there was a lower abundance of grazers on the N16961 strain compared to
228 the A1552 strain biofilms (Fig. 3A) but the difference was not as pronounced as in the
229 experiments performed in mid summer (Fig 1A).

230 Counts of suspended *V. cholerae* A1552 exposed to *R. nasuta* in the chambers at the
231 end of autumn (Fig. 3B) differed from the previous series of experiments performed
232 in midsummer (Fig. 1B). The abundance of suspended *V. cholerae* A1552 in the
233 chambers containing *R. nasuta* was higher than the abundance of N16961 (9.2×10^5
234 and 5.5×10^4, respectively; Fig. 3B), while in the earlier experiments they were
235 similar (3.6×10^7 and 1.8×10^7, respectively; Fig.1B). In the chambers with *C.*
236 *roenbergensis*, the number of suspended N16961 was higher than that of A1552 (Fig.
237 3B), similar to the previous experiment (Fig. 2B). Again, biofilm biomass did not
238 differ significantly between treatments or strains indicating that the biofilms were not
239 significantly grazed (data not shown).

240 To compare flagellate growth on *Vibrio* biofilms to a non-*Vibrio* biofilm which was
241 previously shown to support growth of the protozoa, we exposed *E. coli* B and *V.*
242 *cholerae* A1552 to both grazers in the field in autumn (Fig. 4). Both *R. nasuta* and *C.*
243 *roenbergensis* numbers were higher on the *E. coli* B biofilms compared to the *V.*

cholerae biofilms after 10 days in the field (Fig. 4A). While 0.32 ± 0.29 *R. nasuta* mm^{-2} could be detected on the *V. cholerae* A1552 biofilms, *E. coli* biofilms harboured 50 times more. The abundance of *C. roenbergensis* was 2.5 times higher on the *E. coli* biofilms than the *V. cholerae* A1552 biofilms (Fig. 4A). Two-factorial ANOVA revealed that the differences in abundance of *C. roenbergensis* compared to *R. nasuta* were significant ($p < 0.001$). The numbers of suspended *E. coli* were higher than the abundance of planktonic *V. cholerae* in chambers containing *R. nasuta* but lower than the abundance of planktonic *V. cholerae* in chambers with *C. roenbergensis* (Fig. 4B). In this series of experiments, the numbers of planktonic *V. cholerae* were higher in the chambers with *C. roenbergensis* than in those with *R. nasuta*, which is opposite to what was previously observed at the end of autumn (Fig. 3B).

Abundance of *R. nasuta* and *C. roenbergensis* on *V. cholerae* biofilms under semi-field conditions

The isogenic *V. cholerae* A1552 *hapR* mutant strain is a GMO and cannot be exposed to the natural marine environment, thus, in order to compare all three *Vibrio* strains, we designed grazing assays in the laboratory under conditions that simulated the field experiments as closely as possible. The abundance of *R. nasuta* and *C. roenbergensis* on the three *V. cholerae* biofilms was monitored for 5 days in the flow cells (Fig. 5). While *R. nasuta* appears on the biofilm in the flow cells in low numbers from day 2 onwards and slowly increases thereafter (Fig. 5A), *C. roenbergensis* is abundant in high numbers from day 1 onwards (Fig. 5B). The difference in abundance of *R. nasuta* on the three different *V. cholerae* strains was significant (H-test $p = 0.02$). *R. nasuta* was, expectedly, most abundant on the *V. cholerae* A1552 *hapR* mutant biofilms as seen previously in laboratory experiments (Matz*, et al.*, 2005) and was not detected in high numbers on either the *V. cholerae* N16961 or the A1552 biofilms

after day 2 (Fig. 5 A). For *C. roenbergensis,* there were differences in the abundance of the grazer on the *Vibrio* strains for the first 3 days, but abundances thereafter were similar on all strains (Fig. 5B).

Effect of glucose concentration on persistence of *V. cholerae* biofilms under grazing by *R. nasuta*

Growth of *R. nasuta* on *V. cholerae* biofilms supplemented with high and low carbon concentrations revealed that biofilms grown under low carbon concentrations (0.001 %) supported a lower abundance of grazers than those grown at a higher glucose concentration (0.1 %) regardless of strain (Fig. 6). Growth of *R. nasuta* on the A1552 biofilms was 2.8 times higher if the biofilms were grown on the higher glucose concentration while for the *hapR* mutant strain, the increase was 1.5 times more and for N16961, 1.6 times higher. A two factorial ANOVA revealed significant influences of the strain ($p = 0.003$) and the glucose concentration ($p = 0.002$) on the growth of *R. nasuta*. A posthoc test revealed significant differences between the growth of *R. nasuta* on A1552 compared to growth on the isogenic *hapR* mutant biofilm ($p = 0.004$) and growth on N16961 compared to A1552 *hapR* biofilms ($p = 0.014$). No significant differences in growth on A1552 and N16961 were found. Growth of the flagellates on medium with either high or low glucose supplied with heat-killed bacteria was not significantly different (data not shown). The biofilm biomass was not significantly different on the two glucose concentrations (data not shown).

DISCUSSION

Most studies investigating predator-prey interactions in biofilms have been performed in laboratory settings under strictly controlled conditions. Our previous results indicated that *V. cholerae* A1552 wild type biofilms grown in 24 well microtiter

293 plates were toxic to the benthic grazer, *R. nasuta* and resulted in flagellate death,
294 while feeding on the *hapR* QS mutant biofilm resulted in positive growth (Matz, *et*
295 *al.*, 2005), indicating that a factor regulated by HapR is responsible for protozoan
296 killing. The current study was designed to investigate whether *V. cholerae* biofilms
297 grown *in situ* where there is a large dilution effect due to surrounding seawater could
298 inhibit protozoan growth, i.e. whether the toxicity seen in the laboratory was an
299 artefact due to concentration effects of the microtiter experiments. In these field
300 experiments, we used the biofilm feeder *R. nasuta* as well as *C. roenbergensis* which
301 is primarily a suspension feeder but also attaches to the biofilm. Results presented
302 here reveal that *V. cholerae* A1552 wild type biofilms prevent predation associated
303 loss of biofilm biomass in the marine environment where there is a large dilution
304 effect due to surrounding seawater and thus support the concept that the antipredator
305 activity observed in the microtiter experiments is ecologically relevant.

306 **Protozoan abundance varied on biofilms of different *V. cholerae* strains in the**
307 **field as well as under semi-field conditions**

308 The abundances of *R. nasuta* and *C. roenbergensis* varied on different *V. cholerae* and
309 non-*Vibrio* strains and at different times of the year. While the abundance of *R. nasuta*
310 was significantly lower on *V. cholerae* N16961 than on the wild type strain A1552 in
311 summer (Fig. 1A, $p < 0.01$)), the difference in abundance on the two strains in autumn
312 was not significant (Fig. 3A). The predominately planktonic grazer, *C. roenbergensis,*
313 appeared on the N16961 biofilms in higher numbers in midsummer and late autumn
314 (Fig. 2A and 3A). In general, the numbers of *C. roenbergensis* on biofilms of all
315 strains was higher than the numbers of *R. nasuta*, indicating that the surface grazing
316 flagellate *R. nasuta* might be more negatively affected by *V. cholerae* than the
317 suspension feeding *C. roenbergensis*. This may be due in part to the fact that *C.*

318 *roenbergensis* can escape the biofilm and feed on planktonic bacteria but may also
319 indicate that the biofilms affects the grazers differently.

320 The fact that *V. cholerae* N16961 supported lower abundances of the obligatory
321 benthic-feeding *R. nasuta* than A1552 was surprising as N16961 is a QS mutant and
322 thus, it was expected that the QS-regulated antiprotozoal activity (seen in A1552 in
323 previous laboratory studies) would not be expressed. This suggests that there may be a
324 QS-independent pathway for expression of traits that lead to biofilm persistence,
325 however other differences between the strains or nutritional quality may also account
326 for this difference in grazer abundance, as these strains are not isogenic. It has been
327 previously shown that *P. aeruginosa* expresses QS-regulated lethal factors which play
328 a key role in grazing protection of late biofilms, while QS-independent upregulation
329 of the type III secretion system is important as an immediate response to predation
330 (Matz*, et al.*, 2008).

331 Due to the unexpected result of higher numbers of *R. nasuta* on the wild type *V.*
332 *cholerae* strain A1552 than on the QS mutant strain, we compared growth of the
333 grazer on a non-*Vibrio* strain, *E. coli* B, in the field. Both *R. nasuta* and *C.*
334 *roenbergensis* occurred on the *E. coli* biofilms in higher abundances than on the *V.*
335 *cholerae* A1552 strain (Fig. 4A) but the differences in abundance were not significant.

336 Under semi-natural conditions, the same trend for low grazer abundances on the *V.*
337 *cholerae* A1552 and N16961 strains when compared to the A1552 *hapR* mutant strain
338 was observed (Fig. 5). While abundances on the A1552 *hapR* biofilm were higher for
339 both *R. nasuta* and *C. roenbergensis*, the number of grazers on biofilms of the
340 N16961 strain was similar to the A1552 strain. Thus the results obtained in the field
341 and under semi-field conditions were similar. The N16961 strain has a frameshift

342 mutation in the *hapR* gene and thus was considered to be more susceptible to losses to
343 predation than the wild type A1552. Matz *et al.* (2005) showed that the QS dependent
344 *hapR* gene controls antiprotozoal factor(s) that when secreted prevented flagellate
345 grazing. The field experiments reported here indicate that that this activity might be
346 ecologically relevant and that there may be *hapR* independent defensive mechanisms
347 expressed *in situ.* Previous results have shown that *P. aeruginosa* uses both QS-
348 dependent and QS-independent mechanisms for predation resistance and that these
349 mechanisms operate under different physiological and environmental conditions
350 (Matz*, et al.*, 2008), therefore, the same types of responses may be expressed by *V.*
351 *cholerae* during grazing.

352 **Grazing resistance increases as carbon levels decrease**

353 The differences in protozoan growth on the biofilms in mid summer and autumn may
354 be due to differences in DOC levels in the seawater. Thus, we investigated the
355 influence of prey grown on different nutrient concentrations on grazer numbers. When
356 the glucose concentration was higher, the flagellate growth rates were at least 1.5
357 times higher for all three *V. cholerae* strains, while in controls (flagellates with heat
358 killed bacteria) there was no effect of glucose concentration on flagellate growth rate.
359 The greatest difference in growth rates between the high and low glucose biofilms
360 was for the A1552 strain. Interestingly, there was a difference in numbers of grazers
361 on the *hapR* mutant biofilms grown under different nutrient concentrations indicating
362 that under low nutrient conditions, QS-independent grazing resistance occurs.

363 It is known from planktonic studies that in nutrient rich environments with a high
364 abundance of metazooplankton, the phagotrophic protists experience a high grazing
365 pressure (e.g. Jürgens, 1994, Corno*, et al.*, 2008) allowing the bacterial community to
366 increase in numbers. This is in part due to sloppy feeding and from the excretion of

367 the recycled zooplankton nutrients, which then become available for the bacterial
368 community (e.g. Lampert, 1978, Pernthaler*, et al.*, 1997, Corno & Jürgens, 2006).
369 Thus, when nutrient levels are high in the environment, grazing pressure on the
370 bacterioplankton decreases while available nutrient increases resulting in rapid growth
371 of the bacterioplankton (Cole*, et al.*, 1988, Simek*, et al.*, 2003).

372 In contrast, in nutrient poor environments, the impact of grazers on the bacterial
373 community is greater, resulting in significant changes in the composition of the
374 bacterial community (Jürgens*, et al.*, 1999, Hahn & Höfle, 2001). The greater
375 effectiveness of prevention of grazing losses of *V. cholerae* grown under low nutrient
376 concentrations suggests an adaptation to higher grazing pressure in natural food webs
377 with less available nutrients. In fact, top-down control (of the grazers) is generally
378 more pronounced in resource-limited areas (Simek*, et al.*, 2003). This pattern of
379 defense metabolite production fits with the resource availability hypothesis which
380 states that in environments with low resource availability, plants with low growth
381 rates and high levels of defense will be favored, while in environments with high
382 resource availability, plants with fast growth and lower defense levels will be favored
383 (Coley*, et al.*, 1985). Thus, when nutrients are available, *V. cholerae* is able to grow
384 more quickly than it is eliminated by predation, but when nutrients are limited,
385 resources may be shifted from growth to defense metabolites.

386 The results presented here show that *V. cholerae* biofilms are protected from grazing
387 losses *in situ* where there are large dilution effects due to the surrounding seawater
388 environment. While the biofilm biomass remained stable under grazing pressure, the
389 planktonic biomass increased for both strains when the benthic feeder, *R. nasuta* was
390 present on the biofilm. This may be due to cells leaving the biofilm for the planktonic
391 phase to 'escape' predation and to increased nutrient availability due to nutrient

392 recycling by predator feeding. The lower abundances of flagellates on N16961
393 biofilms was unexpected as this strain has a frameshift mutation in *hapR*, which has
394 been shown to be required for the antiprotozoal activity of the A1552 strain expressed
395 in the laboratory (Matz, *et al.*2005). These data indicated that there is potentially QS-
396 independent antipredation activity exhibited by this strain, but does not rule out the
397 possibility that there may also be other strain differences that account for the grazing
398 protection. This work is the first to show that the protection against grazing losses
399 expressed by *V. cholerae* is ecologically relevant and further highlights the
400 advantages of surface-associated growth in environmentally relevant contexts. The
401 data also clearly show that *V. cholerae* expresses traits which prevent grazing induced
402 loss of biomass that are regulated by the cell-cell signaling pathway, *hapR*, as well as
403 in response to environmental conditions (e.g. nutrients) and thus, such traits could
404 play important roles in the persistence of *V. cholerae* in the environment within
405 predator resistant biofilms.
406

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525 Table 1. Environmental chamber field experiments

	Exp. I	Exp. II	Exp. III	Exp. IV
Date	11-21/01/08	02-12/03/09	08-18/05/09	04-14/05/08
Bacterial strains	V.c. A1552[a]	V.c. A1552	V.c. A1552	V.c. A1552
	V.c. N16961	V.c. N16961	V.c. N16961	E.c. B[b]
Grazers	*C. roenbergensis*	*C. roenbergensis*	*C. roenbergensis*	*C. roenbergensis*
	R. nasuta		*R. nasuta*	*R. nasuta*
No. replicates[c]	3 each	4 each	3 each	3 each
Exposure time	10 days	10 days	10 days	10 days

526 [a]V.c. represents *V. cholerae* strains

527 [b]E.c. represents *E. coli*

528 [c]Number of chambers containing individual bacterial strains and grazers. Each

529 chamber held 1 bacterial strain and 1 grazer.

530

531 **FIGURE LEGENDS**

532 Figure 1. Abundance of (A) *R. nasuta* on *V. cholerae* A1552 and N16961 biofilms
533 and (B) abundance (CFU mL^{-1}) of suspended *V. cholerae* A1552 (white bar) and
534 N16961 (black bar) from environmental chambers after 10 days co-culture in the
535 marine environment. Chambers were co-inoculated with the flagellate *R. nasuta*
536 (grazed) or not (non-grazed). Shown are mean values (± SD, n=3). Note the
537 logarithmic y-scale. (A) ** T-test $p < 0.01$, (B) *** 2-way ANOVA $p < 0.001$.

538 Figure 2. Abundance of (A) *C. roenbergensis* on *V. cholerae* A1552 and N16961
539 biofilms and (B) abundance (CFU mL^{-1}) of suspended *V. cholerae* A1552 (white bar)
540 and N16961 (black bar) from environmental chambers after 10 days co-culture with
541 the protozoan in the marine environment. Chambers were co-inoculated with the
542 flagellate *C. roenbergensis* (grazed) or not (non-grazed). Shown are mean values (±
543 SD, n=4). Note the logarithmic y-scale, (B) * 2-way ANOVA $p < 0.05$.

544 Figure 3. Abundance of (A) the flagellates *R. nasuta* and *C. roenbergensis* on *V.*
545 *cholerae* A1552 (white bar) and N16961 (black bar) biofilms and (B) the abundance
546 (CFU mL^{-1}) of suspended *V. cholerae* A1552 (white bar) and N16961 (black bar)
547 from environmental chambers after 10 days co-culture with the protozoan in the
548 marine environment. Shown are mean values (± SD, n=3). Note the logarithmic y-
549 scale. 2-factorial ANOVA revealed no significant differences.

550 Figure 4. Abundance of (A) the flagellates *R. nasuta* and *C. roenbergensis* on *V.*
551 *cholerae* A1552 (white bars) and *E. coli* B (black bars) biofilms in environmental
552 chambers after 10 days in the marine environment. *R. nasuta* appeared on the
553 *V.cholerae* A1552 biofilms in very low abundances (0.32 ± 0.29 Ind mm^{-2}). 2-
554 factorial ANOVA revealed highly significant differences between the grazers *** $p <$

555 0.001. (B) Abundance (CFU mL^{-1}) of suspended *V. cholerae* A1552 (white bar) and
556 *E. coli* B (black bar) in environmental chambers after 10 days in the marine
557 environment. Shown are mean values (± SD, n=3).

558 Figure 5. Abundance of the flagellates *R. nasuta* (A) and *C. roenbergensis* (B) *on V.*
559 *cholerae* A1552 (white bar), the A1552 *hapR* mutant strain (black bar) and N16961
560 (striped bar) biofilms in the flow cells of the semi-field set-up over 5 days. Shown are
561 mean values (± SD, n=4). One-factorial ANOVA revealed no significant differences
562 for *C. roenbergensis* on the different *V. cholerae* strains on day 5. H-test (Kruskal-
563 Wallis-Test) showed significant differences for *R. nasuta* on day 5 ($p = 0.02$). A U-
564 test (Mann-Whitney) revealed significant differences between the strains A1552 and
565 *hapR* ($p < 0.04$) and N16961 and A1552 *hapR* ($p < 0.04$) and no significant difference
566 between the A1552 wild type and N16961 strains ($p = 1$).

567 Figure 6. Growth rates of *R. nasuta* on *V. cholerae* A1552 wild type and *hapR* mutant,
568 and N16961 biofilms grown in seawater with two different glucose concentrations for
569 3 days. Sterile filtered seawater was supplemented with 0.1% glucose (white bar) or
570 0.001% glucose (black bar) as a carbon source, respectively. Shown are mean values
571 (± SD, n=3). A 2-factorial ANOVA showed significant differences in strain ($p = 0.03$)
572 and glucose concentrations ($p = 0.02$). A Tukey-HSD posthoc-test revealed
573 significant differences between A1552 wild type and *hapR* mutant ($p = 0.004$) and
574 N16961 and A1552 *hapR* biofilms ($p = 0.014$).

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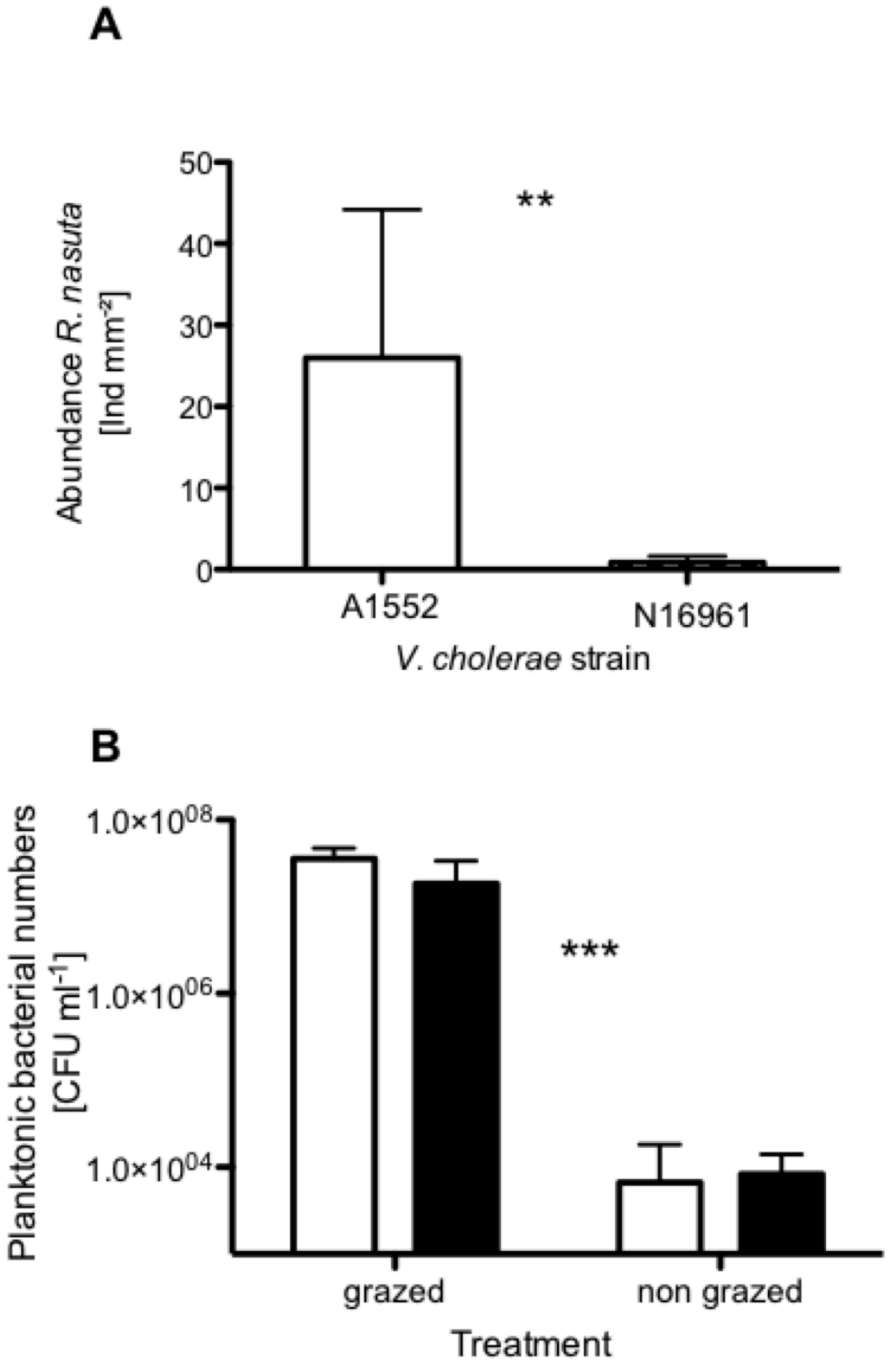
577 Figure 1.



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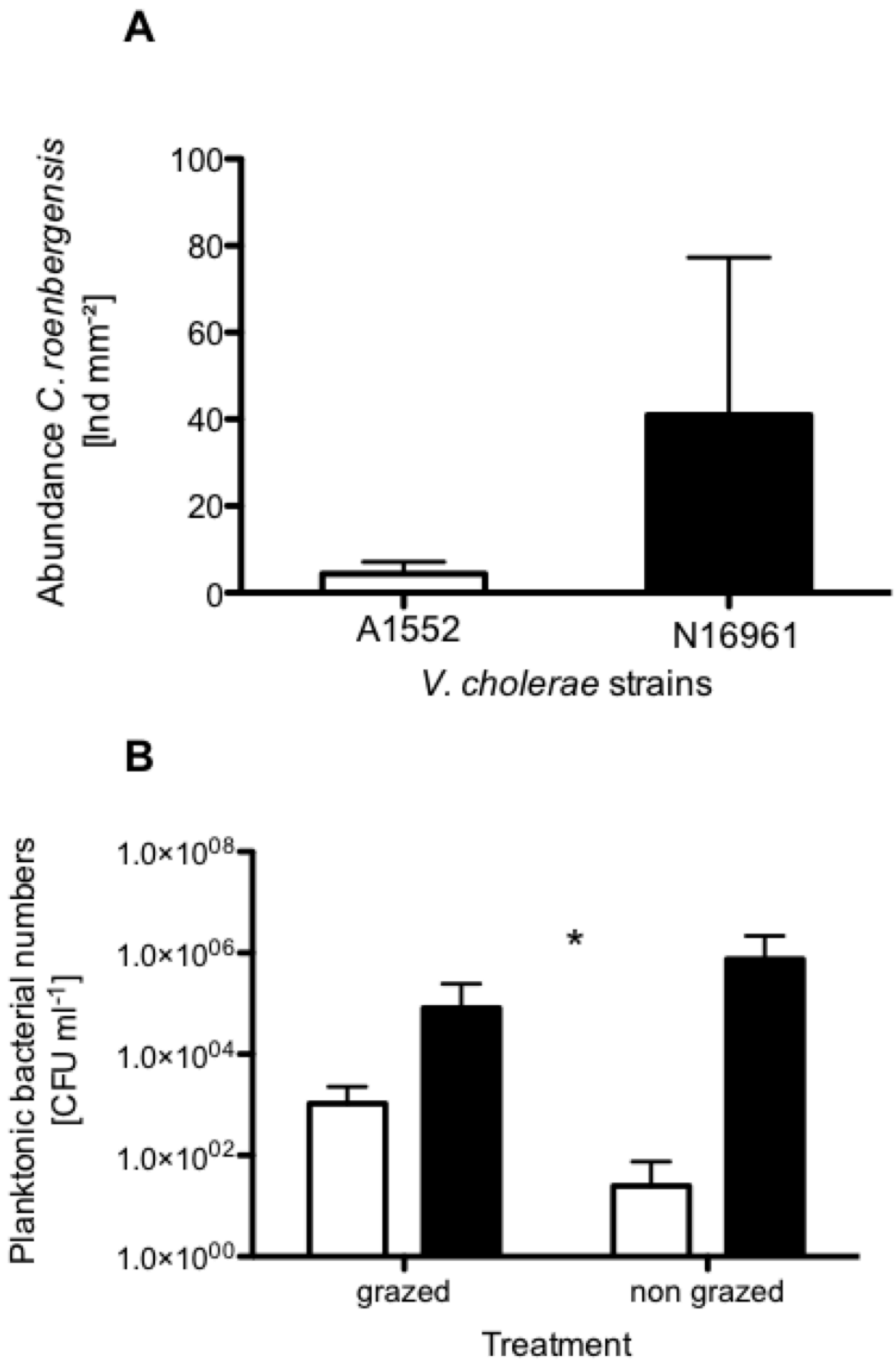

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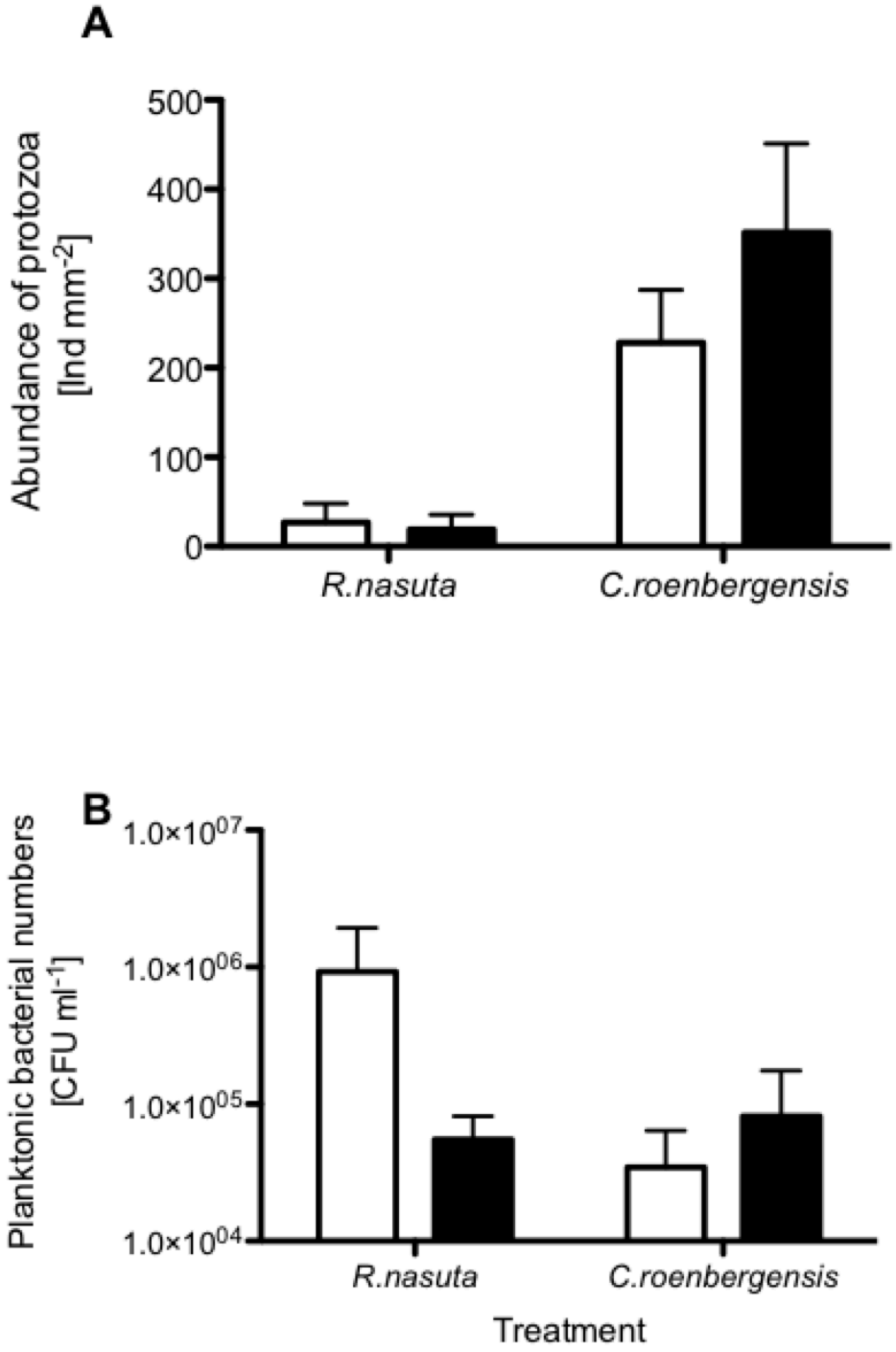

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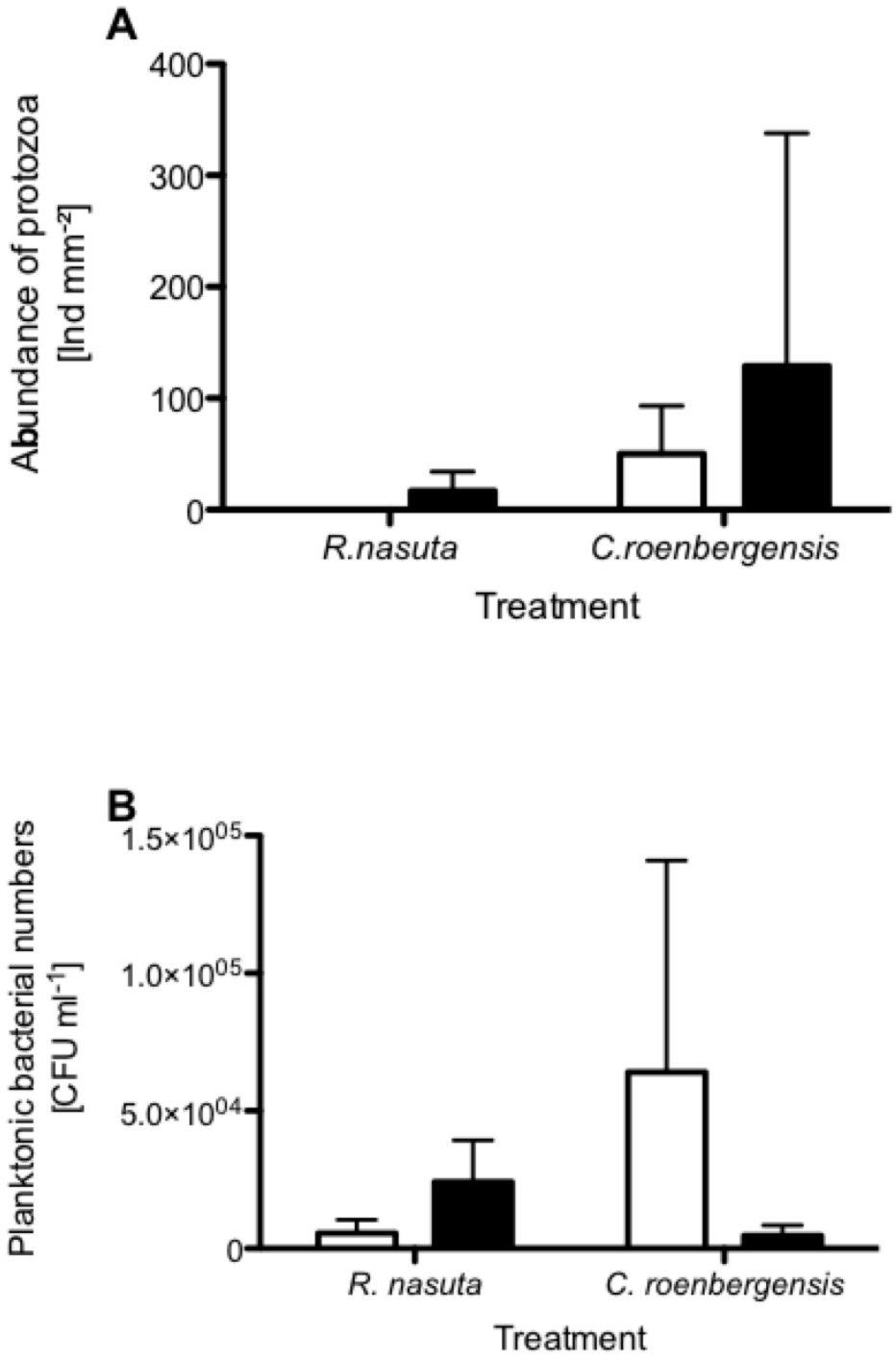

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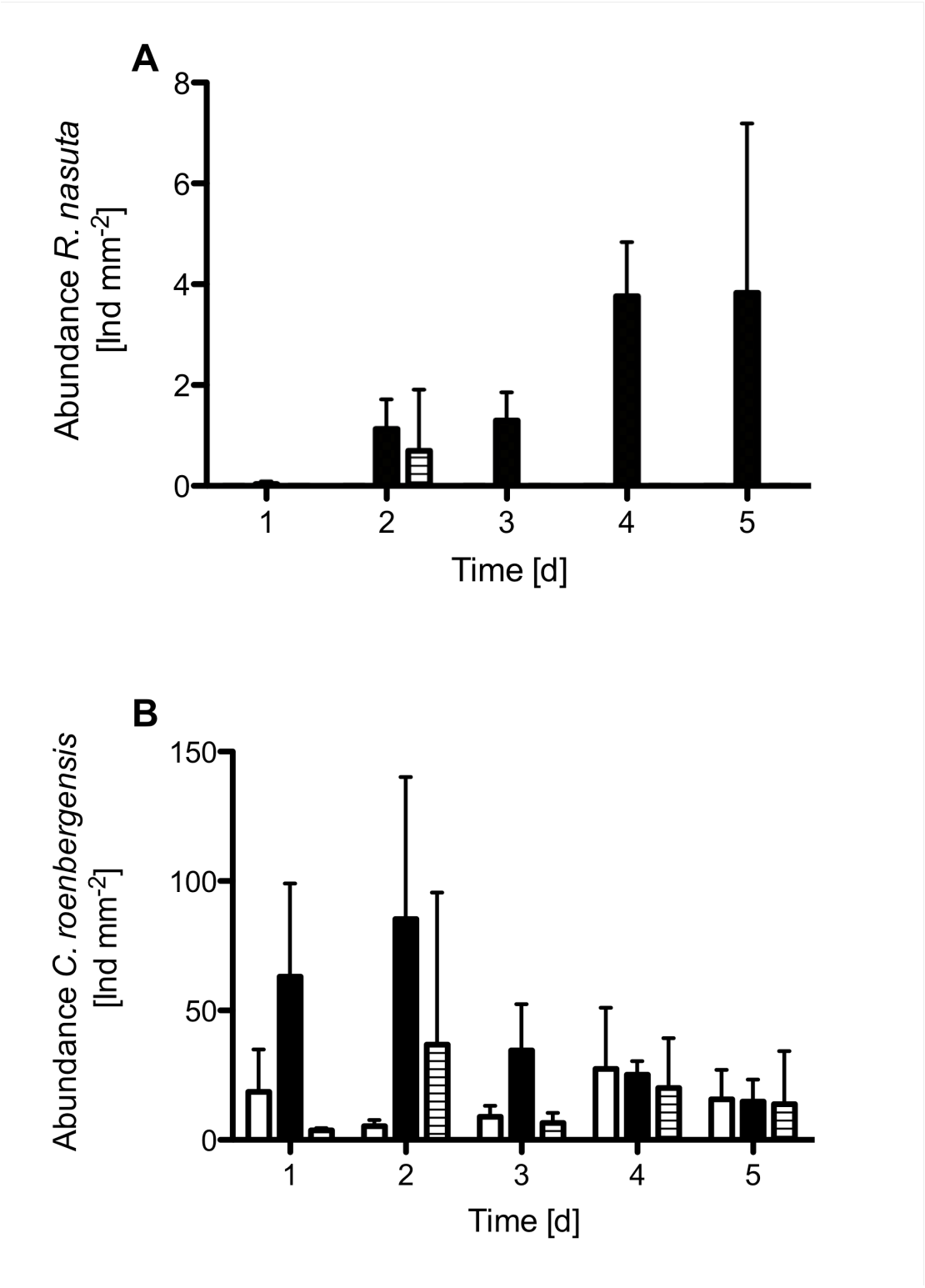

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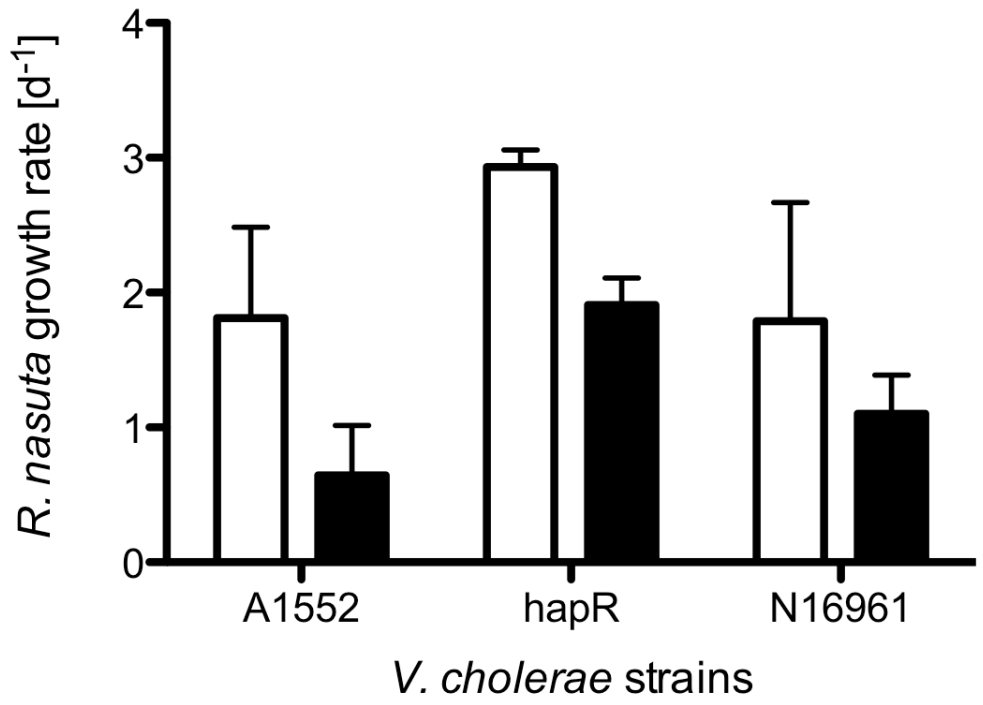

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