Bacterial diversity related to the toxic impact of chemicals

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Abstract

Hypothesis: A group of bacteria having a high phylogenetic diversity displays a higher variation in sensitivity to toxic chemicals than a group having lower phylogenetic diversity.

Fifty-one bacterial strains belonging to different phyla were exposed to 14 different chemicals. It was found that the variation in sensitivity towards chemicals was at the same level in a group of bacteria from the same species as in a group with different species from the same genus. Increasing the phylogenetic diversity to a group of bacteria belonging to different species from the same phylum also increased the variation in sensitivity whilst increasing the phylogenetic diversity to bacteria from different phyla did not increase the variation in sensitivity any further. Thus, the phylogenetic diversity of a population is thus not *per se* a sufficient measurement of the plausible stability of a microbial population when being exposed to a toxic chemical.

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Introduction

A high biodiversity is generally regarded as intrinsically good in an ecosystem (1), (24). The greater the variation in the species response, the lower the species richness needed for the ecosystem to become redundant (36). A high biodiversity increases the possibility for a species to replace an extinct species with a similar function making the ecosystem more stable (25), and also contributes to a greater possibility of fruitful genetic exchange between species. A certain level of diversity is required to reach stability in a microbial community (3). The preservation of a large biodiversity also ensures the genetic resources for future generations (16). Micro-organisms, in particular bacteria, represent a major part of the biodiversity on earth (9).

Biodiversity measurements in a community consisting of higher organisms is mostly based on the quantification of specific species (23). It is often not possible to count specific species in microbial communities as a large proportion of bacteria are either not culturable by traditional methods (34), or are unknown in the literature (20). Instead, the biodiversity of bacterial communities is often expressed as functional, genetic or phylogenetic diversity. The endpoints of biodiversity measurements are structure, richness, evenness and composition of the community (34). Methods of measuring functional diversity includes Biolog^{TM} plates (29), (37), where the pattern of utilisation of 95 different carbon sources are compared, and PhP^{TM}-plates (19), where the rate and pattern of utilisation of 48 different carbon sources are compared. Other parameters that can be measured include enzyme activities (35), mineralisation kinetics of compounds added to soils (28) or SIR – Substrate Induced Respiration (10). Analysis of the genetic diversity can be performed using molecular techniques, which have the advantage of being non-culture dependent. Hybridisation techniques as well as PCR-based methods have been applied to natural bacterial communities (20), although they rarely can be used for species identification but rather for detection of microbial groups because their resolution is too low (28). PLFA (PhosphoLipid Fatty Acid) analysis has also been used to track changes in a bacterial community (28), (17). This method is also used for quantification of the abundance of some phylogenetic groups of bacteria and fungi (35). Assessment of the phylogenetic diversity can be performed using traditional cultivation methods (2), but this has the disadvantage in that only culturable bacteria are identified. Species variability within

Kommentar [JG1]: Expressed?

Kommentar [JG2]:

a microbial community can be estimated from knowing “the total number of individuals in the community and of the most abundant members of that community” (9).

In most studies of the ecotoxic effects on a microbial community, the functional rather than the phylogenetic biodiversity has been assessed. Functional biodiversity is easier to measure (28) , (37) and in many cases is regarded as being more relevant (28), (6). It has also been concluded that a functionally diverse system is more stable than a system with lower diversity (6), but information regarding the relationship between functional and genetic/pylogenetic diversity is scarce (28), (34), (38). To our knowledge, no previous study has been made on what impact the phylogenetic diversity has on the stability of a certain group of microorganisms when exposed to toxic chemicals. Several studies have been made on the effect of different chemicals on specific bacteria or groups of bacteria. For example, Brim et al investigated the phylogenetic diversity of strains, mainly belonging to genus *Ralstonia*, with operons czc (confers resistance to cadmium, zink and cobalt) and ncc (confers resistance to nickel, cadmium and cobalt) isolated from a heavy-metal contaminated environment (5). Macnaughton et al found that oil contamination promoted the growth of α-proteobacteria and Flexibacter-Cytophaga-Bacteroides whilst the eucaryotic biomass decreased (22) and Frostegård et al analysed the PLFA-patterns in metal contaminated soils (14). The studies have been performed using different methods and the results are, unfortunately, difficult or impossible to compare (32), (8). Molecular methods have been used to measure the genetic diversity of a community, but species identification it not necessary for drawing conclusions. For example Moffett et al assessed the bacterial diversity in zinc contaminated soil by counting the number of different OTUs (operational taxonomic units) and their relative abundance (27). A relatively simple way of tracking changes in a community is to track the G+C-content. Different species have different and specific G+C-content and, as the species composition changes, then the total G+C-content changes. This can be used on its own or in combination with community DNA hybridisation, which is used to quantify to what extent two populations are similar (20).

In this study, we assessed the hypothesis that bacteria in a group having a high phylogenetic diversity would exhibit a higher variation in their response to toxic

chemicals than bacteria in a group having a lower phylogenetic diversity. If this hypothesis is correct, a bacterial community with a high phylogenetic diversity would be more stable when exposed to chemical pollution than an ecosystem with a low phylogenetic diversity, according to the "insurance hypothesis" developed by Yachi and Loreau (36).

Materials and methods

Bacterial strains

51 different bacterial strains were used. All could be grown over-night on nutrient broth and reacted with the growth indicator tetrazolium red; these criteria were necessary for the toxicity test employed. The strains selected were divided into four groups

1. Bacterial strains from three different phylogenetic groups, mainly Gram+ and proteobacteria. See Table 1. The proteobacteria are the most physiologically diverse of all bacterial phyla (7) and the diversity of its subdivisions, the α-, β- and γ-proteobacteria, were also examined.
2. Eight bacterial strains from one genus with high metabolic diversity – *Enterococcus*
3. Eleven bacterial strains from one species with high metabolic diversity – *Escherichia coli*
4. Thirteen bacterial strains from one species with low metabolic diversity – *Staphylococcus aureus*

The strains in group 1 have been identified with 16S DNA sequencing and classified into the different phyla as defined by Bergey's Manual of Systematic Bacteriology (4). The *Enterococcus* strains have been identified by PCR and *E.coli* and *S.aureus* strains with classical microbiological methods. The metabolic diversity was defined by means of the PhP-system (21).

Table 1 Strains used in the study. The different phylogenetic groups are indicated. The NCIMB = National Collections of Industrial, Food and Marine Bacteria (www.ncimb.co.uk)

Name	**NCIMB-number**	**Phylogenetic group**
Sphingobacterium multivorum	12559	flavobacteria
Arthrobacter aurescens	8912	Gram +
Arthrobacter citreus	8915	Gram +
Brevibacillus parabrevis	7577	Gram +
Curtobacterium sp	10352	Gram +
Microbacterium laevaniformans	9659	Gram +
Brevundimonas diminuta	9393	α-proteobacteria
Ensifer adhaerens	12342	α-proteobacteria
Phyllobacterium rubiacearum	12128	α-proteobacteria
Rhizobium radiobacter	9042	α-proteobacteria
Comamonas testosteroni	8955	β-proteobacteria
Aeromonas sobria	1105	γ-proteobacteria
Citrobacter freundii	12203	γ-proteobacteria
Erwinia perscina	13181	γ-proteobacteria
Pantoea agglomerans	12126	γ-proteobacteria
Pseudomonas aurantiaca	10068	γ-proteobacteria
Pseudomonas chlororaphis	9392	γ-proteobacteria
Pseudomonas mendocina	10541	γ-proteobacteria
Serratia rubidaea	4	γ-proteobacteria
Shewanella putrefaciens	10471	γ-proteobacteria
Vibrio harveyi	1280	γ-proteobacteria

Table 2 Strains from group 2-4. All *Enterococcus* were isolated from sewage water. ATCC= American Type Culture Collection, Manassas, USA, NCTC = National Collection of Type Cultures, London, UK

Enterococcus		***E.coli***			***S. aureus***	
Species		**Denotation**	**Comment**		**Denotation**	**Comment**
E.faecalis		NCTC 11601			B37	Sepsis patient
E.faecalis		NCTC 11603			R23	Healthy carrier
E.casseliflavus		ATCC 35401			R69	Healthy carrier
E.pseudavium		Nic ref 2	Nicaraguan infant		CowanS	
E.faecalis		Nic ref 9	Nicaraguan infant		8824	Internal reference strain
E. faecalis		DS 17			Wood 46	
E.faecalis		R2	Calf in Mozambique		BB	Healthy carrier
E.gallinarum		CN13	University of Barcelona		AW	Healthy carrier
		EC C600	University of Barcelona		ATCC 25293	
		EC J2:5	Swedish infants		Orsa	Internal reference strain
		EC J12:16	Swedish infants		6538	Internal reference strain

Chemicals

Fourteen chemicals were tested; EDTA, caffeine, sodium fluoride, lithium sulphate, quinidine sulphate, acryl amide, pentachlorophenol, hydrogene peroxide, glyphosate, 3,5 dichlorophenol, chlorohexidine, nicotine, trichlosane, metimidazol and sodium azide.

Six of the chemicals (caffeine, sodium fluoride, lithium sulphate, quinidine sulphate, pentachlorophenol and nicotine) are found on the MEIC-list, a list of 50 chemicals that have been studied extensively both *in vivo* and *in vitro* (12). The remaining eight chemicals have been selected because of their well known toxic effects; EDTA s a metal chelating agent removing essential metal ions from the growth medium, acryl amide is a known genotoxicant and caused ecotoxic injuries when used in tunnel construction in Hallandsåsen, Sweden in 1992, hydrogen peroxide is a strong oxidizer and often used as disinfectant, glyphosate is the active compound in the herbicide Round Up (also named Rambo), 3,5-dichlorophenol is commonly used as a standard

toxicant when evaluating ecotoxicity tests, chlorohexidine and trichlosane are antibacterial agents in toothpaste (sic!) and hand-disinfectants, metimidazol is a cytochrome P450 inhibitor and sodium azide is used for pest control in agriculture.

Toxicity test assay

The toxicity testing was performed using a modified version of the MARA-method, described by Gabrielson et al (15). The *Enterococcus spp* and *Echerichia coli* were grown on nutrient agar plates over night at 37°C, *Staphylococcus aureus* on blood agar at 37°C and the other strains on nutrient agar plates at 28°C. Bacteria were transferred from an agar plate using a 1µl loop into 3 ml Brain Heart Infusion (BHI)-broth (Difco Labs, Detroit, USA) in a test tube. The tubes were incubated over night with shaking (2 rps) at 37°C or 28°C. After incubation the cultures were centrifuged (50 rps for 5 min), the supernatant discarded and the pellet was resuspended in 8 ml LB-broth (Difco Labs, Detroit, USA). In order to obtain constant OD, the bacterial suspensions were measured by transferring 100 µl into the wells of a 96-well round bottomed polystyrene microplates (Greiner Labortechnik, Frickenhausen, Germany, Catalog No. 650101) and reading at 620nm.

Microplates were prepared for the assay by the addition of 100 µl growth medium (LB-broth (Difco Labs, Detroit, USA) adapted to pH 7.5 with 0.01M phosphate buffer, 0,5% dextrane T500, 0.75% glucose and 0.01% of growth indicator tetrazolium red (2,3,5-triphenyltetrazolium chloride, Sigma T-8877, St. Louis, MO, USA)) per well in rows A-G. To wells in row H, 150µl growth medium containing the toxicant under test was added. 50µl was transferred from each of the wells in row H to the adjacent wells in row G so that a 3x dilution was obtained. This was repeated to row B. Row A was left as a positive growth control containing no toxicant. These volumes were adjusted accordingly if different dilutions were required. 12.5µl of bacterial suspension were added to each well, one strain per column 1-11. Column 12, left as a medium control without bacteria.

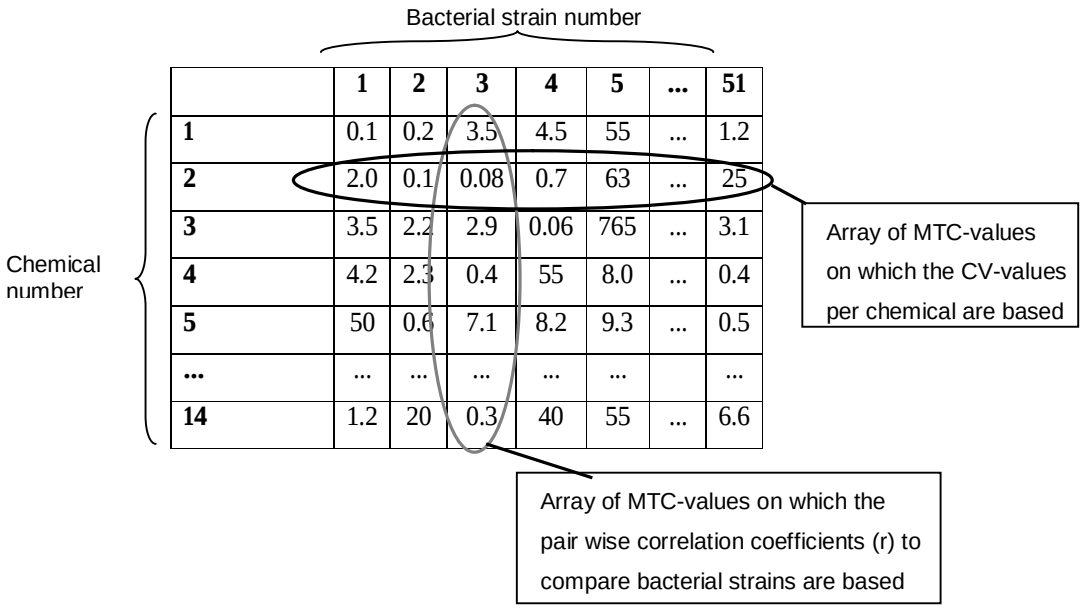
All plates were incubated in a moist chamber at the appropriate temperature (28°C and 37°C as above) and were read after 18h. The plates were scanned and the scans analysed as described in Gabrielson et al 2003 (15) (available from PhPlate Microplate Techniques AB, Stockholm, Sweden, www.phplate.se), to obtain

inhibitory concentrations for each chemical on each bacterial strain. All tests were performed in duplicates.

Analysis of Data

The toxic effect of each chemical was expressed as Microbial Toxic Concentration - values (MTC) (15). The MTC-value for a strain is equivalent to the IC_{50}-values (i e the Concentration at which 50% Inhibition of growth is obtained) when the reduction of the size of the pellets formed produce a perfect slope along the chemical gradient. This is seldom obtained and is compensated for when the MTC-value is calculated.

Figure 1 A table with fictitious MTC-values. Examples of two **arrays** are marked with ovals. The **correlation coefficients** are based on the pair wise comparisons of the arrays along the columns (vertical oval). The coefficients of variation (**CV**-values) are based on the arrays along the rows (horizontal oval).



	1	**2**	**3**	**4**	**5**	**...**	**51**
1	0.1	0.2	3.5	4.5	55	...	1.2
2	2.0	0.1	0.08	0.7	63	...	25
3	3.5	2.2	2.9	0.06	765	...	3.1
4	4.2	2.3	0.4	55	8.0	...	0.4
5	50	0.6	7.1	8.2	9.3	...	0.5
...	...	...	...	...	...		...
14	1.2	20	0.3	40	55	...	6.6

The 14 MTC-values obtained for each strain constitute an array (vertical oval in Fig 1). The dendrograms in figures 2 and 3 are based on the comparison of these arrays and were calculated with the UPGMA-method (33). The correlation coefficients of each pair of strains are given on the x-axis in each dendrogram, and thus the further to the right the linkage the more similar each pair is in their resistant pattern towards chemicals under test.

The **heterogeneity** in Table 3 is $1\text{-}r_{mean}$, where r_{mean} equals the mean correlation coefficient between the arrays of MTC-values (vertical oval in Fig 1) for any two strains in each group. The higher the heterogeneity is the larger the difference between the arrays of inhibition values within the respective group of bacteria.

The **variability** in Table 3 is defined as the mean CV% (Coefficient of Variation)-value from the 14 chemicals. The CV-value for each chemical is based on the array of MTC-values (horizontal oval in Fig 1) for the strains in each group. The CV% is the standard deviation for a given set of data divided by the mean value for that set of data and multiplied with 100. A low CV-value means low variability and that the strains have a similar sensitivity towards the test chemicals, whereas a high CV-value means a high variability and that the strains have different sensitivities towards the test chemicals.

The **reproducibility** is calculated using the correlation coefficient. The higher the correlation coefficient between the arrays obtained for two bacterial strains (vertical oval in Fig 1) run in duplicate, the more reproducible the test is.

Results

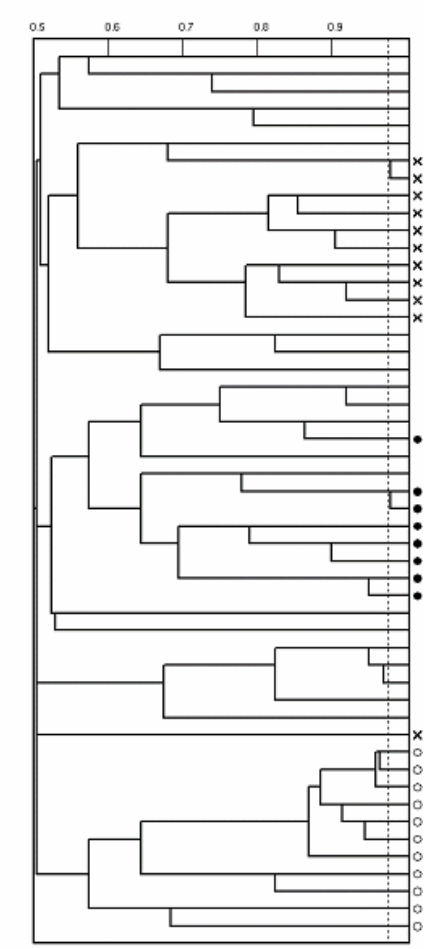


Figure 2 Dendrogram of all 51 strains clustered together. Strains of *S.aureus, Enterococcus* and *E.coli* cluster in separate groups without mixing with the other species with the exception of one strain of *S.aureus* and one of *Enterococcus*. This means that the resistance patterns are similar within each group of bacteria. **X**=*S.aureus*● = *Enterococcus*○ =*E.coli*

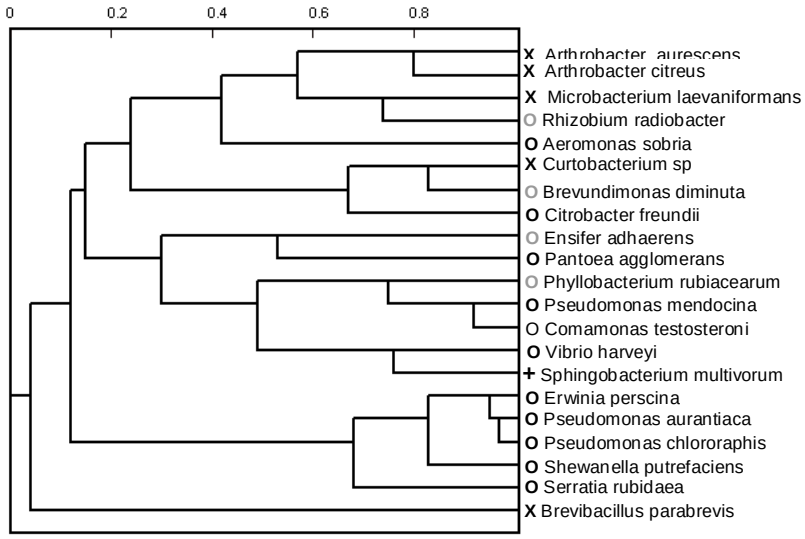


Figure 3 Strains clustered and marked according to their phylogenetic group. The strains cluster together with other strains from the same phylogenetic group only to a low extent. **X** = Gram positives, **+** = flavobacteria, O = α-proteobacteria, O = β-proteobacteria, **O** = γ-proteobacteria

Figure 2 shows the result from a comparison between the arrays of inhibition values from all bacteria (duplicates are excluded), clustered in a dendrogram. Bacteria from the same genus/species, i e *Staphylococcus aureus, Enterococcus spp* and *Escherichia coli*, cluster together with each other rather than with other bacteria. On the other hand, as can be seen in Figure 3, bacteria in a specific phylogenetic group do not necessarily cluster together. Some strains, such as the γ-proteobacteria *Erwinia perscina*, *Pseudomonas spp, Shewanella putrefaciens* and *Serratia rubidaea*, cluster together, whereas other γ-proteobacteria, such as *Citrobacter freundii* and *Aeromonas sobria* show a very low similarity to these strains. The result is confirmed in Table 3 where the heterogeneity and the variability are at the same level for *E.coli*, *Enterococcus spp* and *S.aureus*, whilst these increase for the different phylogenetic branches. Thus, the difference in sensitivity is low between bacteria from the same genus. As phylogenetic diversity increases, both the difference between the strains and the variation in sensitivity increases up to the phylum level. Mixing strains from different phyla did not seem to increase either the difference in sensitivity, or the variation in sensitivity towards a given chemical. This indicates that the phylogenetic relation between two strains provides little information on their similarity in sensitivity towards different toxic chemicals.

Table 3 Numerical evaluation of the results. The heterogeneity is a measurement of the similarity between the arrays of inhibition values from the bacteria in each group. The variability is a measurement of how similar the strains react to each chemical. The higher the heterogeneity and variability are the higher the diversity within the group is. The values in row "Total" are based on the results from all strains in Figure 3.

	Heterogeneity	**Variability**
Gram +	0.78	89
a - **proteobacteria**	0.68	93
g - **proteobacteria**	0.81	98
Total	0.85	116
S.aureus	0.38	46
E.coli	0.32	49
Enterococcus	0.37	48

Reproducibility

The mean correlation coefficient between the duplicates in group 1 is 0.94 and in a dendrogram the duplicates cluster together. The correlation coefficients between the duplicates of the strains in groups 2-4 are lower (data not shown), but they still have a higher correlation coefficient to other strains within the group than to bacteria in other groups. It was previously known that these species were not ideal for this test method. This does not affect the outcome of the assay, though, since it can be concluded that these strains respond very similar to the chemicals tested. Thus the conclusion remains valid regardless of which duplicates are used for the calculations.

Discussion

As evaluation the phylogenetic diversity in a natural population is a difficult and labour intensive task (20), (18), (9), this study was performed using pure cultures of known bacterial strains. The study was performed at four levels of bacterial diversity 1) bacteria from different phylogenetic groups 2) bacteria from one genera having a high metabolic diversity (*Enterococcus*) 3) bacteria from a single species group also having a high metabolic diversity (*E.coli*) and 4) bacteria from a single species group having a low metabolic diversity (*S.aureus*). In total 51 bacterial strains were tested against 14 chemicals.

It was expected that a higher phylogenetic diversity within a bacterial group would result in a greater variation in the toxic effect on the individual strains within that group. This hypothesis is supported by studies indicating that the toxicity of a chemical is dependent on the membrane composition of the bacteria involved (32). However, bacteria from different phylogenetic groups may exhibit the same sensitivity towards a chemical as a result of different metabolic pathways and receptors etc *kolla ref* (30), (13). On the other hand, Miller et al observed that phylogenetically similar cyanobacteria do not show the same tolerance to sulphide (26).

Up to a certain level of genetic diversity, i e within the same phylum, our results indicate that the hypothesis was correct since the variation in toxic response to the chemicals increased with a higher level of phylogenetic diversity. However, above that level, i e including bacteria from different phyla, the variation in toxic response increased only to a minor degree. The same tendency was observed when similarities in sensitivity between the strains were compared. It can be concluded, therefore, that a broad phylogenetic diversity within a single phylum in an ecosystem is important for stability but increasing this diversity to include bacterial strains from a different phyla may not increase the towards toxic chemicals.

The importance of the metabolic diversity of a microbial population was studied by Degens et al (11), who found that a low metabolic diversity of a microbial community reduces the resistance to stress or disturbance. Thus the variation in sensitivity should be greater in the *S.aureus* group than within the Enterococci in this study. However, the results in Table 3 indicate that the two groups have the same variation in sensitivity.

The toxicity of a chemical is largely dependent on the environment in which the test is performed (32), (31). In this study, a strict *in vitro*-system with standardized conditions was used; it follows that the results from the single strains cannot be transferred to *in vivo*-conditions without caution. However there is no reason to believe that the conclusions regarding the importance of phylogenetic diversity are not also valid under other conditions. Furthermore, no account of the fact that in many

cases it is derivates or metabolites rather than the chemicals themselves that are toxic to the bacteria has been taken into consideration (32). The selection of strains is biased towards those strains giving a prompt response when grown over night in nutrient broth and which react with tetrazolium red. This means that strains in this study have similar nutrient requirements and also have similar enzymes mediating electron transport and oxidative phosphorylation since these are the enzymes included in the reduction of tetrazolium red producing the red precipitating indicative of growth.

Conclusion

A high phylogenetic diversity does not necessarily correspond to a high functional diversity in a bacterial community. The variation in the sensitivity towards toxic chemicals increased with increased phylogenetic diversity up to the phylum level. Above that level, i e including strains from different phyla, the variation did not increase. The phylogenetic diversity of a population is thus not *per se* a sufficient measurement of the stability of a microbial population when exposed to toxic chemicals.

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