

### 3. Use of microbial communities as bioindicators for land-use systems

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#### 3.1 Introduction

The release of industrial and municipal waste products in freshwater ecosystems has become a dramatic issue for the environment and for human health. Freshwater contaminants are mostly associated with the particles transported by water, attached to their surfaces through covalent as well as ionic chemical bonds. In slow-flowing water, particles can be deposited by gravity into sediments, where particular biological and chemical properties lead to microbial communities to form a complex biofilm in oxic as well as anoxic conditions. Thus, freshwater sediments can host Bacteria, Archaea and other microorganisms capable of a plethora of different metabolisms, ranging from anaerobic respiration to fermentation, dehalogenation, oxygenic and anoxygenic photosynthesis and others.

These microbial communities react to any compounds released into the freshwater system by increasing or decreasing of taxa, and by genetic richness and diversity. Microbial communities are, indeed, able to provide an effective and integrated measure regarding the presence and effects of toxic xenobiotics in water.

#### 3.2 Biomonitoring in a freshwater environment

The standard chemical-physical analysis of freshwater environments has been historically based on the monitoring of parameters such as the Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), measure of Total Suspended Solids and determination of the concentration

of metals, nutrients, organic compounds or xenobiotics. These parameters provide information about the concentrations of a particular contaminant in an ecosystem, but they do not inform on the additive, antagonistic and synergistic effects of such compounds and elements on the biota (McGeoch 1998; Fränzle 2006). Moreover, an analytical approach gives insight on the sampling time, and does not permit detection of sporadic release or presence of pollutants. Analytical investigations cannot reflect the integration of numerous environmental variables because freshwater environments are usually strikingly complex, in which multi-sources of numerous contaminants are often rapid and difficult to estimate any hydrological changes. In this context, a study of bioindicators can be the best choice to assess ecosystem quality.

Bioindicators are biological processes, species or groups of species used to monitor biotic and abiotic variations of an environment during a certain span of time. Bioindicators show the cumulative impact of pollutants on biota, providing relevant long-term information on the environmental status or trends (McGeoch 1998). An ideal bioindicator should show a measurable and proportional response to environmental stresses. It has to be widely spread in the study area and stable, despite moderate environmental variations. It should have a low mobility, to avoid its movement in long distances, far from the pollution source. Finally, it has to be easily sampled and classified to avoid excessive time, i.e. the use of highly specialised personnel and prohibitive analytical costs.

Basically, there are two different approaches in biomonitoring. In the first case, organisms, which already exist in the environment, are observed and analysed to provide information about the environmental status (Boothroyd & Stark 2000; Parr & Mason 2003). In the second case, bioassay organisms either are used in the laboratory to test for an example of toxicity via an environmental sample, or directly introduced on-site to monitor the overall environmental quality (Girotti et al. 2008). For example, periphyton, a complex matrix of algae, fungi, protozoa, metazoa and heterotrophic microbes diffused in almost all aquatic ecosystems, can be considered as

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pointer multi-assemblage of organisms. It is commonly attached on rocks and on other submerged substrates, playing an important ecological role in freshwater food webs (Rosemond et al. 1993). Periphyton can accumulate many types of pollutants, ranging from heavy metals to bacterial pathogens. These pollutants can remain stable and protected for a long time in the biological matrix (Ács et al. 2003). Algae respond to an environmental stress, such as nitrogen and phosphorous surplus, by decreasing their diversity and richness, or changing their taxonomic composition, or varying their biomass. Other organisms, such as aquatic invertebrates, are commonly used to assess freshwater quality because of their long-term presence in sediments and sensitivity to changes in water or habitat quality. The presence and absence of taxa or variations in their richness and diversity are related to the occurrence of organic pollution levels (Boothroyd & Stark 2000; Parr & Mason 2003).

As mentioned above, bioassays may be an alternative approach in biomonitoring. For instance, measure of the mortality level of *Daphnia magna*, a freshwater zooplankton species belonging to the subphylum Crustacea, is widely used to determine the pollution level of freshwater samples, since *D. magna* is easier affected by ingestion of xenobiotics than other organisms. Moreover, it is a good candidate for bioassay analyses to monitor residuary waters, due to its short generation time, high rate in multiplication, and easiness of manipulation and maintenance in laboratory conditions. Furthermore, its physiological answer to toxicity can be evaluated in a relatively short time (Villegas-Navarro et al. 1999; Emmanuel et al. 2004).

Microorganisms are considered good bioindicators because they respond quickly to physical and chemical environmental changes. Bacteria, fungi and Archaea are strictly interconnected with the surrounding environment, because of their high surface area-to-volume ratio. Bacteria are more abundant in a microsite compared to other microorganisms and their assay is becoming ever more rapid and economically advantageous. Their analysis is particularly advantageous since a huge amount of individuals can be

harvested and processed from a very small volume of sample, minimising the sampling disturbance.

Bacteria can be used as bioindicators at different levels: gene, population and communities. They have evolved resistance mechanisms against contaminants, which involve specialised functional genes. The abundance and occurrence of the above-mentioned genes can provide evidence of the presence of certain xenobiotics such as antibiotics, heavy metals and polycyclic aromatic compounds (PAH), which might be difficult to identify through routine measurements. In agricultural areas characterised by copper pollution, it was observed a widespread presence of bacterial strains characterised by the occurrence of the *cop*-gene family, which confers resistance to copper. On the contrary, the authors did not detect *cop*-genes in the samples collected in non-polluted sites (Altimira et al. 2012). Fuel combustion, waste incineration, coal gasification and petroleum refining processes produce a large amount of PAH that can be detected through the analysis of PAH-specific ring hydrolysing dioxygenases (RHD), in which the involved genes are a part of the cleavage of the ring in an aromatic hydrocarbon (Figure 1a) (Kumar & Khanna 2010).

A number of studies have reported that there is a correlation between antibiotic-resistance genes and the levels of antibiotic concentrations in the environment (Wu et al. 2010; Allen et al. 2010). Wu et al. (2010) found a link between tetracycline concentration and the presence of tetracycline resistance (*tet*) genes in the vicinity of nine swine farms located in three cities in China. Potentially heavy metals and others toxic compounds such as quaternary ammonium compounds, antifouling agents and detergents may select for genes encoding antibiotic-resistance (Figure 1a) (Berg et al. 2005; Singer et al. 2006). Genes encoding heavy metals resistance can be located together with antibiotic-resistance genes. Bacteria may have also an unspecific mechanism of resistance common to different substances, for example, the multivalent pumping systems prevent the intracellular accumulation of structurally diverse xenobiotics (Figure 1b) (Piddock 2006; Poole 2005). A high concentration of antibiotic-resistant genes were detected

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in agricultural soil treated with copper and in freshwater microcosms with a high concentration of heavy metals (Berg et al. 2005; Stepanauskas et al. 2006).

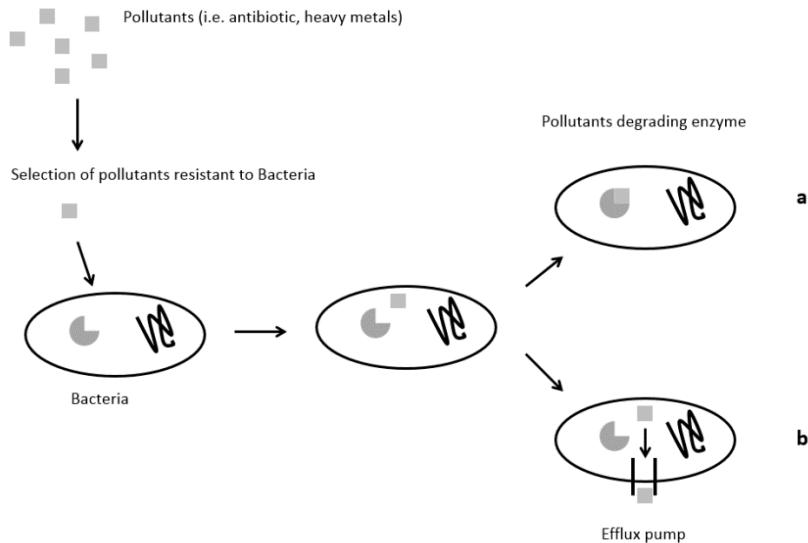


Figure 1 – Pollutants select Bacteria that can adopt different survival strategies. [a] Inactivation of the pollutants via modification or enzyme degradation process. [b] Some Bacteria possess an efflux pump located on the cellular membrane with the ability to extrude pollutants out of the cell (Borruso 2014).

In specific cases, the genes that confer resistance to antibiotic and heavy metals can be integrated in different types of mobile genetic elements named plasmids, transposons and integrons. Plasmids are small, circular and double-stranded molecules of DNA, not essential for cell life, and capable of replicating independently. They are, mainly, responsible for the spreading of antibiotic- and heavy metal-resistant genes among microorganisms (intercellular mobility or horizontal gene transfer), which are often taxonomically distant (Heuer & Smalla 2007). Transposons are segments of DNA that facilitate the transfer from one genetic locus to another one in the same cell (intracellular mobility) or among cells (conjugative transposons) (Hall & Collis 1995). Integrons are genetic elements able to capture, carry and

express genes (known as gene cassettes) associated with antibiotic and heavy metal resistance. Integrons are not self-mobilisable, but they are usually located in composite transposons or on plasmids facilitating their mobility (Nemergut et al. 2008).

Borruso (2014) analysed the presence of Class I integron and the associated genes cassettes in the city of Zhangye, Gansu Province, northern China. Sediments associated to *Phragmites australis* roots in freshwater channels characterised by a high level of pollution were studied, and the authors reported the presence of integrons carrying various gene cassettes in all polluted sites. Differently, integrons and relative gene cassettes were not detected in the unpolluted area used as control. These results indicate that Class I integrons could be a promising bioindicator in freshwater environments affected by a broad spectrum of pollutants. As for the Eukaria, microorganisms are extensively used to assess the environmental quality as "tester organisms". The bioluminescent Bacteria *Pseudomonas fluorescens*, *Vibrio fischeri* and *Vibrio harveyi* have been broadly used to detect the level of xenobiotics such as pesticides, heavy metals or organic compounds in a sample. The light that is normally emitted by the Bacteria decreases in presence of toxic substances, since pollutants inhibit or disrupt the cellular metabolism. This test can be used for analyses of short- and long-term effects of contaminants (Girotti et al. 2008).

The presence and abundance of faecal coliforms are indicators of microbiological water quality, since they are indicative of faecal contaminations and of the possible presence of enteric pathogens. This group includes *Klebsiella* sp., *Escherichia* sp., *Citrobacter* sp. and *Enterobacter* sp., associated to the intestine of warm-blooded animals and easily found in their faeces (APHA 1995). Although extensive research literature has focused on monitoring specific bacterial species or a limited bacterial population to assess the health of freshwater ecosystems, the use of the entire microbial community for environmental monitoring has been receiving attention only recently.

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A few works have demonstrated that the entire bacterial community is a promising tool to predict freshwater health status, granted, its sensitivity to the presence of contaminants and environmental stresses. As already stated, bacterial communities have the ability to change their taxonomic and physiological features according to environmental stresses and to contaminants they are exposed to (Lear & Lewis 2009; Sun et al. 2012; Borruso 2014). Biomonitoring at the community level integrates numerous taxa that bioindicate a broader aspect of freshwater environments, underlying the occurrence of different types of disturbance. This approach is more robust because it reflects changes for multiple species, including rare species types. On the contrary, a single taxon, which may be a limited bacterial population, may potentially remain unaffected by the same disturbance. The bacterial community composition of six estuaries, three with a high level of anthropic impact and three less impacted ones, was analysed by Sun et al. (2012). They found a differentiation in the microbial community composition between polluted and moderately polluted samples. The differences among the microbial communities composition in the same-site results were limited, confirming that they do not differ if exposed with same-environmental variables. Similar outcomes were obtained in a study in which the authors found a link between land use and the microbial communities associated to stream sediments. The bacterial community structures analysed in samples collected in the rural and urban area showed striking differences (Lear & Lewis 2009).

#### 3.3 Methods to assess microbial community diversity and structure in freshwater sediments

There is a variety of techniques to study freshwater microbial diversity. Traditional methods are based mainly on culturing methodologies that use a variety of culture media designed to select several different microbial taxa. Culture-based methods are important to isolate and study bacterial strains, but they are not the optimal tool to evaluate the overall microbial diversity, given that conditions they offer are usually selective for a particular population of microorganisms. It is estimated that less than 1 % of the known bacterial species can be isolated by using traditional techniques, since

the vast majority of microorganisms are not able to grow due to the lack of required environmental conditions that cannot be simulated in the laboratory (Curtis et al. 2002). Other Bacteria are intrinsically not cultivable, due to physiological constraints such as quorum sensing growth limitations or the necessity to grow in co-culture with other species.

In the last few decades, several biomolecular methods have been developed to study uncultivable microorganisms, allowing a new perspective for the analysis of microbial community diversity and structure. This approach is based on the Polymerase Chain Reaction (PCR). DNA extracted from the sample is analysed to detect microorganisms. The most common approach is the PCR amplification of the 16S rRNA conserved gene. In particular, this gene is broadly used because of its noteworthy features, namely its essential function, evolutionary properties and characteristic of having highly conserved as well as species-conserved regions. Moreover, 16S rRNA gene sequences are commonly used as a housekeeping genetic marker to study bacterial phylogeny and taxonomy, mainly due to the fact that it is present in all Bacteria and the fragment is large enough (1,500 bp) for bioinformatic purposes (Woese 1987; Neefs et al. 1993).

Once DNA has been amplified, a crucial point is the separation of the amplified fragment from the non-target sequences. Different techniques are available for sequence separation. Cloning libraries involve the ligation of the amplified genes into a plasmid vector and the transformation of *Escherichia coli*, followed by the screening of the obtained clones. Alternatively, fragments with the same size but different sequences can be separated via the use of Denaturing Gradient Gel Electrophoresis (DGGE). This technique allows the analysis of different microbial communities simultaneously on the same gel. Sequences are separated because of the different dissociation behaviour of the DNA fragments. After the run, it is possible to cut the single bands and sequence them (Muyzer et al. 1996). The sequence identification is done by comparing them with those of known organisms in a large database such as the Ribosomal Database Project (RDP) (<http://rdp.cme.msu.edu/>) and GenBank (<http://www.ncbi.nlm.nih.gov/>).



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Electrophoretic profiles can be used to represent the investigated microbial structure by displaying the community in a band or peak profile that can be used for statistical comparison. Each band or peak represents a taxon. This approach is useful to assess microbial communities' differences among samples. If applying automated methods, fragments are marked with a fluorescent chromophore, separated through capillary electrophoresis and detected by a "CCD camera" after being excited by a laser-light. Among these methods, Terminal-Restriction Fragment Length Polymorphism (T-RFLP) implies that the 16S rRNA gene is amplified by using two specific primers with two different fluorophores, and then digested with a restriction enzyme. The laser will detect only the terminal fragments obtained after the restriction. The different sizes of the digested terminal fragments of the 16S rRNA gene represent the different taxa, and in some cases at the genus level (Liu et al. 1997).

In addition, the microbial structure can be studied with another automated method called Automated Ribosomal Intergenic Spacer Analysis (ARISA), based on the investigation of the amplified intergenic region between the 16S and 23S rRNA genes (ITS). Being able to detect differences up to a single-nucleotide, the technique shows a high resolution, up to the sub-species level, and reproducibility is guaranteed by instrumental automatism (Fisher & Triplett 1999; Cardinale et al. 2004). Although, automated techniques can be used to analyse microbial community profiles, offering a huge amount of information with respect to traditional techniques, they do not sufficiently describe microbial diversity in depth. Next generation sequencing is becoming a routinely used technique able to provide deeper insights into complex microbial life.

Pyrosequencing is a flexible, parallel-processing and easily automated method for DNA sequencing. It has a higher throughput and coverage of phylotypes compared to other techniques. One of the primers used to amplify the fragment of interest is modified with biotin. The fragment is mixed with the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase, the substrates adenosine-5-phosphosulfate (APS) and luciferin.

Later, the four nucleotides are added one at a time, iteratively, in the nucleic acid polymerisation reaction. Pyrophosphate (PPi) is released during the ATP-conversion operated by ATP sulfurylase, and light is emitted, while luciferin is converted into oxyluciferin; this latter reaction is catalysed by luciferase. The light produced emits a signal, detected by a camera, proportional to the number of nucleotides incorporated during DNA synthesis. The process is repeated with each one of the four nucleotides (dAGP, dGTP, dCTP and dTTP) until the DNA sequence of the single stranded template is synthesised. The sequential collection of images taken by the camera is analysed to measure the light intensity in order to work out the amount of a specific dNTP incorporated in a given attempt. The image analysis permits for the calculation of a number of sequences per bead (Margulies et al. 2005; Sogin et al. 2006).

### **3.4 Bacterial bioindication to assess water quality in different land-use systems: Two case studies in northern China**

Northern China has a dry climate, abundant sun radiation, strong winds and little precipitation, concentrated in a restricted period of the year. The territory is characterised by a number of arid biomes, i.e. deserts, semi-deserts, steppes and mountain ecosystems (MWR 2004) – for more topographical information see Chapter 2. In the last 50 years because of the continuously growing population, the rapidly expanding industry and the increase of productive farms, the length of the water channel system has increased by more than 350 % and water demand by more than 250 % (Ringler et al. 2010). Moreover, Fu et al. (2004) reported that climatic change is causing a dramatic decline of the runoff water, followed by a decline of water quality because of the continuous release of several inorganic and organic toxic compounds. Pollution is exacerbated by the loss of cultivable land due to desertification, erosion, salinisation and heavy metal pollution (Kim 2007). The use of fertilisers and pesticides in agriculture has led to an increased presence of nitrogen, phosphate and heavy metals – such as Cd, Pb, Cu and Zn. In addition, the development of metallurgical industries has caused a further escalation in heavy metal pollution (Su et al. 1994; Wang et

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al. 2001; Cheng 2003) that negatively influences the quality of crops, atmosphere, water and human and animal health (Zhang 1999; Liao 1993).

Zhangye, located in Gansu Province, is situated nearby the Heihe River. The water sources of the Heihe River are the glaciers of Qilianshan Mountain, south of the region. The city is an oasis in an arid region characterised by an urban environment, numerous streams, fertile soil and reed stands. Zhangye is growing in various aspects including mining, production of building materials, electric power, metallurgy, machinery assembly, transportation and agriculture. In recent years, water demand has been dramatically restricted due to the excessive water use in socio-economic systems while environmental pollution has caused a decrease in water quality (SBZC 2003). Moreover, since the water system is not well organised and the irrigation methods are inefficient, desertification is increasingly causing conflicts between local communities (MWR 2004) due to the loss of suitable land (Peidong et al. 2007). About 95 % of the water is currently used for agriculture and, in particular, for the cultivation of crops that need a high amount of water.

Borruso (2014) analysed sediment samples associated to the rhizosphere of *P. australis*, in Zhangye, collected in channels exposed to different land uses. Microbial communities, not only resulted in being extremely different in the polluted and unpolluted sites, but they also differed according to the type of pollution (i.e. heavy metals and nutrients; Photograph 1). Furthermore, samples not affected by pollutants showed a bacterial community structure highly similar to the one of the samples collected in a similar natural area in Inner Mongolia (considered as an out-group). The similarity among samples from very distant unpolluted areas of different channels could indicate that, in absence of stressors, the rhizosphere effect is the major driver of bacterial diversity. Therefore, the rhizosphere of *P. australis* can be seen as a normaliser of the bacterial community structure, given that it does not vary between different geographic areas (Figure 2).

The Hetao Irrigation District, located in the western part of Inner Mongolia, has a typical continental climate with moderate precipitation throughout the

year and very cold winters and very dry summers. The Hetao Irrigation District hosts the largest farmland drainage and irrigation system of the Yellow River Basin. About 78 % of the water is used for the agriculture in particular for maize, wheat and sunflower cultures (Barton 2005; Fejes et al. 2008). The system is composed of 20,000 branch irrigation channels, which enter into the main drainage channel and finally into Wuliangshuai Lake (Barton 2005; Fejes et al. 2008). Wuliangshuai Lake has an area of 33,348 km<sup>2</sup> and a capacity of 2.5×10<sup>8</sup> - 3.0×10<sup>8</sup> m<sup>3</sup>. Half of the lake's surface is covered by macrophytes and in particular, *P. australis* as the most dominant species (Barton 2005). The intensive use of fertilisers in the Hetao area has resulted in a large nutrient load in the irrigation water system and eventually in Wuliangshuai Lake. The fertiliser drained into the Wuliangshuai Lake increased from 60,000 t in 1980 to 600,000 t in 2000 (Yu et al. 2007). The eutrophication and pollution of Wuliangshuai Lake is very serious. It is estimated to be 18,750 t y<sup>-1</sup> of COD, 2,350 t y<sup>-1</sup> of BOD, 10<sup>6</sup> t y<sup>-1</sup> of phosphates and 1,673 t y<sup>-1</sup> of nitrates (Barton 2005; Fejes et al. 2008).

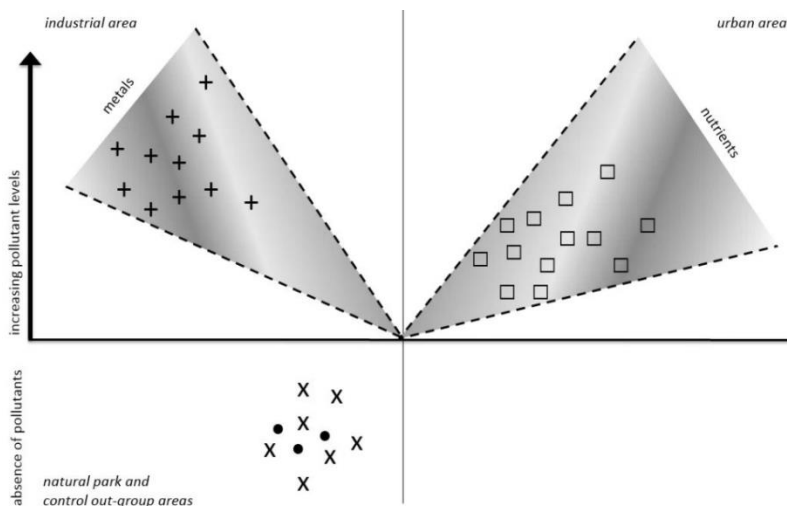


Figure 2 – A multivariate analysis of ARISA profiles via ordination analysis. Metals (Cu, Zn, Pb, Cd, As, Cr, Hg, Mn, Al and Ni) and nutrients (N and P) are influenced by different microbial communities. Industrial area [+], urban area [□], natural park area (x) and out-group [•]; adapted from Borruso (2014).

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Sediments associated to *P. australis* were collected from the main drainage of Hetao Irrigation District along a transect of around 250 km. The microbial community structure results differentiated according to a clear biogeographical scale. Samples from the secondary and first part of the main drainage channels of the Hetao tended to group together. The third part of the main drainage channel showed a distinctive bacterial community, probably due to the effect of the entrance of polluted water from Bayannur, urbanised Linhe area, where chemical industries are located. The latter part of the main drainage channel as well as Wuliangshuai Lake were characterised by very different bacterial communities less influenced by metals and agricultural nutrients. Microbial communities from the samples of the lake outgoing channel showed distinctive profiles, originated from those of the lake. A close relationship between the microbial community structures analysed by ARISA and the geography of the sampling sites was found. Accordingly, we found that water characterised by the observed chemical pollution or by supposed organic pollution clustered differently from those in areas that are more natural.

### 3.5 Conclusion

The remarkable developments made within biomolecular sequencing techniques and innovative fingerprinting analysis has allowed microbiologists to deeper analyse environmental samples. They can now overcome problems arising from bacterial strains which are not unculturable in a laboratory environment. Microbial bioindicators, not excluding other novel available methodologies, are a useful tool in context, characterised by environmental factors that cannot be directly measured. Examples include compounds derived by pesticides or toxic waste containing a number of interacting pollutants. Microbial communities should be considered as one of the first environmental parameters to monitor in order to have a fast response into ecosystem health and its relating factors.

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