## Growth Promotion and Inhibition Induced by Interactions of Groundwater Bacteria

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<td><strong>Date Submitted by the Author:</strong></td>
<td>23-Jun-2018</td>
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Growth Promotion and Inhibition Induced by Interactions of Groundwater Bacteria

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Keywords: groundwater bacteria, high-throughput screening, microbial interactions, secondary metabolites
Abstract

Microorganisms can produce a plethora of secondary metabolites, some acting as signaling compounds, and others as suppressing agents. As yet, the potential of groundwater microbes to produce antimicrobial compounds to increase their competitiveness against other bacteria has not been examined. In this study, we developed an AlamarBlue® based high-throughput screening method that allowed for a fast and highly standardized evaluation of both growth inhibiting and promoting metabolites. With this technique, 149 screened bacterial isolates were grown in monocultures and in 1402 co-cultures. Co-cultivation did not increase the frequency of growth inhibition against the two tested model organisms (S. aureus 533R4 and E. coli WA321) compared to monocultures. Mainly co-cultivation of Proteobacteria induced growth inhibition of both model organisms. Only slightly increased growth promotion of S. aureus 533R4 was observed. Growth promoting effects on E. coli WA321 were observed by supernatants from co-cultures between Bacteroidetes and Firmicutes. With the standardized screening for both, growth inhibiting and promoting effects, this method will enable further studies to elaborate and better understand complex interspecific interactions and networks in aquatic communities as well as in other environments.
Introduction

In nature, bacteria exist as part of a community where they are constantly interacting with their own and other bacterial species. Bacterial growth and performance depend highly on these interactions (Hibbing et al. 2010; Braga, Dourado and Araújo 2016). Thus, bacterial interactions play an important role in the microbial community composition and functioning (Pande and Kost 2017). Bacteria frequently release primary and secondary metabolites into their environment and thereby interact with other microbes. Syntrophic relationships, where the primary metabolism of one organism is directly built on the primary metabolic products of another, are well-described phenomena occurring in the environment (Dolfing 2014; Pande and Kost 2017). Secondary metabolites, like antibiotics or growth factors, can have important ecological functions as they can both negatively or positively influence the growth of other bacteria in the same environment (Hibbing et al. 2010; Cornforth and Foster 2013; Pande and Kost 2017). In this way, they not only modify their niche but can also affect the performance and secondary metabolite production of other bacteria nearby (Seyedsayamdost et al. 2012; Pande and Kost 2017).

One of the most complex ecosystems, harbouring a large diversity and density of bacteria is the soil, with prokaryotic abundances ranging from $10^7$ to $10^{10}$ cells/g soil (Uroz et al. 2010; Akob and Küsel 2011). In a heterogeneous and nutrient-poor soil environment, microorganisms may encounter and interact with numerous taxonomically different neighbours in different microsites in the soil. The production of antimicrobial compounds in the soil can be a useful strategy against competitors for nutrients (Demoling, Figueroa and Bååth 2007; Rousk and Bååth 2007; Rousk, Demoling and Bååth 2009). Recent studies focusing on soil bacteria revealed that the production of secondary metabolites is often a direct result of interactions with other microorganisms in their environment (Garbeva et al. 2011; Traxler et al. 2013; Tyc et al. 2014, 2017). For example, bacteria of the genus Streptomyces, which produce over 70% of all naturally occurring antibiotics, have been found to exhibit more inhibitory effects in pairwise interactions (Kinkel et al. 2014). Genomic studies revealed that in many microorganisms, gene clusters for secondary metabolite production are only expressed
during certain biotic interactions or after receiving signals from neighbouring microorganisms (Scherlach and Hertweck 2009; Cornforth and Foster 2013). Nonetheless, the production of secondary metabolites is assumed to be energetically expensive and therefore might be reduced or abandoned under nutrient limited or competitive conditions.

In freshwater ecosystems, research on the production of antimicrobial compounds is rare and has been restricted to studies with freshwater sponges (Keller-Costa et al. 2014) or surface water ecosystems. For example, almost 70 % of bacteria isolated from the Amazon river were producing antimicrobial compounds against at least one of the seven tested indicator strains (Motta, Cladera-Olivera and Brandelli 2004). Groundwater, as a major reservoir for freshwater on our planet, is still insufficiently investigated in the context of microbial interactions via secondary metabolites. These subsurface environments are characterized by a generally low nutrient content since no light driven primary production is present (Akob and Küsel 2011). With approximately $10^5$ to $10^7$ cells/L (Opitz et al. 2014), the abundance of bacteria in groundwater is at least three orders of magnitude lower than in surface water. Due to the lower cell numbers and the accompanying lower chances of direct cell contact, one could assume that interactions between bacteria in an aquifer are less likely to be of relevance. However, under nutrient-limited conditions in the subsurface, bacteria might significantly benefit from trading metabolites and may cooperate with respect to cost extensive production of exo-enzymes or secondary metabolites. In fact, groundwater communities are often dominated by bacteria with small, streamlined genomes of around 1 Mbp or less (Hug et al. 2016; Solden, Lloyd and Wrighton 2016) with reduced metabolic capabilities. Hence, the majority of these bacteria are proposed to live in symbiotic or parasitic association with other bacteria in the aquifer (Luef et al. 2015; Hug et al. 2016; Castelle et al. 2017) and are therefore not yet culturable. The dominance of these bacteria with life strategies depending on inter- and intraspecific interactions, is pointing towards the decisive role of microbial interactions in groundwater ecosystems.
The aim of this study was to develop a standardized high-throughput method to screen bacterial isolates in liquid cultures for both growth inhibiting and promoting effects. The newly developed method is particularly suited for aquatic microbes and is applied here to test for the production of growth influencing metabolites during cultivation of monocultures and pairwise co-cultivation of groundwater bacterial isolates. We hypothesized that pristine groundwater is the perfect environment for microorganisms to evolve metabolic interactions that affect other microorganisms both negatively and positively.

Materials and Methods

Origin of groundwater isolates

In the frame of this study, we used bacterial isolates obtained from pristine groundwater of a limestone aquifer. The aquifer is located in the Hainich region in northwest Thuringia where a groundwater monitoring transect, following a downhill slope, was established within the CRC AquaDiva (Küsel et al. 2016). Several groundwater wells access two superimposed aquifer assemblages that are either characterized by alternating sequences of fractured limestones (aquifers) and marlstones (aquitards). The used isolates were obtained from the lower, oxic aquifer. This groundwater is characterized by a dissolved oxygen concentration of around 6 mg/L, a pH of 7.2 to 7.5, a dissolved organic carbon concentration (DOC) of around 2 mg/L, 10 mg/L nitrate, and 0.1 mg/L ammonium (Küsel et al. 2016).

Isolation of groundwater bacteria and phylogenetic analysis

In order to obtain a set of representative bacterial isolates, several cultivation approaches using different media had been performed. For this study, we selected a subset of bacteria that were all able to grow in the same media, designed to reflect the low nutrient conditions of the groundwater. Ultimately, 149 bacterial isolates were selected and cultivated on a medium modified after (Reasoner and Geldreich 1985) (0.6 g/L K$_2$HPO$_4$ x 3H$_2$O, 0.1 g/L MgSO$_4$ x 7H$_2$O, 0.06 g/L Sodium pyruvate, 0.1 g/L...
peptone, 0.1 g/L Casein hydrolysate, 0.1 g/L Yeast extract, 0.1 g/L Dextrose, 15 g/L Agar-Agar, ultrapure), here further called S2P medium.

The phylogenetic affiliation of all isolates was determined by PCR amplification of the bacterial 16S rRNA gene fragment using the primer pairs Bac8F/907R (Lane 1991; Turner et al. 1999) amplifying ~899 bp from the 16S rRNA gene. Amplifications were performed on a thermocycler (Peqlab, primus96) with the following settings: initial denaturation temperature of 95 °C for 5 min, followed by 30 cycles of 94 °C for 45 seconds, 52 °C for 60 seconds and 72 °C for 60 seconds and a final elongation at 72 °C for 10 minutes. Sequencing of the obtained PCR product was done using primer Bac8F (Macrogen, the Netherlands).

Subsequently all sequences were aligned in ARB (Ludwig et al. 2004), representative species of each bacterial family were picked from the SILVA database (release 123) (Pruesse et al. 2007) and the phylogenetic tree was calculated with the ARB neighbor-joining function (1000 bootstraps) and visualized using the interactive tree of life website (Letunic and Bork 2016). In order to estimate the abundance of every isolate within the groundwater bacterial community, we compared every isolate with a previously published 16S rRNA gene amplicon dataset (Schwab et al. 2017) on family level.

Target Organisms

*Escherichia coli* WA321 (DSM 4509) and *Staphylococcus aureus* 533R4 Serovar 3 (DSM 20231) were used as target organisms to screen for the presence of growth inhibiting or growth promoting compounds, following Tyc et al. (2014). The target organisms were pre-cultured from −80 °C glycerol stocks on LB agar plates (LB-Agar, Carl Roth Germany) (Sambrook and Russell 2001) and incubated at 37 °C. For the screening, single colonies of each target strain were picked from plate and incubated at 37 °C 24 h in 25 mL LB medium (LB medium Lennox, Carl Roth GmbH + Co. KG, the Netherlands) prior to each screening assays.
Preparation of 96-well source plates

96-well microtiter plates (Greiner bio-one B.V., Alphen a/d Rijn, the Netherlands, Cat# 655180) were used as source plates that contained all 149 bacterial isolates. Each well was filled with 300 µL liquid S2P medium, and the bacterial isolates were inoculated in rows 3 to 10 manually by picking cells from a single colony of each bacterial isolate with an autoclaved wooden toothpick. The rows one and two as well as 11 and 12 were used for the negative and positive controls, containing 30 µL liquid S2P medium and 70 µL LB medium with AlamarBlue® solution (10 % v/v) with or without target organism, respectively, within the actual screening run. In total, six different source plates (A-F) were produced and used for the screening. All source plates were generated in triplicates. The plates were incubated at room temperature for six days before the screening. In order to generate back-ups of each source plate, 50 µL of 50 % (v/v) glycerol were added to prepare the plates for long-term storage at -80 °C.

High-throughput Interaction Assay

A Genetix QPix 2 colony picking robot (Molecular Devices, UK Limited, Wokingham, United Kingdom) was used for the high-throughput interaction assay. The Genetix QPix 2 robot was mounted with a bacterial 96-pin picking head and programmed to replicate the provided source plates A-F in triplicates into 96-well microtiter plates supplemented with 300 µL per well liquid S2P medium. To maximize the number of co-cultures all plates were combined with each other in their original as well in an inverted orientation. The inoculated screening plates (mono- and co-cultivation plates) were incubated at 21 °C for six days.

Screening for growth inhibitory or promoting effects of cell-free supernatants

After six days, the 96-well microtiter plates were centrifuged at 5000 rpm for 40 minutes at room temperature on a Sigma 3-14K laboratory centrifuge (SIGMA Laborzentrifugen GmbH, Germany) in order to retrieve cell-free supernatants from the bacterial isolates grown in monoculture or in co-culture.
For the screening, 96-well microtiter plates were prepared by dispensing 30 µL of the prepared cell-
free supernatant into the screening plates. Then, a volume of 70 µL of the target organisms (either
*E. coli* WA321 or *S. aureus* 533R4) grown in liquid LB Medium (Carl Roth, Germany) plus 10 % (v/v) 
AlamarBlue® solution (Invitrogen™, Germany, cat# DAL1025) was added to each well, resulting in a
total volume of 100 µL per well. The target organisms were added at a density of OD 0.004,
corresponding to 6.4 x 10^6 CFU/mL (*E. coli* WA321) or 4.0 x 10^5 CFU/mL (*S. aureus* 533R4). The empty
rows 1 and 2 were used as negative and positive controls. All Plates were incubated at 37 °C for 4
hours according to the manufacturers manual. The AlamarBlue® solution was used to monitor the
growth of the target organisms (Tyc et al. 2016), as a color change from blue to pink indicates
metabolic activity. The metabolic activity was monitored using fluorescent measurements on a 
BioTek Synergy™ HT Multi-Mode Microplate Reader (Beun de Ronde Life Sciences, the Netherlands)
applying excitation/emission wavelengths of 530 nm and 590 nm, respectively.

*Data analysis and statistics*

The relative growth of the target organisms in treatments with isolate or co-culture supernatant was
compared to the growth of the control of the respective target organism without added
supernatants. The supernatant of an isolate or co-culture was defined to have an inhibiting effect
when the average of all triplicates showed 70 % or less growth (lower fluorescence values) and to
have a growth promoting effect when there was on average 130 % or more growth compared to the
control (higher fluorescence values). To determine whether a particular taxonomic group, or the
combination of two specific taxonomic groups, had a significantly higher tendency to provide effects
on the growth of the target organisms, Fisher’s exact test (Fisher 1922) was used for the calculation
of p-values as

\[
p = \frac{(a + b)! \times (a + c)! \times (b + d)! \times (c + d)!}{(a! \times b! \times c! \times d!)}
\]

with \(a\) and \(b\) denoting the numbers of co-cultures tested including the taxonomic group(s) with \((a)\)
and without \((b)\) the desired effect, \(c\) and \(d\) denoting the numbers of co-cultures excluding the
taxonomic group(s) with \((c)\) and without \((d)\) the desired effect, and \(n\) denoting the total number of co-cultures tested.

To display different levels of significance, \(p\)-value thresholds of \(< 0.05\) (*), \(< 0.01\) (**) and \(< 0.001\) (***) were used. Fisher’s exact test was used in a likewise manner to determine whether the tendency of isolates showed an effect on the target organisms was significantly different from those of co-cultures.

\[\text{Results}\]

\textit{Growth inhibiting and promoting effects of groundwater isolates}

The selected 149 bacterial groundwater isolates represented 17 different bacterial families of the following four phyla: Actinobacteria (5), Bacteroidetes (35), Firmicutes (9), and Proteobacteria (11 Alphaproteobacteria, 10 Betaproteobacteria, 79 Gammaproteobacteria) (Genbank accession numbers MG980417-MG980565, Fig. 1; Fig. S1, Tab. S1). These bacterial families were shown to cover 2.6 % of the diversity present in the lower aquifer and represent 3.52 % of the relative abundance of the groundwater bacterial community (Tab. S1), as revealed by amplicon sequencing (Schwab et al. 2017).

We used the high-throughput screening approach to test for growth inhibiting and growth promoting effects of our isolates by transferring monoculture supernatants to the two target organisms \(E.\ coli\ WA321\) and \(S.\ aureus\ 533R4\). In most cases where changed growth of the target organisms was observed, isolates had an inhibitory effect. For example, when grown in monoculture 52 isolates showed inhibiting effects on the growth of the gram-negative \(E.\ coli\ WA321\) target organism, and 61 isolates on the growth of the gram-positive \(S.\ aureus\ 533R4\) (Fig. 2A). Growth promoting effects on \(E.\ coli\ WA321\) were only observed with 18 isolates, and only one isolate (#134 belonging to the family of \textit{Pseudomonadaceae}) was promoting the growth of \(S.\ aureus\ 533R4\) (Fig. 2A).
To test whether co-cultivation would show additional effects of the culture supernatant on the growth of the target organisms, we established 1402 random bacterial co-cultures with the isolates. Interestingly, the fraction of co-cultures showing inhibitory effects on growth was significantly lower on both target organisms (\( p < 0.001 \), Fisher’s exact test) than the fraction of monocultures. Less than 20% of co-cultures led to inhibitory effects on \textit{E. coli} WA321, and for \textit{S. aureus} 533R4, this number was below 10% (Fig. 2B). All phyla showed a significantly lower frequency of growth inhibition on \textit{S. aureus} 533R4 (\( p < 0.01 \) to \( p < 0.001 \)). For \textit{E. coli} WA321 all phyla, except Proteobacteria, showed a significantly reduced frequency of inhibition (\( p < 0.01 \) to \( p < 0.001 \)) in co-cultures. For growth promoting effects, the observed differences were not as clear. While for \textit{E. coli} WA321, a significantly lower number (\( p < 0.001 \)) of co-cultures stimulated growth, for \textit{S. aureus} 533R4, the number of co-cultures leading to a growth promotion increased from 0.7 to 2%, although the difference was not significant (Fig. 2B). Hence, none of the investigated bacterial groups showed an increased frequency of growth-affecting activities in co-culture compared to monoculture.

The applied fluorometric assay furthermore allowed us to quantify the growth inhibiting and growth promoting effects exhibited by the bacterial supernatants, and to compare them between mono- and co-culture. The strength of both, growth inhibition and promotion, did not differ between mono- and co-cultures. Supernatants that had inhibitory effects on the two target organisms on average reduced their growth almost by half (Fig. 3). The maximal inhibitory effect observed even led to a four to five times reduced growth of \textit{E. coli} WA321. Co-cultures that had a growth promoting effect increased the growth of the target organisms by a factor of 1.5, however, in one case more than three-fold growth increase was observed for \textit{E. coli} WA321 (Fig. 3).
Identification of key players within interactions

We further investigated whether specific taxonomic groups were capable of conferring growth inhibiting or promoting effects by interaction with other organisms in the co-cultures. Hence, we split the 1402 co-cultures tested based on the taxonomic classification of the two organisms involved.

On phylum level, inhibiting effects on growth were mostly observed for members of the Proteobacteria (Fig. 4). Co-cultures including Gammaproteobacteria revealed inhibiting effects for both S. aureus 533R4 and E. coli WA321 ($p < 0.001$ and $p < 0.001$). This equally applied to Betaproteobacteria for S. aureus 533R4 ($p < 0.05$) and to Alphaproteobacteria for E. coli WA321 ($p < 0.01$). In particular, co-cultures of Alpha- and Betaproteobacteria had a significantly increased frequency of growth inhibiting effects on both target organisms (S. aureus 533R4: $p < 0.01$; E. coli WA321: $p < 0.01$; Fig. 4).

On family level, mainly co-cultures between Pseudomonadaceae isolates showed a significantly increased growth inhibition frequency (S. aureus 533R4: $p < 0.05$; E. coli WA321: $p < 0.001$).

Supernatants derived from co-cultures between Caulobacteraceae and Pseudomonadaceae isolates ($p < 0.001$), Oxalobacteraceae and Pseudomonadaceae ($p < 0.05$) as well as co-cultures between Flavobacteriaceae and Pseudomonadaceae isolates ($p < 0.01$) led to growth inhibitory effects on E. coli WA321; co-cultures between Caulobacteraceae and Comomonadaceae ($p < 0.01$) as well as Pseudomonadaceae ($p < 0.05$) significantly inhibited the growth of S. aureus 533R4.

The growth of S. aureus 533R4 was not significantly promoted by any of the tested phylum-phylum interactions (Fig. 4A), whereas the growth of E. coli WA321 was promoted mostly by co-cultures between Bacteroidetes and Firmicutes ($p < 0.01$). Within these phyla, especially co-cultures between Sphingobacteriaceae and Paenibacillaceae led to a more frequent growth promotion ($p < 0.05$).
Discussion

The exploration of new antimicrobial substances is of wide interest, not only in the context of discovery of novel compounds, but also to understand microbial interactions in various ecosystems. Classical techniques to screen for antimicrobial production have been carried out on solid agar plates using an overlay assay combined with visual screening for zones of inhibition (Tyc et al. 2014; Balouiri, Sadiki and Ibnsouda 2016) and thus, no analysis of growth promoting effects was possible. Furthermore, the evaluation of these screenings is time consuming and strongly dependent upon a subjective evaluation. The newly developed high-throughput screening applied in this study is a fast and highly standardized method to detect growth inhibiting as well as growth promoting effects induced during mono- and co-cultivation of bacteria in liquid medium. Thereby, it provides new insights into microbial interactions in groundwater and opens up a new door to interaction studies in other environments.

The bacterial isolates obtained in this study represent up to 2.1% of the bacterial community of the oxic aquifer which is high compared to the usual 0.25% of the microorganisms that are assumed to be culturable from freshwater environments (Amann, Ludwig and Schleifer 1995). However, a high percentage of phylogenetic groups of groundwater microbes lack cultured representatives. This is especially true for Candidate Phyla, such as the superphylum Cand. Parcubacteria, which constitute up to 50% of the microbial community in the groundwater of the pristine limestone aquifers and other groundwater ecosystems (Schwab et al., 2017; Luef et al., 2015). With their reduced genomes of around 1Mbp (Luef et al. 2015) and reduced metabolic capacities, it is believed that their dependence on interaction explains why these microbes could not be cultivated in monocultures. Thus, our cultivation approach can only provide a limited view of microbial interactions occurring in groundwater.

Inhibitory effects dominated in both monocultures (6% promotion versus 38% inhibition) and in co-cultures (3% promotion versus 13% inhibition) for both target organisms used in this study.
suggests that interactions between groundwater bacteria are more antagonistic in nature than mutualistic. Overall, co-cultivation of groundwater bacteria did not increase their potential to affect the growth of the tested target organisms, although almost half of the tested bacteria showed an effect in mono-culture. Only growth promotion on *S. aureus* 533R4 increased from 0.7 to 2% in co-cultures, whereas growth promotion on *E. coli* WA321 and growth inhibition on both model organisms decreased by 50-75%. The limited effect of co-cultures on growth affecting activities might be caused by the competitive situation for nutrients inside a co-culture, which prevents bacteria from investing in the production of secondary metabolites. In contrast to *Cand.* Parcubacteria that rely on metabolic interactions due to their reduced genomes (Luef et al., 2015), groundwater bacteria like the isolates tested in this study, might not activate their full secondary metabolite potential in the presence of other microorganisms but rather invest on efficient uptake mechanisms for nutrients.

Limited growth-affecting activities of groundwater isolates incubated in co-culture are in disagreement with previous findings obtained with soil bacterial isolates (Garbeva *et al.* 2011; Traxler *et al.* 2013; Kinkel *et al.* 2014; Tyc *et al.* 2014). A high percent of soil bacteria reveals antimicrobial activity only when tested in interactions (Tyc *et al.*, 2014). In soil ecosystems, microorganisms are present in spatial proximity, whereas groundwater bacteria barely have physical contact with other bacteria. As exchange of secondary metabolites is dependent upon diffusion to the partner organism, this strategy might not be effective in pristine groundwater with very low microbial abundance (Opitz *et al.*, 2014).

Nonetheless, our screening revealed that the groundwater microbiome seems to harbour a potential to produce secondary metabolites of both growth promoting and inhibiting effects. On the phyla level, growth promoting effects were mostly conferred by bacteria belonging to the phyla of Bacteroidetes and Firmicutes. In particular, supernatants derived from bacteria belonging to the families of *Sphingobacteriaceae* and *Paenibacillaceae* showed growth promoting effects on the used
target organisms. These families are known producers of diverse secondary metabolites such as nonribosomal peptide synthetases (NRPs), lipopeptides (LPs), polyketide synthases (PKSs), PKS–NRPS hybrid compounds and others (Pawlowski et al. 2017; Shen et al. 2017), though a growth stimulating effect has not yet been described. Proteobacteria, especially Pseudomonadaceae, well-known producers of antimicrobials (Raaijmakers, Weller and Thomashow 1997; de Bruijn et al. 2007; Matthijs et al. 2007; Silby et al. 2011), were key players in interactions with inhibitory effects on the target organisms. In our pristine groundwater habitat bacteria belonging to the family of Pseudomonadaceae make up to 1.5 % of the total microbial community (Schwab et al. 2017) and could be therefore key players in interactions.

The complexity of microbial interactions in numerous environments has been poorly investigated and has so far often been focusing on the identification of antimicrobial substances. With our high-throughput screening method, growth promoting effects can also be quantified, which can be applied to improve our understanding of microbial interactions in diverse environments. Studies on the microbiomes of different environments has increased exponentially during the last decade, revealing the tremendous diversity and complexity of microbial communities. At the same time, our knowledge about metabolic interactions which shape microbial communities, is still rudimentary. More empirical work is required to understand microbial metabolic interactions of different environmental origins and high-throughput methods, such as the one described in this study, might be very useful.

**Funding**

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) CRC 1076 "AquaDiva" as well as a Strategy and Innovation Grant from the Free State of Thuringia (41-5507-2016) and the Leibniz Research Cluster InfectoOptics (SAS-2015-HKI-LWC). Climate chambers, used to isolate
bacteria under controlled temperature conditions, were financially supported by the Thüringer Ministerium für Wirtschaft, Wissenschaft und Digitale Gesellschaft (TMWWDG; project B715-09075).

Acknowledgement

We thank Robert Lehmann, Heiko Minkmar and Falko Gutmann for sampling and filtration of the groundwater, as well as Martina Herrmann for helpful discussions and support. This is NIOO-KNAW publication number XXXX.
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Figure 1

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Figure 2

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Fig 3

Boxplot showing relative growth in co-cultures (%) for S. aureus and E. coli.
Fig. 4

155x165mm (96 x 96 DPI)
Figures

Figure 1 Overview of the 149 bacterial groundwater isolates belonging to sixteen different bacterial families within the phyla Actinobacteria (green), Bacteroidetes (blue), Firmicutes (purple) as well as Alpha- (red), Beta-(orange) and Gamma-(yellow) Proteobacteria.

Figure 2 Relative fractions of growth promotion and inhibition in the tested monocultures (n=149) and co cultures (n=1402). For both target organisms the relative occurrence of growth influencing effects decreases in the tested co-cultures.

Figure 3 Relative growth of the two target organisms S. aureus 533R4 and E. coli WA321 in co-cultures that were evaluated to have growth inhibiting (blue) and growth promoting (pink) effects.

Figure 4 Heat map showing growth inhibiting (blue shades) and growth promoting (purple shades) effects of cell-free supernatants derived from co-cultures on phylum level on (A) S. aureus 533R4 and (B) E. coli WA321. P-values derived from Fisher’s Exact Ratio test are indicated by asterisks, phyla that show an overall higher frequency of growth promotion or inhibition are highlighted through coloured boxes.

Supplemental

Figure S1 Phylogenetic affiliation of the 149 isolates used in this study. Actinobacteria (green), Bacteroidetes (blue), Firmicutes (purple) as well as Alpha- (red), Beta-(orange) and Gamma-(yellow) Proteobacteria. The two target organisms are highlighted in bold.

Figure S2 Setup of the six source plates A-F. In order to be able to generate more combinations with less abundant bacterial families some isolates can be found on multiple plates.

Table S1 Overview of the isolates used in this study, their Genbank accession numbers and their phylogenetic affiliation.

Table S2 Relative abundance of the bacterial families in the lower aquifer (Schwab et al., 2017). Listed are exclusively bacterial families of those isolates were obtained and used in the framework of this study.

Table S3 Screening results of co-cultures on phylum level with the respective results of fisher’s exact test for both growth inhibition and promotion.

Table S4 Screening results of co-cultures on family level with the respective results of fisher’s exact test for both growth inhibition and promotion.
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Overview of the isolates used in this study, their Genbank accession numbers and their phylogenetic affiliation.

Genbank Phylogeny

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| Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae |
| Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae |
| Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae |
| Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae |
| Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae |
| Firmicutes; Bacilli; Bacillales; Paenibacillaceae |
| Bacteroidetes; Bacteroidia; Flavobacteriales; Flavobacteriaceae |
| Bacteroidetes; Bacteroidia; Flavobacteriales; Flavobacteriaceae |
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**Table S2** Relative abundance of the bacterial families in the lower aquifer (Schwab et al., 2017). Listed are exclusively bacterial families of those isolates were obtained and used in the framework of this study.

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al., 2017). Listed are exclusively bacterial families of those isolates were obtained and used in the fra
mework of this study.
Table S3  Screening results of co-cultures on phylum level with the respective results of Fisher's exact test for both growth inhibition and promotion.

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For Peer Review

Screening results of co-cultures on phylum level with the respective results of Fisher’s exact test for both growth inhibition and promotion.

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**E. coli WA321**

*screening results*
Table S4: Screening results of co-cultures on fish mortality with the respective results of Fisher’s exact test for both-growth inhibition and promotion.

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