

SCIENTIFIC REPORTS



OPEN

Predators and nutrient availability favor protozoa-resisting bacteria in aquatic systems

A. Andersson^{1,2}, J. Ahlinder³, P. Mathisen¹, M. Hägglund³, S. Bäckman³, E. Nilsson³, A. Sjödin³  & J. Thelaus³

The long co-existence of bacteria and protozoa has led to the development of bacterial protozoa resistance strategies, which are suggested to serve as drivers for the evolution of pathogenic bacteria. However, the ecological mechanisms underpinning selection for protozoa-resistance in aquatic bacteria are poorly known. To assess the role of nutrient availability and predation-pressure on selection for protozoa-resisting bacteria (PRB), an enrichment-dilution experiment was designed using laboratory microcosms containing natural lake water. PRB was monitored by screening 16S rRNA amplicon sequence data for reads assigned to bacteria that previously has been shown to resist degradation by amoebae. To estimate the effects of the microbial food web dynamics (microscopy of; heterotrophic bacteria, phytoplankton, protozoa and rotifers) and physicochemical variables on the PRB abundance in the study system, a joint species distribution modelling approach was used. The predation-pressure (ratio between predator and bacterial biomass) had a positive effect on the abundance of the PRB genus *Mycobacterium*, while perturbation (enrichment and dilution) favored the PRB genus *Pseudomonas* that dominated the bacterial community in the disturbed systems. Our results show that PRB with different ecological strategies can be expected in water of high and intermediate nutrient levels and after major disturbances of an aquatic system.

The potential for transmission of pathogenic bacteria from environmental reservoirs to susceptible hosts depends on the pathogens' environmental distributions and the hosts' behaviour. Knowledge of pathogens' environmental persistence is limited, largely because it is difficult to study these microbes in their natural environments. However, a better understanding of pathogens' long-term fates in natural ecosystems is needed to accurately assess risks of exposure and design effective strategies for responding to emerging diseases^{1,2}.

The long co-existence of bacteria and bacterivorous protozoa in aquatic and terrestrial ecosystems has led to the evolution of antipredator strategies in many bacterial groups³. Bacteria that can survive protozoan predation, *i.e.* protozoa-resisting bacteria (PRB), frequently exhibit traits such as morphological adaptation (e.g. elongation, aggregation, and filament or biofilm formation), increased growth rates, motility, toxicity and ability to replicate or avoid degradation in eukaryotic cells using specific outer membrane structures^{4–9}. Interactions between bacteria and bacterivores have been suggested to serve as a driver or selective force for pathogen evolution^{3,10–14}. Pathogenic bacteria contain virulence factors that enable them to colonize a niche in their hosts, evade or suppress the host's immune response, enter and exit host cells, or obtain nutrition from the host. Several of these abilities are similar to those of PRB; it is widely believed that many traits that render bacteria pathogenic in susceptible hosts were shaped by evolutionary forces outside the context of human-pathogen interactions, and should be seen as colonization factors that produce "accidental virulence"¹⁵.

Bacteria in natural aquatic systems are exposed to diverse stressors that vary over time and over spatial scales. In addition to protozoan predation and viral lysis, aquatic bacterial communities are exposed to bottom-up forces such as limited nutrient availability^{16–18}. Consequently, these communities experience highly variable conditions, creating fluctuations in their selection dynamics *i.e.* variation in the bottom-up and top-down control of the microbial communities^{19,20}. The heterogeneity of the aquatic environment may create niches that enable the co-existence of bacteria with different defense traits. Therefore, PRB could theoretically occur in any aquatic

¹Department of Ecology and Environmental Science, Umeå University, SE-901 87, Umeå, Sweden. ²Umeå Marine Sciences Centre, SE-905 71, Hörnefors, Sweden. ³Division of CBRN Defence and Security, Swedish Defence Research Agency, FOI, SE-901 82, Umeå, Sweden. Correspondence and requests for materials should be addressed to A.A. (email: agneta.andersson@umu.se)

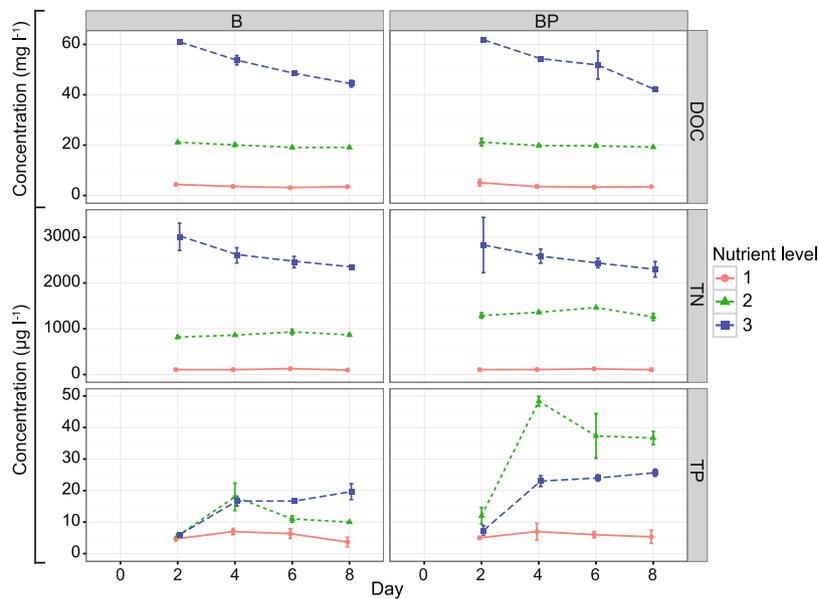


Figure 1. Concentrations of dissolved organic carbon (DOC), total nitrogen (TN) and total phosphorus (TP) during the experiment. Treatments: B = bacteria, BP = bacteria + predators and nutrient level = 1 (diluted), 2 (*in situ*), or 3 (enriched). Error bars indicate standard deviations.

system. However, the ecological mechanisms underpinning selection for protozoa-resisting bacteria with varying ecological strategies are poorly known.

We performed a controlled experiment with the aim to elucidate effects of bottom up (nutrient level) and top-down forces (predation) on the occurrence of PRB genera in natural aquatic microbial communities. The presence of PRB was monitored by amplicon sequencing of the 16S rRNA gene. PRB were defined as amoeba-resisting bacteria¹³, which comprises bacteria shown to resist amoeba degradation with varying ecological strategies (amoeba associated and free-living). We hypothesized that increased nutrient load would increase the predation-pressure on the bacteria, leading to selection for PRB.

Results

Dynamics of nutrients, plankton and predation-pressure on bacteria. The dissolved organic carbon (DOC) and total nitrogen (TN) concentrations for the highest and lowest nutrient levels (levels 3 and 1) differed ~20–30-fold, while TP concentrations differed by a factor of ~10 (Fig. 1). At nutrient level 3, the DOC and TN concentrations decreased over time, but at lower levels (2 and 1), DOC and TN were stable throughout the experiment. TP increased between days two and four and then remained relatively stable.

The biomass of autotrophic and mixotrophic plankton was lowest at nutrient level 1 and highest at levels 2 and 3 (Fig. 2). Initially their biomasses were highest in BP2, but decreased over time. Despite incubation in darkness, the biomasses of autotrophic and mixotrophic plankton increased in B3. Heterotrophic predator biomass were high in BP2, intermediate in BP3, and very low or absent in BP1 (Fig. 2). Heterotrophic predators in the BP2 and BP3 included rotifers, amoebae, ciliates and heterotrophic nanoflagellates (HNF) (Fig. 3). The B treatments were generally free of predators, although HNF were detected in B2 and B3 (Fig. 3).

The bacterial biomass increased steadily over time in BP3, but gradually decreased from an initially high level in BP2 (Fig. 2). Similar patterns occurred in B2 and B3. Conversely, the bacterial biomass remained low throughout in both nutrient level 1 treatments (B1 and BP1).

Initially the predation-pressure on bacteria in BP2 and BP3 were equally high (Fig. 2). However, over time the pressure increased in BP2. Towards the end of the experiment, the predation-pressure on bacteria was 20 times higher in BP2 than in the other treatments.

Bacterial community composition. The 16s rRNA amplicon sequencing yielded 12,509,951 reads, 11,176,076 (89.3%) of which remained after OTU picking. The reads were clustered into 6,463 OTUs. After filtration, 10,882,635 reads remained, clustered into 544 OTUs; the number of reads per sample ranged from 19,471 to 243,297 (Appendix A). The rarefaction curves, representing the estimated diversity within the different treatments, reached an asymptote at a sequencing depth equal to the minimum number of reads per sample (Suppl. Figure 2). This indicates that the sequencing depth was sufficient for further analysis and most of the samples' expected diversity was captured.

Eleven major phyla (including sub-classes of Proteobacteria) were identified: Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Cyanobacteria, Deltaproteobacteria, Firmicutes, Gammaproteobacteria, Gemmatimonadetes, Planctomycetes and Verrucomicrobia (Suppl. Figure 3). Sub-classes of the phylum Proteobacteria were included because most of the reads (39.0–97.8% per sample) were classified to that phylum. Betaproteobacteria was the dominant group at the lowest nutrient levels (1 and 2), but Gammaproteobacteria dominated at nutrient level 3.

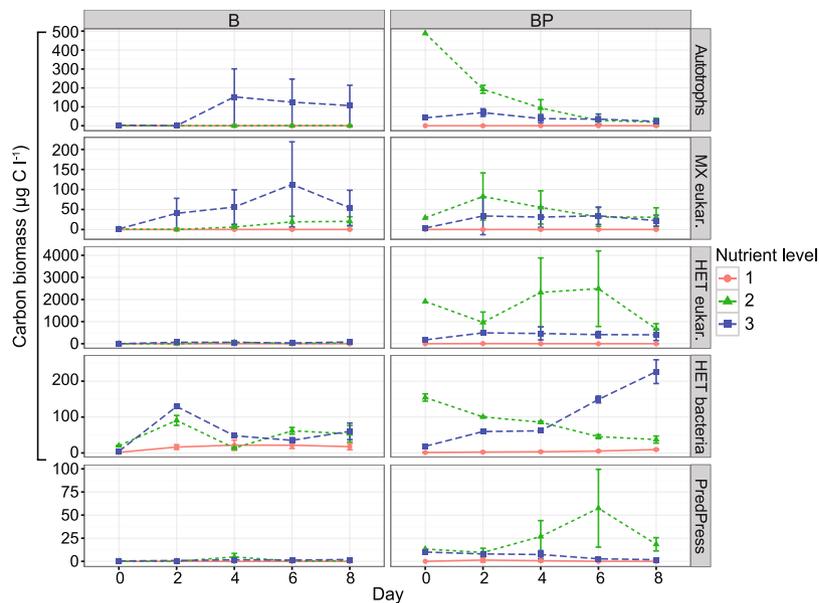


Figure 2. Carbon biomasses of autotrophic plankton (Autotrophs), mixotrophic plankton (MX eukar.), heterotrophic predators (HET eukar.), heterotrophic bacteria (HET bacteria) and predation pressure on bacteria (PredPress) during the experiment. Treatments: B = bacteria, BP = bacteria + predators and nutrient level = 1 (diluted), 2 (*in situ*), or 3 (enriched). Error bars indicate standard deviations.

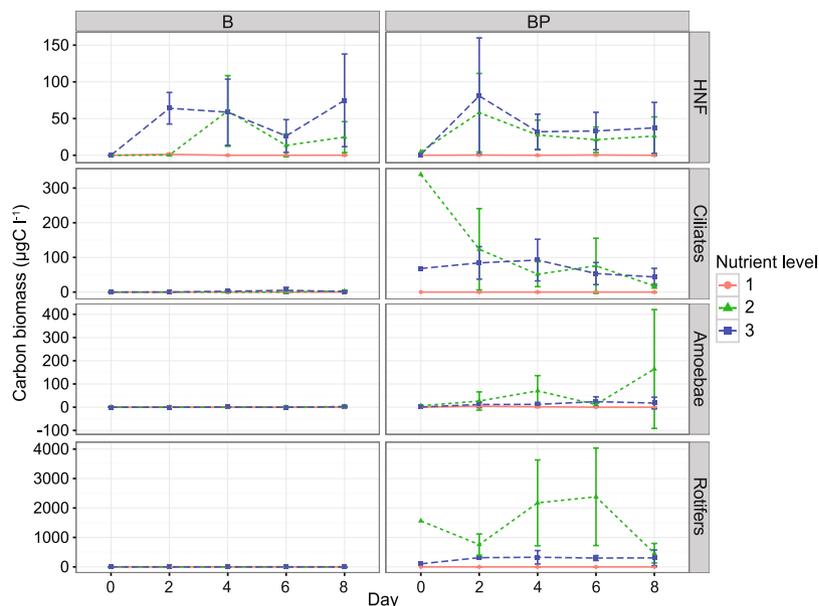


Figure 3. Biomass concentrations of heterotrophic predators, *i.e.* heterotrophic nanoflagellates (HNF), ciliates, amoebae and rotifers during the experiment. Treatments: B = bacteria, BP = bacteria + predators and nutrient level = 1 (diluted), 2 (*in situ*), or 3 (enriched). Error bars indicate standard deviations.

The bacterial community's alpha diversity was higher in the undisturbed *in situ* cultures (nutrient level 2) than in the diluted or enriched treatments (estimate 5.25, SE 0.72, $p = 2.57E-9$). Presence of heterotrophic predators resulted in higher alpha diversity in BP2 and BP3 than in other treatments (estimate 4.72, SE: 0.72, $p = 9.74E-9$).

Factors promoting the occurrence of protozoa-resisting bacteria (PRB). OTUs associated with three genera of PRB were identified from the 16S rRNA sequence data: *Pseudomonas* (Gammaproteobacterium), *Rickettsia* (Alphaproteobacterium) and *Mycobacterium* (Actinobacterium). The proportion of reads assigned to *Mycobacterium* and *Rickettsia* increased over time in BP2 (Fig. 4), which had the highest predation-pressure (Fig. 1). However, these PRB genera constituted a relatively small proportion of the total bacterial reads (~0.1%) in all samples. *Pseudomonas* was promoted in the enriched treatments B3 and BP3 (Fig. 4). In these samples, *Pseudomonas* quickly became dominant, constituting 30–50% of the total bacterial reads. A PCA biplot

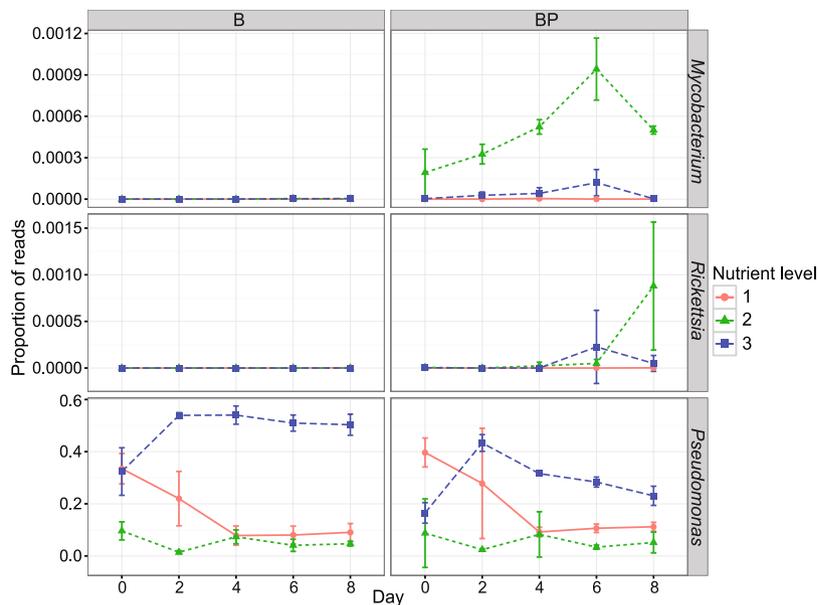


Figure 4. Proportion of reads assigned to protozoa-resisting bacterial (PRB) genera *Mycobacterium*, *Rickettsia* and *Pseudomonas* during the experiment. Treatments: B = bacteria, BP = bacteria + predators and nutrient level = 1 (diluted), 2 (*in situ*), or 3 (enriched). Error bars indicate standard deviations.

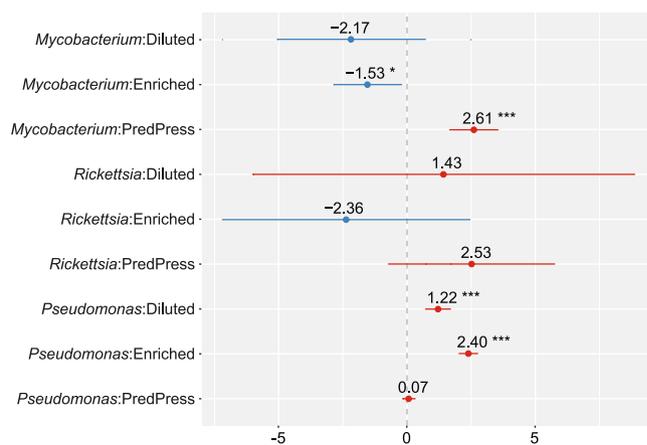


Figure 5. Summary statistics of the joint species distribution model (JSDM) analysis for experiment days 2–8, including effect sizes and 95% effect size confidence intervals for the nutrient and predation pressure (PredPress) parameters. Blue and red colors indicate negative and positive effect directions, respectively. Statistical significance at the $\alpha = 0.05$, $\alpha = 0.01$ and $\alpha = 0.001$ levels is denoted by *, ** and ***, respectively. Treatments: Diluted (nutrient level 1), Enriched (nutrient level 3).

of measured variables indicated that *Pseudomonas* was linked to high DOC and TN concentrations and low predation-pressure (Suppl. Figure 4A), while *Mycobacterium* and *Rickettsia* were linked to heterotrophic nano- and microplankton and high predation-pressure, TP and alpha diversity. Pearson correlation analysis showed that *Pseudomonas* correlated negatively with *Rickettsia* and *Mycobacterium* (Suppl. Figure 4B).

The JSDM analysis showed that predation-pressure had a strong positive effect on *Mycobacterium* abundance ($\hat{\beta}_{51} = 2.61$; 95% CI: 1.68, 3.55) (Fig. 5). This corresponds to a greater than 13-fold predicted increase in abundance if the predation-pressure ratio increased by 50% while all other conditions were unchanged. The same analysis revealed a negative association for *Mycobacterium* abundance with both dilution and enrichment compared to the undisturbed condition (Fig. 5). The association with dilution was weak ($\hat{\beta}_{211} = -2.17$; 95% CI: -5.05, 0.71) but that with enrichment was more obvious ($\hat{\beta}_{213} = -1.53$; 95% CI: -2.85, -0.22). The relatively rare occurrence of *Rickettsia* resulted in wide estimated 95% CI ranges for all parameters: none of the corresponding parameters was statistically significant. However, given that *Rickettsia* was only detected in predator-rich samples, predation-pressure seemed to be a favorable predictor ($\hat{\beta}_{52} = 2.53$; 95% CI: -0.71, 5.76). The biomasses of both amoebae (Fig. 3) and *Rickettsia* peaked on the last day of the experiment (Fig. 4), suggesting potential co-occurrence. Both dilution and enrichment had strong positive effects on the abundance of *Pseudomonas* ($\hat{\beta}_{231} = 1.22$; 95% CI: 0.73, 1.70 and $\hat{\beta}_{233} = 2.40$; 95% CI: 2.04, 2.76). This implies that going from the undisturbed

in situ state to the enriched state without changing any other predictors would increase the *Pseudomonas* abundance ten-fold. Similarly, a change from the undisturbed *in situ* state to the diluted state would increase abundance three-fold. Predation-pressure was shown to be less important ($\hat{\beta}_{53} = 0.07$; 95% CI: $-0.15, 0.30$).

The JSMD analysis revealed a negative correlation between the occurrence of *Pseudomonas* and the two other PRB genera ($r = -0.27$ and $r = -0.30$ for *Mycobacterium* and *Rickettsia*, respectively), but there was a positive correlation between *Mycobacterium* and *Rickettsia* ($r = 0.60$). These inferred correlations were obtained after adjusting for treatment effects, time and replicate dependencies, and are therefore lower than the corresponding Pearson correlations (see Suppl. Figure 4B). There was, on average, a predicted decay in the abundance of PRB in the diluted samples over time that manifested as a negative slope effect. In all other cases, the predicted abundance of PRB genera increased over time. A JSMD analysis of only the last-day data resulted in parameter estimates that almost always included zero within the 95% CI (results not shown). This result suggested that all the data from day's two to eight should be included in the modelling. Excluding rotifers in the calculation of predation pressure on bacteria, yielded similar results in the JSMD analysis (Suppl. Figure 5). However, one difference was that the predation pressure had less distinct influence on the occurrence of *Mycobacterium*, indicated by a wider 95% confidence interval of the regression coefficient (Suppl. Figure 5).

Discussion

Contradicting our hypothesis, the predation-pressure did not increase in parallel with the nutrient load in natural lake water. This was unexpected because a previous field study identified a weak but general increase in predation-pressure with increasing productivity²¹. Here, the predation-pressure increased >1000-fold from the diluted to the undisturbed *in situ* state, and >100-fold from the diluted state to the highest nutrient load. The DOC concentrations in the experiment ranged from ~3 to 50 mgC l⁻¹, with carbon concentrations in the diluted, *in situ*, and enriched treatments corresponding to those of drinking water or oceanic water, eutrophied waters and hypertrophic aquatic systems^{22–24}, respectively. Predation-pressure had a strong positive effect on *Mycobacterium*, while nutrient availability had a negative effect on this bacterial genus. The opposite was observed for the opportunistic *Pseudomonas*, which was greatly promoted by nutrient enrichment. Our results thus imply there is a threshold beyond which the predation-pressure on bacteria does not increase further. This may be due to changes in the bacterial community composition that could arise if predation-resistant bacteria have negative effects on their predators.

Mycobacterium was favored by *in situ* conditions with nutrient concentrations matching those found under eutrophic conditions. The predator biomass, the predation-pressure on bacteria, and the alpha diversity of bacteria were all high under these circumstances. The genus *Mycobacterium* is divided into the tuberculosis and non-tuberculous complex, both of which have obligate or facultative intracellular lifestyles²⁵. Species within the *Mycobacterium tuberculosis* complex (MTC) cause tuberculosis in mammals and humans, predominantly as a result of direct host-to-host contact^{26,27}. Non-tuberculous mycobacteria (NTM) are responsible for community-acquired and focal health-care associated infections, and have been isolated from natural waters, water distribution systems, drinking water, soil, food, biofilms, aerosols and dust^{28,29}. In keeping with previous studies, our results show that top-down control of the microbial community favors the occurrence of mycobacteria^{25,30–33}. Rotifers and protozoa are both potential predators of bacteria. Omnivory is very common among both ciliates and rotifers^{34–36}, so it is likely that they fed on both bacteria and HNF in the *in situ* and enriched treatments. In the present study, it is not clear whether the *Mycobacterium* were growing intracellularly in the protozoa or saprozoically (extracellularly), as shown for *Mycobacterium avium* in co-culture with amoeba³⁷. Nevertheless, our results indicate that predatory protozoa have important effects on the persistence and dissemination of *Mycobacterium* in aquatic systems as a source of human infection³².

The obligate intracellular *Rickettsia* spp. appeared to be promoted by high predation-pressure, but this could not be proven by statistical analysis. *Rickettsia* spp. are obligate intracellular bacteria associated with eukaryotes, mainly arthropods, and range from being harmless to etiologic agent of severe disease in humans^{38,39}. In our study, *Rickettsia* showed a marked covariation with *Mycobacterium*, indicating that they have similar ecological niches in the system. This suggests that both genera exist in association with protozoa and other predators, and are therefore favored under systems subject to strong top-down control. Notably, under *in situ* conditions, the abundance of both *Rickettsia* and amoebae peaked on the last day of the experiment (Figs 3 and 4). This may indicate that *Rickettsia* depend on amoebae as hosts^{39,40}. It would be interesting to further elucidate if *Rickettsia* use specific species or groups of species as hosts in natural systems.

Pseudomonas spp. dominated the enriched treatment constituting 20–40% and 50–60% of the bacterial sequences in the presence and absence of protozoa, respectively. Bacteria from this genus can grow on a wide range of organic compounds due to their vast metabolic capabilities^{41,42}. Consequently, they can colonize diverse environmental niches and species within this genus are among the most widespread opportunistic pathogens, causing infections in hosts including insects, plants, animals and humans⁴². Several *Pseudomonas* strains respond to grazing by inducing resistance traits such as colony and biofilm formation and activation of the type III secretion system, which mediates the secretion of effector toxins^{43–46}. Pathogenic *Pseudomonas aeruginosa* has been shown to kill the amoeba *Acanthamoeba castellanii* by producing toxins mediated by the type III secretory system^{45,47}. The opportunistic ability to compete for nutrients and fast growth together with the ability to form biofilms and micro-colonies enables *Pseudomonas* to monopolize the bacterial community in disturbed systems. In our study, nutrient enrichment greatly increased the abundance of *Pseudomonas*, which in turn reduced the general predation-pressure on bacteria. This is consistent with previous findings showing that *Pseudomonas* is promoted in eutrophic and disturbed systems such as streams receiving effluents from hospital waste and fertilized soils^{48,49}.

The definition of PRB is based on amoeba-resisting bacteria (ARB), i.e. bacterial taxa that resist uptake by amoebae and can survive, grow inside, and exit free living amoeba after internalization^{13,38}. These ARBs include

established pathogens such as *Legionella pneumophila*, *Mycobacterium avium*, *Francisella tularensis* and *Rickettsia* sp. Advancements in genome sequencing have made it possible to monitor the presence of traits associated with bacterial pathogenicity, i.e. virulence genes and pathogenicity islands, in complex environmental samples. Bacterial traits linked to pathogenicity (protein secretion systems, toxins and pathogenicity islands) are known to be widespread in marine bacteria, being present in up to 8% of oceanic metagenomic data⁵⁰. Previous studies have related the presence of virulence genes to environmental factors such as productive waters, polycyclic aromatic hydrocarbons, and pH, but have not linked these genes to the occurrence of pathogenic bacterial taxa or associated them with infectious disease^{50,51}. This suggests that bacterial gene homologues associated with virulence in humans commonly enhance persistence in natural environments. We therefore suggest that analysis of PRB genera, could be useful to gain knowledge on ecological drivers that influence the long-term fates of pathogens in natural ecosystems.

This study shows that the generative model may be useful in risk assessment frameworks because it integrates environmental data (i.e. microbial food web dynamics and physiochemical data) with predictive modeling of bacterial abundance in aquatic systems. We have shown that the relationship between organic load and predation-pressure on bacteria is non-linear: there seems to be a tipping point beyond which PRB with extracellular lifestyles start to control the predators. Above this threshold, disturbances enable opportunistic bacteria such as strains within the *Pseudomonas* genus to monopolize the system, causing very little energy to be transferred up the food web. Conversely, the high predation-pressure on bacteria associated with undisturbed *in situ* conditions promotes other types of PRBs, including the slow-growing intracellular *Mycobacterium* spp. and *Rickettsia* spp., which depend on eukaryotic cells for survival and replication. Our results thus indicate that waters of all nutrient states can harbor PRB, but that bacteria with different ecological strategies can be expected in water of high and intermediate nutrient levels and after major disturbances of the aquatic system.

Material and Methods

Microcosm experiment. A microcosm experiment was performed using water from a small eutrophic lake in southern Sweden (Suppl. Material 1). The experiment consisted of six triplicated treatments, including three different nutrient levels (1 - diluted, 2- *in situ* and 3- enriched) with or without predators (BP and B, respectively) (Suppl. Figure 1). The dilution treatment was designed to mimic oligotrophic conditions, while the enrichment treatment was intended to simulate hypertrophic environments. Bacterial composition and plankton biomass were analyzed on day 0, 2, 4, 6 and 8, while chemical analyses were performed on day 2, 4, 6 and 8.

Chemical analyses. TP and TN were measured using a Braan & Luebbe TRAACS 800 autoanalyzer, according to standard analytical methods⁵². Dissolved organic carbon (DOC) was analyzed in water filtered through a 0.22 μm Supor Membrane Syringe Filter (non-pyrogenic; Acrodisc[®]) and acidified to 18 mM HCl, final concentration. Samples were analyzed with a Shimadzu TOC-5000 instrument.

Bacterial abundance and biomass. Samples for bacterial counts were preserved in 0.1% glutaraldehyde (final concentration), frozen at $-80\text{ }^{\circ}\text{C}$ ⁵³ and analyzed with a BD FACSVerse[™] flow cytometer (BD Biosciences). Samples were stained with SYBR Green I (Invitrogen) at a final concentration of 1:10 000⁵³. As internal standard, 1 μm microspheres (Fluoresbrite plain YG, Polysciences) were added to each sample. The bacterial carbon biomass was calculated using a conversion factor of 20 fg C cell⁻¹ according to Lee and Fuhrman⁵⁴.

Nano and microplankton abundance and biomass. Samples were preserved with alkaline Lugol's solution (2% final concentration) and analyzed using Utermöhl technique. Briefly, samples (10 ml) were added to sedimentation chambers and plankton allowed to settle for 12 hours before being counted with a Nikon inverted microscope (TMS) using phase contrast. Nanoplankton were counted in one transect at 400 times magnification, while microplankton were generally counted in half a chamber at 100 times magnification. Generally >100 cells per sample were counted. Phytoplankton, protozoan, and rotifer biovolumes were calculated from cell geometries as described by Olenina *et al.*⁵⁵ and Ruttner-Kolisko⁵⁶. Carbon biomasses were calculated according to Menden Deuer and Lessard⁵⁷. Plankton were grouped according to nutritional strategy: autotrophy, mixotrophy or heterotrophy.

Results of chemical analyses and carbon biomasses of heterotrophic bacteria, phytoplankton, and predators were visualized using the R package ggplot2, version 2.1.0^{58,59}.

DNA extraction. Four ml of each water sample were centrifuged at $16\ 000 \times g$ for 1 h, 3.9 ml of the resulting supernatant were discarded, and DNA was extracted from the remaining volume using the SoilMaster DNA Extraction Kit according to the manufacturer's recommendations for environmental water samples (Epicentre Biotechnologies, Madison, WI, USA). The resulting DNA pellet was resuspended in 60 μl of TE buffer and either frozen and stored or immediately subjected to PCR analysis. Sample preparation, PCR reaction preparation, and thermal cycling were performed in different rooms.

Amplicon preparation and sequencing. Amplicons were generated targeting the V4 region of the bacterial 16S rRNA gene⁶⁰. Briefly, the V4 region was amplified using primers F515 (5'-GTCCAGCMGCCGCGGTAA-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3'). Forward and reverse primers were modified to incorporate a 12 bp Golay error-correcting barcode that enabled sample multiplexing with both primers⁶⁰. Amplicons were prepared in triplicate and the resulting PCR products were pooled and analyzed on 1% agarose gel pre-stained with GelRed (1:10,000, GelRed[™] Nucleic Acid Gel Stain, Biotium). Concentrations were determined with a Qubit dsDNA BR Assay Kit on a Qubit fluorometer (Invitrogen). The amplicons were pooled at equimolar concentrations and purified using the UltraClean PCR Clean-Up Kit (MoBio), then sequenced on the MiSeq platform

(Illumina) with 500 bp paired-end reagent kits according to the manufacturer's recommendations (MiSeq System User Guide, Part # 15027617 Rev. C).

Amplicon sequence analysis. The Quantitative Insights Into Microbial Ecology (QIIME) pipeline⁶¹, version 1.7, was used to process the sequenced data. Reads were quality-filtered based on their Phred scores and remaining adapter sequences were removed with Cutadapt⁶². Overlapping read pairs were merged using FLASH⁶³. Merged reads were matched to the reference database Greengenes version 13.8⁶⁴ and clustered into operational taxonomic units (OTUs) through a closed-reference OTU picking approach using UCLUST⁶⁵. Reference sequences were obtained by searching Greengenes for OTUs with at least 97% similarity to the target sequences⁶⁶. Taxonomies were assigned to all OTUs within the OTU-picking workflow, which also includes removal of chimeric sequences using uchime⁶⁷. Source code of scripts used in this work can be found in Appendix B.

A representative set of sequences was obtained in which each OTU was represented by its reference sequence from Greengenes. The representative set was aligned using the PyNAST method⁶⁸ to the Greengenes core reference alignment. A phylogenetic tree was inferred using an approximately-maximum-likelihood method implemented in the software FastTree 2.1.3⁶⁹. Singletons and low abundant OTUs with frequencies below 0.005% of the total reads were removed⁷⁰. OTUs observed in at least three samples were retained. Alpha diversity was determined using the metric of phylogenetic diversity⁷¹. Differences in alpha diversity between nutrient and protozoa levels were estimated using a linear model fitted within R.

PRB were identified as previously suggested by Bertelli and Greub (Amoeba-resisting bacteria, 13). In short, the Bertelli and Greub definition includes; bacteria that has been isolated from amoebae or shown growing within amoebae, bacteria shown to resist amoebae phagocytosis *in vitro* and intracellular and fastidious bacteria¹³. Reads assigned to OTUs of PRB genera were extracted from the sequence data and concatenated at genus level for further analysis after removing singletons and low abundant OTUs.

Statistical analyses. *Predation-pressure.* We tested whether the predation-pressure on bacteria was a key factor for the occurrence of PRB. Predation-pressure on bacteria was calculated in two different ways:

- 1) Ratio between the carbon biomasses of heterotrophic protozoa+eukaryotes and heterotrophic bacteria:

$$\text{Bact}_{\text{PredPress}} = (\text{Protozoa}_{\text{Het}} + \text{Metazoa}_{\text{Het}}) / \text{Bacterial}_{\text{Het}}$$

- 2) Ratio between the carbon biomasses of heterotrophic protozoa and heterotrophic bacteria^{72,73}:

$$\text{Bact}_{\text{PredPress}} = \text{Protozoa}_{\text{Het}} / \text{Bacterial}_{\text{Het}}$$

Principal component analysis. To visualize the treatments' effects, principal component analysis (PCA) was performed using data from the last day of the experiment (day 8), and the R package ade4⁷⁴. Variables included were proportion of reads assigned to PRB, TN, TP, DOC, alpha diversity, and carbon biomasses of heterotrophic bacteria, autotrophs, mixotrophs, heterotrophic protozoa, and metazoa. The alpha diversity and proportion of reads assigned to PRB were square root transformed, while the other variables were log transformed. If the data contained zeros, a value of 0.001 was added prior to log transformation. Furthermore, Pearson correlations between the proportions of reads assigned to different PRB on the last day of the experiment were visualized with a heat map.

Joint species distribution model. Inferential insights into effects of nutrient availability and protozoan predation on the abundance of PRB were drawn by fitting a joint species distribution model (JSDM)⁷⁵, based on a generalized linear mixed model (GLMM) framework, implemented in the lme4 package (version 1.1-12) in R⁷⁶. Total abundance of PRB sequence data was included as the (multivariate) response variable, while total sequence abundance was included as a covariate to control for differences in sampling depth. Thus, we modelled the 16S rRNA sequence data as compositional rather than absolute abundance data. To access residual correlations among PRB reads, a multivariate random intercept at each sample was included in the model. Nutrient level (diluted, *in situ* and enriched) was included in the model as a group-level predictor (coded with $n = 1, 2, 3$, respectively).

The model was set up hierarchically to adjust for dependencies over time and among replicates. Each treatment group (i.e. combination of experimental predictors, such as enrichment, dilution, and/or protozoan filtering) was treated as a random effect with varying intercept and slope: this constituted the second level of the hierarchy, which ensured that the time dependency was acknowledged. The replicates for a given combination of time point and treatment were modelled with a random intercept, and constituted the first level in the model. For each sample $i = 1, \dots, 72$, for each PRB $j = 1, 2, 3$, for each treatment group $k = 1, \dots, 6$, and for each combination of treatment and time point $l = 1, \dots, 24$, the multilevel model can be written as:

$$g(m_{ijkl}) = \beta_{0i} + \beta_{1j} + \beta_{2jn} + \beta_{3k[l]} + \beta_{4l[i]} + x_{5i} \beta_{5j} + x_{6t} \beta_{6k[l]} + u_{ij}$$

$g()$ is the log link function defining the mean of the linear function of predictors, m_{ijkl} is the PRB abundance, β_{0i} is the effect of the total sample sequence abundance for sample i , β_{1j} is the intercept for the j :th PRB, β_{2jn} is the n :th nutrient level effect on PRB j , $\beta_{3k[l]}$ is the varying intercept of the k :th treatment for sample i in treatment k , $\beta_{4l[i]}$ is the varying intercept of the triplicate for group l , β_{5j} is the effect of predation-pressure on PRB j , $\beta_{6k[l]}$ is the varying slope of treatment k at time $t = 2, 4, 6, 8$ days, x_{5i} is the estimated predation-pressure for sample i , and x_{6t} is the time point when sample i was collected. Varying intercepts and slopes (i.e., β_{3k} , β_{4l} , β_{6k}) are assumed to be normally distributed with mean zero and variance among the PRB abundance in each treatment group, respectively.

The residual u_{ij} is assumed to be multinormally distributed with zero mean vector and an unstructured covariance matrix. The predation-pressure predictor, x_{5p} , was log-transformed and scaled to unit variance prior to the analysis. Default values of the parameters controlling convergence of the glmer function in the lme4 package was used. Confidence intervals (CI) were calculated using the Wald method. To visualize the estimated effect size of the regression coefficients, the R package sjPlot⁷⁷ was used. The data for days two to eight of the experiment was used to fit the JSDM model.

Data availability. Sequence data, data set and source codes in appendix A and B.

References

- Sinclair, R., Boone, S. A., Greenberg, D., Keim, P. & Gerba, C. P. Persistence of category A select agents in the environment. *Appl Environ Microbiol* **74**, 555–63 (2008).
- Turner, W. C. *et al.* Lethal exposure: An integrated approach to pathogen transmission via environmental reservoirs. *Sci Rep* **6**, 27311 (2016).
- Molmeret, M., Horn, M., Wagner, M., Santic, M. & Kwaik, Y. A. Amoebae as training grounds for intracellular bacterial pathogens. *Appl Environ Microbiol* **71**, 20–28 (2005).
- Gurijala, K. R. & Alexander, M. Effect of growth rate and hydrophobicity on bacteria surviving protozoan grazing. *Appl Environ Microbiol* **56**, 1631–1635 (1990).
- Pernthaler, J. *et al.* Contrasting bacterial strategies to coexist with a flagellate predator in an experimental microbial assemblage. *Appl Environ Microbiol* **63**, 596–601 (1997).
- Matz, C. & Jürgens, K. Interaction of nutrient limitation and protozoan grazing determines the phenotypic structure of a bacterial community. *Microb Ecol* **45**, 384–398 (2003).
- Jürgens, K. Predation on bacteria and bacterial resistance mechanisms: Comparative aspects among different predator groups in aquatic systems in *Predatory prokaryotes* Vol 4. (ed. Jurkevitch, E.) 57–92 (Springer, 2007).
- Corno, G. & Jürgens, K. Structural and functional patterns of bacterial communities in response to protist predation along an experimental productivity gradient. *Environ Microbiol* **10**, 2857–2871 (2008).
- Arnold, J. W. & Koudelka, G. B. The trojan horse of the microbiological arms race: Phage-encoded toxins as a defence against eukaryotic predators. *Environ Microbiol* **16**, 454–466 (2014).
- Brown, M. & Barker, J. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. *Trends Microbiol* **7**, 46–50 (1999).
- Casadevall, A. & Pirofski, L. Accidental Virulence, Cryptic Pathogenesis, Martians, Lost Hosts, and the Pathogenicity of Environmental Microbes. *Eukaryotic Cell* **6**, 2169–2174 (2007).
- Adiba, S., Nizak, C., van Baalen, M., Denamur, E. & Depaulis, F. From Grazing Resistance to Pathogenesis: The Coincidental Evolution of Virulence Factors. *PLoS One* **5**(8), e11882 (2010).
- Bertelli, C. & Greub, G. Lateral gene exchanges shape the genomes of amoeba-resisting microorganisms. *Front Cell Infect Microbiol* **2**, 110 (2012).
- Erken, M., Lutz, C. & McDougald, D. The Rise of Pathogens: Predation as a Factor Driving the Evolution of Human Pathogens in the Environment. *Microb Ecol* **65**, 860–868 (2013).
- Pallen, M. & Wren, B. Bacterial pathogenomics. *Nature* **449**, 835–842 (2007).
- Hunter, M. & Price, P. Playing Chutes and Ladders: Heterogeneity and the Relative Roles of Bottom-Up and Top-Down Forces in Natural Communities. *Ecology* **73**, 724–732 (1992).
- Pace, M. & Cole, J. Comparative and experimental approaches to top-down and bottom-up regulation of bacteria. *Microb Ecol* **28**, 181–193 (1994).
- Rosemond, A. D., Pringle, C. M., Ramirez, A. & Paul, M. J. A test of top-down and bottom-up control in a detritus based food web. *Ecology* **82**, 2279–2293 (2001).
- Bohannan, B. J. M. & Lenski, R. E. The relative importance of competition and predation varies with productivity in a model community. *Am Nat* **156**, 329–340 (2000).
- Kalinowska, K., Guśpiel, A., Kiersztyn, B. & Chróst, R. J. Factors controlling bacteria and protists in selected mazurian eutrophic lakes (north-eastern Poland) during spring. *Aquatic Biosystems* **9**, 9–9 (2013).
- Thelaus, J., Forsman, M. & Andersson, A. Role of productivity and protozoan abundance for the occurrence of predation-resistant bacteria in aquatic systems. *Microb Ecol* **56**, 18–28 (2008).
- Thingstad, T. F., Perez, M., Pelegri, S., Dolan, J. & Rassoulzadegan, F. Trophic control of bacterial growth in microcosms containing a natural community from northwest Mediterranean surface waters. *Aquat Microb Ecol* **18**, 145–156 (1999).
- Bayarsarkhan, U., Ruhl, A. S. & Jekl, M. Characterization and quantification of dissolved organic carbon releases from suspended and sedimented leaf fragments and of residual particulate organic matter. *Sci Total Environ* **571**, 269–274 (2016).
- Zielinski, P., Grabowska, M. & Jekatierynczuk-Rudczyk, E. Influence of changeable hydro-meteorological conditions on dissolved organic carbon and bacterioplankton abundance in a hypertrophic reservoir and downstream river. *Ecology* **9**, 382–395 (2016).
- Mba Medie, F., Ben Salah, I., Henrissat, B., Raoult, D. & Drancourt, M. *Mycobacterium tuberculosis* complex mycobacteria as amoeba-resistant organisms. *PLoS One* **6**, e20499 (2011).
- Murray, P. R., Rosenthal, K. S. & Pfaller, M. A. Medical microbiology. 8th Edition. Elsevier: Philadelphia (2016).
- Alexander, K. A. *et al.* Novel mycobacterium tuberculosis complex pathogen, M. Mungi. *Emerg Infect Dis* **16**, 1296–1299 (2010).
- Falkinham, J. O. III. Epidemiology of infection by nontuberculous mycobacteria. *Clin Microbiol Rev* **9**, 177–215 (1996).
- Thomas, V., Herrera-Rimann, K., Blanc, D. S. & Greub, G. Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Appl Environ Microbiol* **72**, 2428–2438 (2006).
- Ben Salah, I. & Drancourt, M. Surviving within the amoebal exocyst: the *Mycobacterium avium* complex paradigm. *BMC Microbiol* **10**, 99 (2010).
- Ghodbane, R., Medie, F. M., Lepidi, H., Nappez, C. & Drancourt, M. Long-term survival of tuberculosis complex mycobacteria in soil. *Microbiology* **160**, 496–501 (2014).
- Delafont, V., Cambau, E., Joyeux, M., Bouchon, D. & Moulin, L. First Evidence of Amoebae – Mycobacteria Association in Drinking Water Network. *Environ Sci Technol* **48**, 11872–11882 (2014).
- Ojha, A. K. *et al.* Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol Microbiol* **69**, 164–174 (2008).
- Zingel, P., Agasild, H., Noges, T. & Kisand, V. Ciliates are dominant grazers on pico and nanoplankton in a shallow naturally highly eutrophic lake. *Microb Ecol* **53**, 134–142 (2007).
- Oganjan, K., Virro, T. & Lauringson, V. Food spectrum of omnivorous rotifer *Asplancha priodonta* in two large European lakes of different trophy. *Oceanol Hydrobiol Stud* **42**, 314–323 (2013).
- Miracle, M. R., Vicente, E., Sarma, S. S. S. & Nandini, S. Planktonic rotifer feeding in hypertrophic conditions. *Int Rev Hydrobiol* **99**, 141–150 (2014).
- Steinert, M., Birkness, K., White, W. E., Fields, B. & Quinn, F. *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Appl Environ Microbiol* **64**, 2256–2261 (1998).

38. Greub, G. & Raoult, D. Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev* **17**, 413–433 (2004).
39. Ogata, H. *et al.* Genome sequence of *Rickettsia belli* illuminates the role of amoebae in gene exchanges between intracellular pathogens. *PLoS Genetics* **2**, e76 (2006).
40. Fritzsche, T. R. *et al.* *In situ* detection of novel bacterial endosymbionts of *Acanthamoeba* spp. phylogenetically related to members of the order Rickettsiales. *Appl Environ Microbiol* **65**, 206–212 (1999).
41. Stover, C., Pham, X., Erwin, A. & Mizoguchi, S. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959–964 (2000).
42. Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B. & Jackson, R. W. *Pseudomonas* genomes: Diverse and adaptable. *FEMS Microbiol Rev* **35**, 652–680 (2011).
43. Matz, C., Bergfeld, T., Rice, S. A. & Kjelleberg, S. Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. *Environ Microbiol* **6**, 218–226 (2004).
44. Matz, C. & Kjelleberg, S. Off the hook—how bacteria survive protozoan grazing. *Trends Microbiol* **13**, 302–307 (2005).
45. Matz, C. *et al.* *Pseudomonas aeruginosa* uses type III secretion system to kill biofilm-associated amoebae. *ISME J* **2**, 843–852 (2008).
46. Galle, M., Carpentier, I. & Beyaert, R. Structure and function of the Type III secretion system of *Pseudomonas aeruginosa*. *Curr Protein Pept Sci* **13**, 831–42 (2012).
47. Abd, H. *et al.* *Pseudomonas aeruginosa* utilizes its secretion type III system to kill the free-living amoeba *Acanthamoeba castellanii*. *J Eukaryot Microbiol* **55**, 235–243 (2008).
48. Magalhães, M. J. T. L. *et al.* Multidrug resistant *Pseudomonas aeruginosa* survey in a stream receiving effluents from ineffective wastewater hospital plants. *BMC Microbiol* **16**, 193 (2016).
49. Udikovic-Kolic, N., Wichmann, F., Broderick, N. A. & Handelsman, J. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proc Natl Acad Sci USA* **111**, 15202–15207 (2014).
50. Persson, O. P. High abundance of virulence gene homologues in marine bacteria. *Environ Microbiol* **11**, 1348–1357 (2009).
51. Soborg, D. A., Hendriksen, N. B., Kilian, M. & Kroer, N. Widespread occurrence of bacterial human virulence determinants in soil and freshwater environments. *Appl Environ Microbiol* **79**, 5488–5497 (2013).
52. Grasshoff, K., Ehrhardt, M. & Kremling, K. *Methods of Seawater Analysis*, 2nd ed. Verlag Chemie, Weinheim, Germany (1983).
53. Marie, D., Simon, N. & Vaulot, D. Phytoplankton cell counting by flow cytometry in Algal Culturing Techniques (ed. Andersen, R.A.) 253–267 (Academic Press, 2005).
54. Lee, S. & Fuhrman, J. Relationship between biovolume and biomass of naturally derived bacterioplankton. *Appl Environ Microbiol* **53**, 1298–1303 (1987).
55. Olenina, I. *et al.* Biovolumes and size - classes of phytoplankton in the Baltic Sea. Baltic Sea Environment Proceedings No. 106, 144pp. Updated list at http://ices.dk/marine-data/Documents/ENV/PEG_BVOL.zip (2006).
56. Ruttner-Kolisko, A. Suggestions for biomass calculation of plankton rotifers. *Hydrobiol Beih Ergebn Limnol* **8**, 71–76 (1977).
57. Menden-Deuer, S. & Lessard, E. J. Carbon to volume relationships for dinoflagellates, diatoms and other protist plankton. *Limnol Oceanogr* **45**, 569–579 (2000).
58. Wickman, H. ggplot2: Elegant Graphic for Data Analysis. Springer-Verlag, New York. (2009).
59. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/> (2016).
60. Caporaso, J. G. *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* **6**, 1621–1624 (2012).
61. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335–336 (2010).
62. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* **17**(1), 10 (2011).
63. Magoč, T. & Salzberg, S. L. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**, 2957–2963 (2011).
64. McDonald, D. *et al.* The Biological Observation Matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. *GigaScience* **1**(1), 7 (2012).
65. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–1 (2010).
66. Drancourt, M. *et al.* 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol* **38**, 3623–3630 (2000).
67. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194–200 (2011).
68. Caporaso, J. G. *et al.* PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**, 266–267 (2010).
69. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* **26**, 1641–50 (2009).
70. Bokulich, N. A. *et al.* Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* **10**, 57–59 (2013).
71. Faith, D. P. Conservation evaluation and phylogenetic diversity. *Biol Conserv* **61**, 1–10 (1992).
72. Thelaus, J., Haecky, P., Forsman, M. & Andersson, A. Predation-pressure on bacteria increases along aquatic productivity gradients. *Aquat Microb Ecol* **52**, 45–55 (2008).
73. Mathisen, P., Thelaus, J., Sjöstedt de Luna, S. & Andersson, A. Rapid adaptation of predation resistance in bacteria isolated from a seawater microcosm. *Aquat Microb Ecol* **78**, 81–92 (2016).
74. Dray, S. & Dufour, A.-B. The ade4 Package: Implementing the Duality Diagram for Ecologists. *J Stat Softw* **22**, 1–20 (2007).
75. Warton, D. I. *et al.* So Many Variables: Joint Modeling in Community Ecology. *Trends Ecol Evol* **30**, 766–779 (2015).
76. Bates, D., Maechler, M., Bolker, B. M. & Walker, S. Fitting Linear Mixed-Effects Models using {lme4}. *J Stat Softw* **67**, 1–48. <https://doi.org/10.18637/jss.v067.i01> (2015).
77. Lüdtke, D. *sjPlot: Data Visualization for Statistics in Social Science*. R package version 2.0.0, <http://CRAN.R-project.org/package=sjPlot> (2016).

Acknowledgements

This project was financed by the Swedish Research Council FORMAS [217–2008–1443], the Swedish Research Council [60276201], the research program EcoChange and the Swedish Ministry of Defence [A404217]. We thank Ylva Nordström, Jonas Forsberg and Sonia Brugel for sampling and laboratory work. Mats Forsman is acknowledged for scientific discussions and comments on drafts of the manuscript.

Author Contributions

A.A., P.M., J.A. and J.T. designed the study. A.A. performed the field sampling and wrote the paper together with M.H., J.A., J.T. and P.M. All co-authors commented on drafts of the manuscript. P.M. performed the laboratory experiment and analyzed samples. S.B. prepared amplicons and ran Illumina sequencing based on a method developed by E.N. and A.S. J.A. and M.H. analyzed the sequencing data.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-26422-4>.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2018