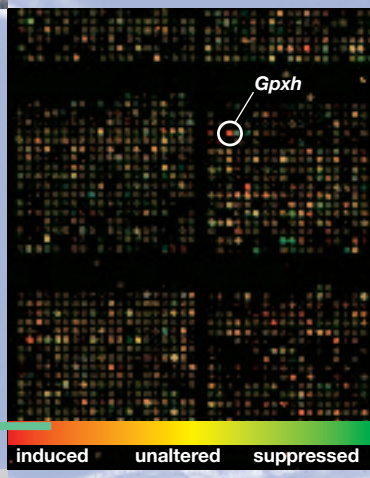
EAWAG

EAWAG news

56e November 2003
Swiss Federal Institute for Environmental Science and Technology (EAWAG),
A Research Institute of the ETH-Domain • CH-8600 Dübendorf

Molecules in Action



EAWAG

EAWAG news 56e • Nov. 2003
Information Bulletin of the EAWAG

From ecosystem via molecule to ecosystem

Molecules in Action

Publisher Distribution and © by:
EAWAG, P.O. Box 611, CH-8600 Dübendorf
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Fax +41-1-823 53 75
http://www.eawag.ch

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Publication Three times yearly in English, German and French. Chinese edition in cooperation with INFOTERRA China National Focal Point.

Cover Photos EAWAG

Design inform, 8005 Zurich

Layout Peter Nadler, 8700 Küsnacht

Printed on original recycled paper

Subscriptions and changes of address New subscribers are welcome! Please find the order form in the middle of this issue.

ISSN 1440-5289

Rik Eggen, head of the department "Environmental Microbiology and Molecular Ecotoxicology"

Molecular biology nowadays plays a vital role in many fields of scientific research. In medicine, for example, pathogenic processes are studied on a molecular level. Subsequent to the identification of the underlying mechanisms, it becomes possible to develop specific drugs. These are either applied as preventive medicine, such as vaccination, or – once the disease has broken out – are used as a cure, acting specifically and with as few side effects as possible. Less known, however, is the fact that molecular approaches are also becoming more and more important in environmental research, too.

The EAWAG is committed to the sustainable use of aquatic ecosystems – rivers, lakes and groundwater. Aquatic ecosystems are very complex and offer habitats for a diversity of living creatures, from single cellular bacteria or algae to multicellular higher plants and animals. The organisms live in permanent interaction with each other and with their environment, which in itself is very dynamic and subject to continuous change. Just think of natural changes such as daily and seasonal fluctuations as an example. Adding to these natural changes are anthropogenic impacts which are continuously increasing due to the steadily growing world population. Problems such as the input of pollutants into aquatic ecosystems, the growing pressure on freshwater resources and the increase of pathogens in surface waters of developing countries can no longer be neglected. There is a need for concepts and approaches which allow the protection of the complex aquatic environment for the future by preventive methods and make it equally possible to deal with acute problems directly and "without side effects". Therefore, the EAWAG is attempting to analyze processes in ecosystems on a molecular level, in order to better understand, predict and prevent the effects of anthropogenic impacts. In this work, we are well aware that important insights for the ecosystem can only be obtained if we do not lose the view of the whole picture of the ecosystem.

To study processes on molecular level, it is essential to perform basic molecular research. The EAWAG is studying such different aspects as the genetic diversity of *Daphnia* in alpine lakes and the mechanisms of action of pollutants on a molecular level. Furthermore, applied science plays an important role at the EAWAG. Here as well, molecular approaches and methods are used more and more. Examples from this field are the development of biosensors for the detection of pollutants, the identification of a bacterium now being used for the removal of nitrogen in wastewater treatment plants and the development of a molecular method for the detection of pathogens in drinking water.

I invite you to enter the world of the molecules and hopefully become convinced that molecular biology offers an essential contribution to the sustainable management of aquatic ecosystems.

Molecular Strategies in the Environment

135 Years of Spell-Binding Research

Since the middle of the last century, molecular biology is its own discipline. It has its roots in microbiology, a discipline traditionally examining life processes and the role of microorganisms in different ecosystems. While today's applications of molecular biology in biotechnology and in the medical field are widely known, molecular approaches to problem-oriented environmental research are still very much in the background. This is not justified since these techniques are highly useful in the solution of current problems.

As early as the middle of the 19th century, scientists began to study the transformation of specific compounds by microorganisms. The driving force for most of the studies was the theory of spontaneous generation which proposed that life could form repeatedly and spontaneously. As an unintended consequence, these studies yielded the first insights into the metabolism of microorganisms. The French scientist Béchamp [1], a contemporary of Louis Pasteur, was as well a proponent of the theory of spontaneous generation. He examined a variety of environmental samples for their capacity to transform specific chemicals, describing for example the formation of methane from ethanol. According to his interpretation, the transformation was accomplished by microorganisms that had newly formed in his flasks; he named these organisms *Microzyma cretae.* It was Pasteur who refuted spontaneous generation with his ingenious experiments. He showed that these observations were, in reality, the growth and enrichment of microorganisms that were already present at the beginning of the experiments.

In the second half of the 19th century, the German scientist Felix Hoppe-Seyler continued the molecular strategy of environmental research. As the first Professor for Physiological Chemistry at the University of Strasbourg, he coupled the molecular understanding of biological processes to energy considerations. He recognized that every biochemical transformation yields energy that can be used by microorganisms for their growth and metabolism. Remarkable is that the now well-known classic theory of thermodynamics was just being developed (Josiah Gibbs did not introduce the term free-energy until 1878) [2]. The original approach used by Hoppe-Seyler was refined in later years.

Radioactive Isotopes as Markers for Metabolic Products

The next major breakthrough occurred in the 1930's and 1940's when the chemist Samual Ruben and the physicist Martin Kamen discovered the radioactive isotopes ^{11}C and ^{14}C [3]. Both scientists immediately recognized the potential that this discovery had for science. Experiments with ^{11}C proved to be difficult since this isotope has a half-life of only 21 minutes. Only when ^{14}C with a half-life of ~5700 years was used, did it became possible to follow the intermediates of biochemical transformations (even in complex systems) and to ascribe assimilation products to specific organisms within an ecosystem. With the use of microautoradiography, organisms that catalyze certain biochemical transformations in an ecosystem can be made directly visible (Fig. 1). Leading the way in this methodology were Louise and Thomas Brock during the 1960's [4].

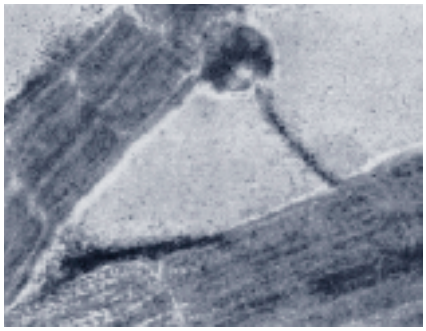


Fig. 1: Autoradiogram of epiphytic bacteria (visible as black colony, lower left) living on marine red algae. The bacteria were fed ^{14}C-glutamate as a carbon source [from 4].

Detection of Specific Microorganisms

During the 1970's and 1980's, biologists, particularly microbial ecologists, concentrated their research on the development of methods for direct identification of microorganisms in complex environmental samples.

Specific compounds: Some compounds are found only in very specific groups of organisms. The electron transfer coenzyme F_{420}, for example, is only found in methanogenic bacteria, with one exception. This compound is particularly interesting because it fluoresces, which allows simple detection of the organism (Fig. 2).

Immunological methods: While the detection of certain bacteria by immunological methods was already widely used in medical microbiology by the late 1970's, this technique was only starting to be employed in environmental research. Already at that time, a wide variety of markers was available for making antibodies bound to specific target organisms visible. The options included radioactive labels, enzymes that catalyze specific reactions (ELISA technique), specific heavy metals, or fluorescent

A. Zehnder, EAWAG

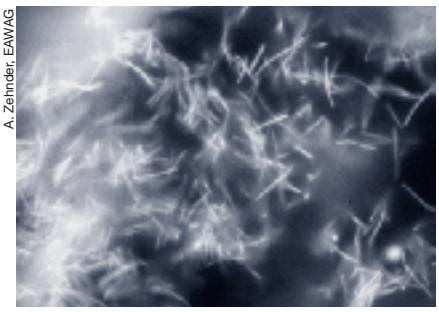
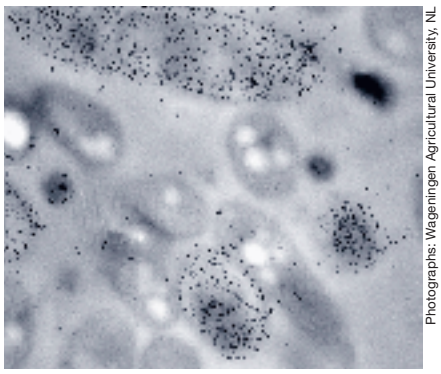


Fig. 2: F_{420}, an electron transfer coenzyme occurring almost exclusively in methanogens. UV light causes this coenzyme to fluoresce. This property allows us to directly identify methanogens in natural populations, such as *Methanobacterium formicicum* in this case.



Photographs: Wageningen Agricultural University, NL

Fig. 3: Gold colloids (black dots) bound to antibodies can be used to immunologically identify bacteria in thin sections prepared for electron microscopy. This figure shows the specific marking of *Methanosaeta concilii*. This acetate degrading organism grew in a biofilm using propionate as a substrate.

dies. Antibodies could even be used to detect individual proteins, usually enzymes, in the complex system of a single cell (Fig. 3 and 4). The disadvantage of immunological techniques is that the organisms or proteins that need to be recognized by the antibodies, first have to be isolated. They are needed for the production of the antibodies.

RNA and DNA probes: A quantum leap was achieved with the development of RNA and DNA probes in the first half of the 1980's. Two requirements had to be met before these methods could be developed: First, RNA had to be recognized and investigated as a universal indicator of relationships between organisms. Carl Woese [5] and Norman Pace [6] were the pioneers in this field. Second, DNA had to be replicated outside the cell in a test tube. In 1985, Kary Mullis [7] achieved this in his discovery of the so-called polymerase chain reaction, or PCR for short (Fig. 5). The probes are constructed such that they recognize specific RNA or DNA sections. Depending on whether the target area is a variable or a conservative region, this method can detect individual strains or entire groups of organisms. If the probes are combined with fluorescent dyes, marked organisms can be made visible directly under the fluorescence microscope. Furthermore, PCR allows us to isolate unknown gene sequences from an ecosystem and to subsequently compare them to known DNA sequences. Because we now have huge data banks on DNA sequences, chances are relatively high that the unknown sequences can be attributed to specific functions or organism groups.

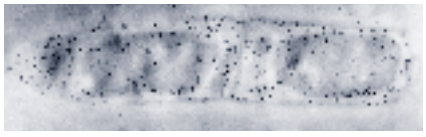


Fig. 4: *Methanosaeta concilii* in an electron microscope thin section. The enzyme carbon monoxide dehydrogenase is immunologically marked with gold colloids. The black dots appear to be evenly distributed throughout the cell, suggesting that the enzyme is soluble in the cell plasma.

What are Negative Impacts on Microorganisms?

In addition to questions about our understanding of processes, i.e., who does what, where and how, an interest in a new set of questions relating to causes and effects of environmental damage has emerged. This also includes problems around toxic chemicals that are released into the environment and can have negative impacts on organisms. The urgent need for solutions in this field has led to enormous progress in analytical chemistry. Today, a wide variety of pollutants can be measured at very low concentrations in complex matrices. In addition, our knowledge of how biological processes are controlled on the molecular level forms the basis for the development of so-called biosensors. They indicate even the lowest pollutant concentrations by the expression of specific enzymes or fluorescent proteins. Furthermore, the sequencing of entire genomes has made it possible to assemble specific gene groups and to apply them to so-called DNA chips. These chips can then be used to study the reaction of these genes to chemicals or other environmental factors.

Over the last century and a half, we have come a long way in understanding molecular processes in an ever-changing environment. The last 20 years, in particular, have been marked by a rapid expansion of our ability to apply our knowledge to the real world. EAWAG has contributed significantly to this progress, and we would like to present a few examples of our work in this issue.

The Effect and the Detection of Environmental Pollutants

Worldwide, arsenic represents one of the most important inorganic pollutants in drinking water. Millions of people in Bangladesh and Vietnam already show symptoms of arsenic poisoning. The detection of arsenic by analytical chemistry is far from trivial and nearly impossible without an infrastruc-

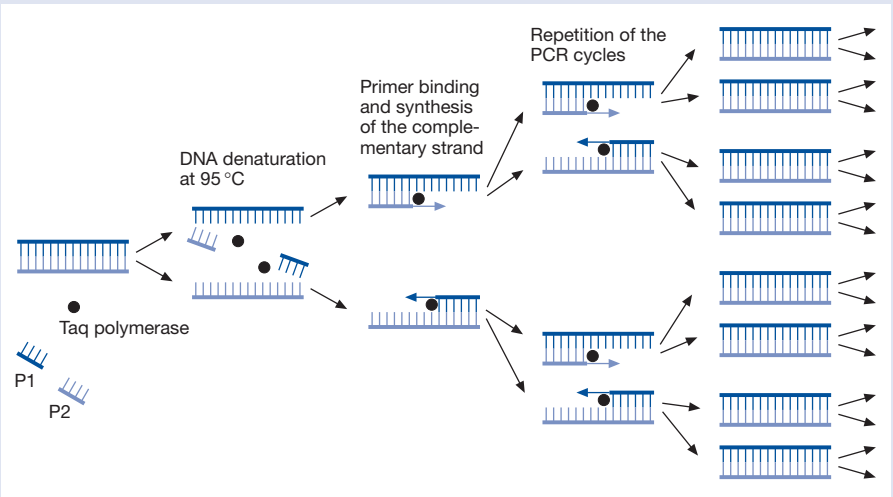


Fig. 5: Replication of a specific DNA fragment by the PCR method. The DNA to be replicated is mixed with two primers (P1 + P2) and the enzyme Taq polymerase. This mixture is subjected to approximately 30 temperature cycles in the PCR thermocycler. One temperature cycle lasts roughly 4 minutes and is made up of three steps. Step 1: the DNA is denatured at 95 °C; step 2: the temperature is lowered up to 37 °C to allow the primers to bind to the DNA; step 3: at 72 °C, the Taq polymerase synthesizes the complementary DNA strand.

ture of analytical laboratories. J.R. van der Meer and J. Stocker (p. 12) have developed a bacterial biosensor that is easy to use and can detect arsenic at the ppb level (one millionth of a gram per liter). The genetically engineered bacteria carry a so-called reporter gene which is activated in the presence of arsenic and causes the production of the corresponding reporter protein. This enzyme catalyses a reaction that releases a blue dye. Depending on the arsenic concentration, more or less enzyme is produced, which in turn releases varying amounts of dye. With a simple paper strip, even an untrained person can easily test a well for arsenic contamination.

For many years, ecotoxicology was dominated by a mostly phenomenological approach. Modern methods of molecular biology allow us to detect the expression of individual genes and, therefore, the production of the corresponding proteins in a very targeted, purposeful manner. When an organism comes in contact with a pollutant, various genes are activated, although this is not externally observable, and the organism continues its normal biochemical activities. The products synthesized by the activated genes aid in the defense against the pollutants inside the cell. In their article on page 15, B. Fischer and R. Eggen show how these defense genes can be used to detect certain pollutants; however, the response of the cell to an environmental pollutant is only half the answer. It is equally important to understand how these pollutants do their damage, i.e., we need information about how the pollutants react with biological structures. B. Escher (p. 9) and her group have investigated a number of so-called reactive chemicals for a wide range of toxic reactions and their primary toxicity mechanism.

Nitrogen Elimination and Clean Drinking Water

A group of engineers and microbiologists (see article by C. Fux and colleagues on p. 20) has examined new ways to remove nitrogen from waste water. A new method known as the Anammox process was discovered in the Netherlands and has proved to be extremely well suited for the treatment of waste water with high ammonia levels. In this process, ammonia is transformed directly to nitrogen using nitrite. A first task was to identify the microorganism responsible for this transformation. This was accomplished using specific gene probes. The practicality of the method was then tested in a pilot reactor, where successful operation cleared the way for the use of the Anammox process in full-scale reactors in wastewater treatment plants.

To date, the biological quality of drinking water is determined by cultivation techniques that are largely based on methods developed in the 19th century. The main problem of these cultivation techniques is the fact that they are rather time-consuming. Results are available after 24 hours at the earliest, often only after 72 hours. Acute contamination can, therefore, not be detected quickly. The PCR method has the potential for significant improvements in this area. Contamination could be confirmed within approximately 4 hours, leading to much shorter response times. A. Rust and W. Köster demonstrate in their article on page 18 how this method can be used in the monitoring of drinking water quality.

Gene Transfer and Genetic Diversity

Two other articles deal with evolutionary aspects. The group of J.R. van der Meer was able to demonstrate how large sections of DNA can be exchanged between bacteria. These DNA sections are referred to as "genomic islands" and can account for more than 10% of the entire genetic material. The receptor bacteria can obtain new capabilities from these transfers, such as the ability to degrade certain pollutants. This so-called horizontal gene transfer allows bacteria to accomplish important evolutionary steps with a high success rate and within very few generations.

M. Winder and P. Spaak (p. 22) examined the diversity of *Daphnia* populations in alpine lakes at varying elevations. The prevailing assumption was that genetic diversity should decrease with increasing elevation, just as the species diversity decreases at higher altitudes. Their results, however, have demonstrated that genetic diversity does not decrease but remains high even at high elevations.

"More is Different"

Rapid progress in molecular methods brings with it the danger of losing the view for the whole system but trying to explain the functioning of an entire ecosystem with a few details. It is important to use molecular strategies in trying to understand processes, and to validate this understanding at the system level. As long ago as 1972, Philip W. Anderson stated in his article "More is Different" that the dissection of a system into individual parts is not enough to help us understand the functioning of the entire system [8]. We need both, and EAWAG is fully aware of this fact.

Alexander Zehnder, microbiologist, director of EAWAG and professor for water protection and water technology at ETH Zurich. His research interests are environmental microbiology and the application of microbial processes in environmental biotechnology. He has recently also become involved in sustainable development, particularly with respect to water resources.

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Genomic Islands and Horizontal Gene Transfer Among Bacteria

Chromosomes are usually thought to be stable molecules, which have to be copied carefully for each of the new daughter cells. Except for a few copying mistakes ("mutations"), not much is happening to the chromosomal DNA. Or is it? Bacterial chromosomes are now known to harbor what is called "genomic islands", regions which can cut themselves out of the chromosome, in some cases travel to other bacterial cells and reinsert into the recipient's chromosome. Their function? Very often, they provide the recipient bacteria with auxiliary capabilities for infecting eukaryotic hosts or for degrading environmental pollutants.

Almost ten years ago, we started to investigate the process of horizontal gene transfer in bacteria (see glossary). Our aim was to estimate how frequent certain types of genes are transmitted between different bacteria in the natural environment. As a model system for our studies, we chose the bacterium *Pseudomonas* sp. strain B13, which was isolated from sewage sludge and which may use 3-chlorobenzoate as sole carbon and energy source (Fig. 1). When this strain was first described in 1974, it was one of few bacterial strains capable of degrading chlorinated compounds. This had attracted considerable attention, since chlorinated aromatics are often polluting substances in the environment. Strain B13 became yet more appealing because of another spectacular feature that we discovered: these bacteria are able to transfer their genes for the 3-chlorobenzoate metabolism spontaneously to other bacterial species, and this even in wastewater treatment reactor microcosms [1]. One of the curious findings, though, was that the rate of horizontal gene transfer seemed to increase in the presence of 3-chlorobenzoate. At that time, we interpreted these results such that 3-chlorobenzoate was favoring the growth of bacteria which had received the genes for 3-chlorobenzoate degradation. Furthermore, we did not have too much idea on how these genes were actually distributed from B13 to other strains.

Genes for 3-Chlorobenzoate Degradation Combined on a Genomic Island

Therefore, we looked at the mechanism of gene transfer in more detail. Roald Ravatn, who did his PhD thesis on this topic, discovered that the "recipient" bacteria had actually received a large DNA fragment of more than 100 000 basepairs from strain B13. This fragment was called the *clc* element (Fig. 2A) and contains the genes for 3-chlorobenzoate degradation [2]. It had become inserted into one or two very specific sites of the recipient chromosome. Strain B13 itself carries two copies of the *clc* element in its chromosome, which didn't seem to be lost after transfer to a new bacterium (Fig. 2B).

Roald Ravatn also identified the factor responsible for cutting the *clc* element out of the chromosome and for subsequent reintegration. It is an enzyme called "integrase". Comparison of the biochemical composition of the integrase from strain B13 with other proteins showed that it was related to integrases from bacterial viruses (bacteriophages), which place their genomes into the chromosomes of the infected cells, and to integrases from so-called genomic islands (see glossary) [3]. The gene for the B13 integrase is situated at the right end of the *clc* element (Fig. 2A).

Since a few years, the discovery of genomic islands has accelerated enormously, mainly because of genome sequencing projects. Large sequencing laboratories determined the complete nucleotide sequence of currently around 100 bacterial genomes. With the complete nucleotide sequence at hand, it could be shown that many bacteria carry genomic islands and even have multiple different copies. The genomic islands are characterized by the presence of a gene for an integrase and a specific site on the chromosome where they have inserted (Fig. 2B). Taking together all available information, we concluded that the *clc* element is a genomic island.

When Do Genomic Islands Move?

Now that we knew that the genes for the 3-chlorobenzoate degradation lie on a genomic island, we turned back to our earlier observation indicating an increased transfer of the *clc* element when 3-chlorobenzoate is present. At this point, Vladimir Sentchilo started his PhD thesis in 1999 considering the question of which environmental or cellular factors regulate the transfer of the *clc* element. Because the transfer of the *clc* element is always preceded by the activation of the integrase gene, our assumption was that we could take the activation of the

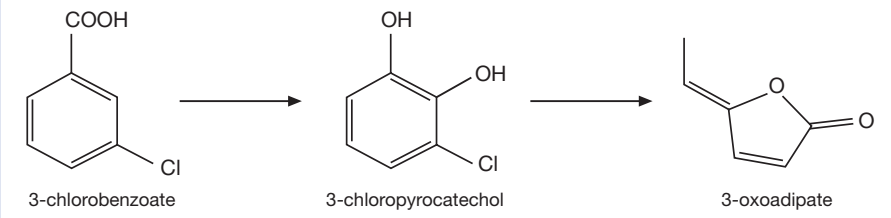

Fig. 1: Specific degradation pathway of the chlorinated aromate 3-chlorobenzoate. The product 3-oxoadipate will be further degraded in the general cellular metabolism.

Glossary

Genomic Islands
Unstable regions on the chromosomes of bacteria, which sometimes transfer themselves from one bacterium directly into the genome of another one. They increase bacterial fitness and can be divided into several subtypes: e.g., "ecological islands" in environmental bacteria and "pathogenicity islands" in pathogenic bacteria with auxiliary functions in infection, toxin synthesis or adhesion [4].

Green Fluorescent Protein or GFP
Reporter protein; those cells in which GFP is synthesized are fluorescent and can be observed under the epifluorescence microscope.

Horizontal Gene Transfer
DNA exchange between bacteria; in contrast to vertical gene transfer signifying the inheritance of a gene from a progenitor. Bacterial reproduction is usually described as asexual, because bacteria have no equivalent of the genetic fusion of two different cells that is characteristic of sexual reproduction in eukaryotes. Nonetheless, bacteria do have the ability to exchange segments of DNA with other bacteria. Because these segments can become fixed in a bacterium's genome and confer new traits, gene exchange among bacteria could be considered to be a form of bacterial sex.

Promoter
Regulating region of a gene in front of the coding region. Activation of the promoter will lead to transcription of the coding region and to subsequent synthesis of the respective protein.

integrase gene as indicator for the subsequent excision and transfer of the *clc* element. Therefore, Vladimir Sentchilo focused on the gene for the integrase and constructed specific reporter bacteria (similar to the arsenic biosensor, see p. 12). These reporter bacteria carried a molecular switch consisting of the integrase gene promoter (see glossary) coupled to the reporter gene for the Green Fluorescent Protein (= GFP, see glossary). Presence of GFP in the cell would thus signify that the promoter of the integrase gene had been activated and that the transfer process will subsequently proceed.

To our astonishment, we observed that only very few cells became fluorescent in a culture of the transgenic strain B13 (Fig. 3), implying that the transfer mechanism was only activated in a small subset of the population. Mainly, however, cells became fluorescent when they were no longer actively growing (i.e., starvation conditions). Strangely enough, though, when cells had been grown in the presence of 3-chloro-

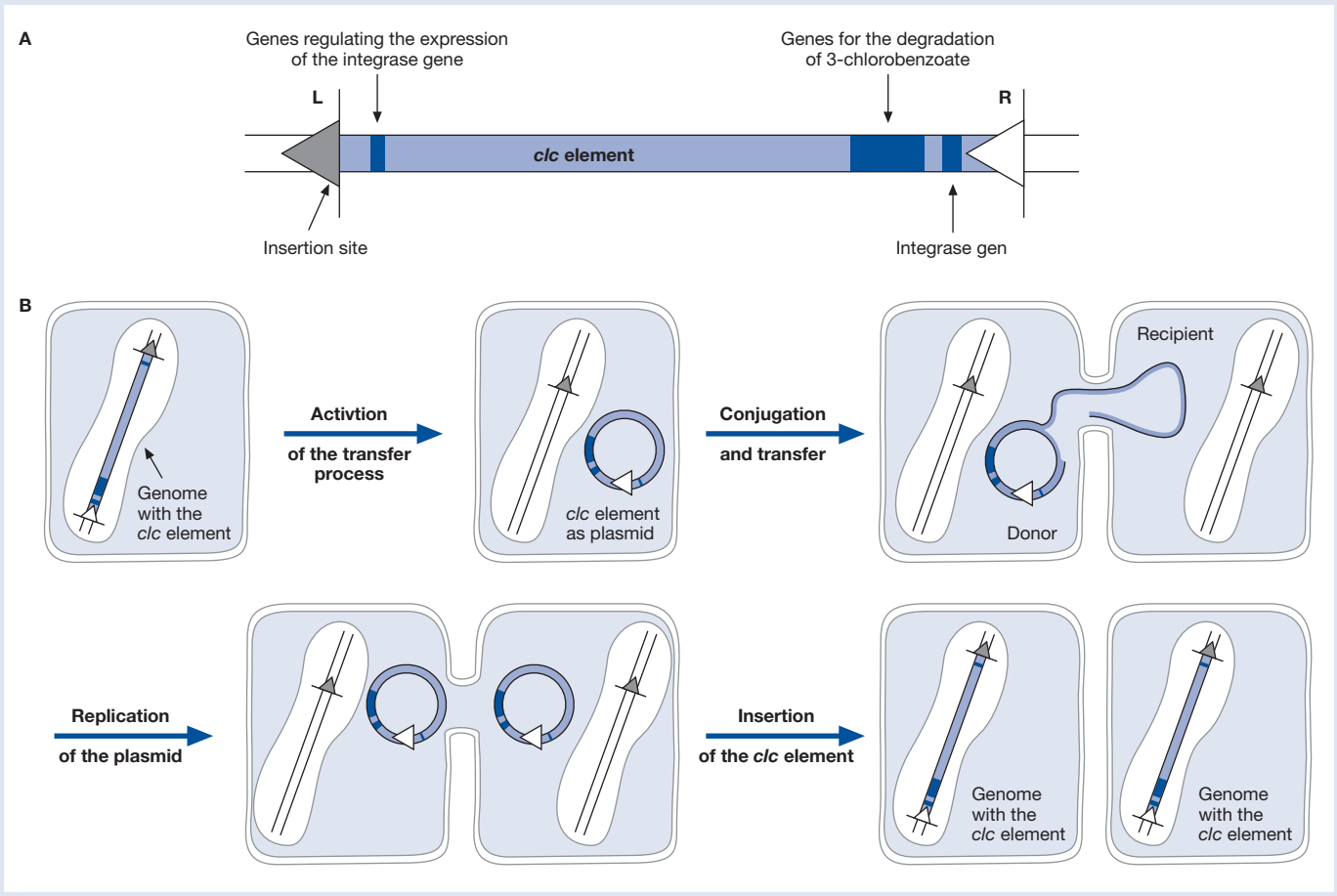

Fig. 2: The *clc* element (A) and its hypothetical life on its own (B). After activation of the transfer process, the *clc* element is cut out of the genome by the integrase and forms a circular molecule (= plasmid) in the bacterial cell. In case this cell comes into contact with another bacterium lacking the *clc* element, the *clc* element will be transferred as single-stranded molecule to the second cell. After replication, the *clc* element integrates at predetermined insertion sites into the genomes of both cells, a process during which the integrase also plays an important role.

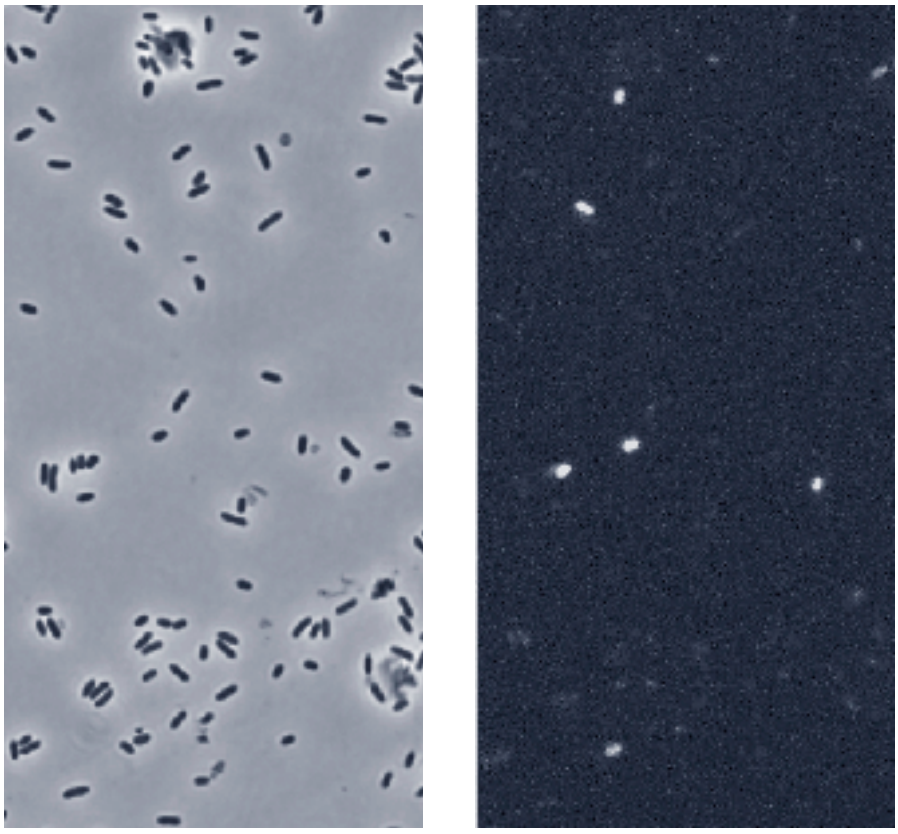

Fig. 3: The transfer process of the *clc* element ist activated only in a small number of bacterial cells of a culture of *Pseudomonas* sp. strain B13. Compare the phase contrast image (left, black on grey) with the same section showing the activated bacteria as bright cells on a black background.

benzoate, the number of fluorescent cells in starvation conditions was higher than when other carbon sources were used. This result confirmed our initial observation and showed, moreover, that 3-chlorobenzoate stimulates the transfer of the *clc* element at a very early stage, i.e., by activating the integrase gene expression. However, it is still unknown why the integrase gene is activated in some bacteria but not in others. Vladimir Sentchilo was also able to identify two proteins which seem to influence the expression of the integrase gene and may perhaps interact with cellular or environmental signals. Interestingly, these two proteins are encoded by the genomic island itself and a database comparison showed similar proteins in a number of other bacteria. In order to better understand the function of the genomic island in strain B13 and its evolutionary relationship to other genomic islands, we are now determining the complete DNA sequence. This is done with the help of the Institute Pasteur in Paris and the Genome Center in Bielefeld, Germany. With this knowledge, we hope to get a better idea of how the transfer of the B13 element and other genomic islands is regulated.

Desirable and Undesirable Implications

If it turns out that certain chemical compounds in the environment, like 3-chlorobenzoate, really act as a trigger for gene transfer, this could have profound influence of the rates of distribution of certain gene functions among bacterial communities. From the perspective of degradation of environmental pollutants, it wouldn't seem too problematic if the genes for their degradation became more widely distributed, since this would result in a faster degradation of the pollutant. However, faster distribution of pathogenicity characteristics providing other bacteria with auxiliary capabilities for infecting eukaryotic hosts might not really be an attractive perspective. It seems that even the genomes of what we usually consider to be the smallest organisms have smaller entities, e.g., the genomic islands with a peculiar life-style of their own.

Jan Roelof van der Meer, microbiologist and head of the group "Molecular Microbiology" in the department "Environmental Microbiology and Molecular Ecotoxikology". Research areas: Evolution, pollutant degradation, biosensor development and microbial ecology.

Coauthors: Vladimir Sentchilo, Muriel Gaillard

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Characterization of Reactive Chemicals Based on their Primary Mode of Action

Dead fish washed on the shore "belly up": who has not seen these pictures? They illustrate dramatically the fatal effects on aquatic life that accidents with environmental pollutants can have. However, our environment is also continuously influenced by chemical pollutants in low concentrations which unfortunately remain unperceived in most cases. It is important therefore to find out how exactly pollutants react in organisms. Thus, the goal of our research is to identify and classify the various primary modes of action of reactive chemicals by means of a bacterial test system.

The ecotoxicological risk of reactive chemicals may be only insufficiently assessed by classical test methods. The reasons for this are that reactive chemicals hydrolyze rapidly and that traditional testing methods often assess only a small part of the broad spectrum of effects that reactive chemicals may cause. Recognizing the mode of action is crucial, however, particularly for reactive chemicals, since the reaction mechanism is one of the major factors determining the risk potential. For this reason, we are currently developing a comprehensive bacterial test system that will cover a wide spectrum of reactive chemicals and their particular modes of action.

Pollutants Damage Biomolecules

Ultimately, all toxic effects can be traced back to primary interactions of the pollutants with three groups of biomolecules: membrane lipids, proteins and genetic material (DNA) [1]. Interactions span the entire range from weak van der Waals forces to specific interactions, such as the formation of hydrogen bonds or mutual attraction between charges, all the way to the formation of chemical bonds (Fig. 1). Weak interactions typically cause nonspecific, reversible effects and are only relevant for hydrophobic pollutants. Specific interactions are observed, for example, in enzyme inhibition, where a pollutant competes in the role of "key fitting" the lock, thus keeping out the actual substrate. Our special interest, however, is focused on reactive chemicals that form covalent – usually irreversible – bonds with a specific target region of the affected biomolecule. Among these reactive chemicals are a large number of compounds with different functional groups such as the reactive oxygen species (see article by B. Fischer, p. 15) and the so-called electrophilic chemicals, which is the focus of this article.

The Cell Arms Itself against Electrophilic Compounds

Electrophilic chemicals are molecules that are electron-poor, due to their electron configuration, and therefore prefer to react with nucleophilic (= electron rich) groups in peptides, proteins or DNA. Preferred targets are the thio groups in proteins and peptides as well as certain oxygen and nitrogen groups in DNA (Fig. 2). In the extreme case, proteins can be damaged by electrophilic compounds to such an extent that they can no longer perform their functions, while reactions between electrophilic compounds and DNA usually cause instability and mutations of the DNA, leading to cancer at the worst. Reactions at both targets may lead to death.

But the cells defend themselves against such attacks. Glutathione, an intracellular tripeptide (Fig. 2), intercepts electrophilic compounds which will subsequently be transported out of the cell. There are also a

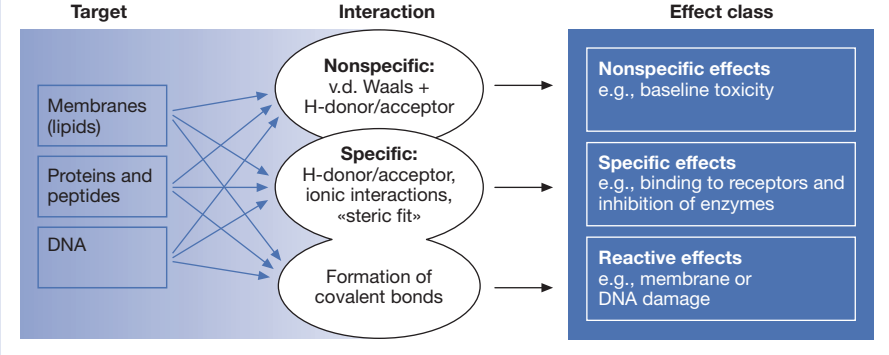


Fig. 1: Classification of toxic effects according to the mode of interaction with biomolecules at the target site.

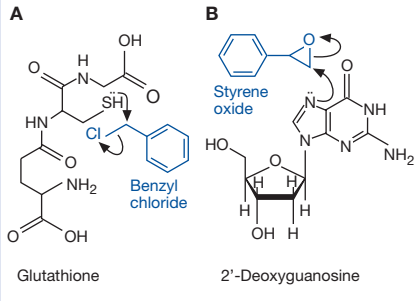


Fig. 2: Two examples of primary modes of action of reactive pollutants. Chemical reactions with proteins (A) or DNA (B) cause toxic effects.

number of repair mechanisms for damage to the DNA, such as proteins that are able to recognize and repair errors in the DNA sequence. However, when pollutants are present in high concentrations and/or over long periods of time, defense mechanisms are overwhelmed and toxic effects begin to manifest themselves.

Evaluation of Different Mutants of *E. coli* and Effect Parameters

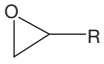
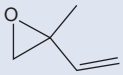
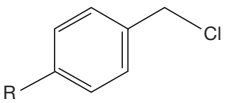
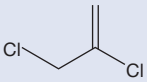
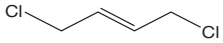
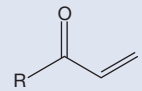
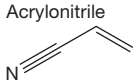
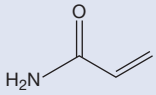
Methods showing the activity of the defense systems are particularly suitable test systems to identify the primary mode of action of electrophilic compounds. One has to take into consideration, however, that the toxicity of electrophilic compounds is not only determined by their chemical reactivity, but also by their concentration at the intracellular target site. The concentration at the target site, in turn, depends on how many electrophilic molecules enter the organism, how the molecules are distributed within the organism, and whether the organism is able to transform the molecule into a nontoxic form. These processes determine the bioavailability of the compound in question. In single-celled organisms, however, one can assume in case of hydrophilic substances that the pollutant concentration at the target site is the same as the extracellular concentration. We therefore have chosen to work with the bacterium *Escherichia coli* as our test organism. An additional advantage of this organism is that numerous mutants of *E. coli* are available.

We evaluated a wide range of *E. coli* strains and a total of 17 electrophilic compounds exhibiting different modes of action (Tab. 1). We measured the following effect parameters: growth inhibition, intracellular glutathione concentrations as well as the occurrence of DNA strand breakage and the activation of various DNA repair mechanisms [2].

Using Two Pairs of *E. coli* Strains as Biosensors

Two pairs of *E. coli* strains have proven to be especially successful for our test purposes. The strains MJF276 (glutathione$^+$) and MJF335 (glutathione$^-$) are genetically identical except for their ability to synthesize glutathione. The second pair is also genetically identical except for the ability to repair DNA damage: in MV4108 (DNA$^-$), several genes encoding for DNA repair systems are mutated, while these genes are intact in MV1161 (DNA$^+$).

Different concentrations of electrophilic compounds were added to liquid cultures of these strain pairs and growth inhibition

Structure		Damaged biomolecules
Epoxides		
		
Styrene oxide	R = phenyl	DNA
2,3-Epoxypropyl benzene	R = benzyl	DNA
2-(4-Nitrophenyl)-oxirane	R = p-nitrophenyl	DNA and proteins
1,2-Epoxybutane	R = C_2H_5	DNA
Epichlorohydrin	R = CH_2Cl	DNA and proteins
2-Methyl-2-vinyloxirane 		DNA and proteins
Reactive organochlorides		
		
Benzyl chloride	R = H	DNA and proteins
3-Methylbenzyl chloride	R = m-CH_3	DNA and proteins
4-Nitrobenzyl chloride	R = p-NO_2	DNA and proteins
2,3-Dichloro-1-propene 		DNA and proteins
trans-1,4-Dichloro-2-butene 		DNA and proteins
Compounds with activated double bonds		
		
Acrolein	R = H	Proteins
Ethyl acrylate	R = O-C_2H_5	Proteins
2-Hydroxyethyl acrylate	R = O-C_2H_4-OH	Proteins
Isobutyl acrylate	R = HO-*sec*-C_4H_9	Proteins
Acrylonitrile 		Proteins
Acrylamide 		Proteins

Tab. 1: The 17 pollutants examined in this study and their primary mode of action.

was measured. Reactive chemicals that primarily attack at the protein level caused differences in growth between the glutathione$^+$ and the glutathione$^-$ strain, while the growth of the DNA$^+$ and the DNA$^-$ strains was not affected (Fig. 3A). Six of the examined compounds, characterized by activated double bonds, fall into this group of chemicals (Tab. 1). In contrast, reactive chemicals that primarily target the DNA clearly cause growth differences in the DNA$^+$/DNA$^-$ strain pair, significantly inhibiting the growth of the DNA$^-$ strain (Fig. 3B). These compounds, which include three of the examined epoxides (Tab. 1), do not induce any growth differences between the glutathione$^+$ and the glutathione$^-$ strain. In addition to these two groups of chemicals, we identified a third group of compounds, characterized by non-specific reactivity since they attack at the protein as well as at the DNA level (Tab. 1). This group of compounds causes growth differences in both strain pairs (Fig. 3C).

Validation and Further Development of the Test System

The results from this study are not only useful in classifying modes of action for environmental pollutants, but they can also be used to describe the effects these pollutants have on aquatic organisms. This becomes evident when EC_{50} values for these compounds obtained in the *E. coli* studies are compared to the EC_{50} values obtained from experiments on aquatic organisms. There is a linear correlation between the different sets of EC_{50} values (Fig. 4). The EC_{50} value is the concentration of a pollutant where a 50% effect occurs – in our case growth inhibition for *E. coli* and algae as well as lethality for daphnids and fish.

The study described here is a first step in assessing reactive chemicals based on their primary mode of action. The goal of our further work is to find ways to incorporate the strain pairs used in this study into ecotoxicological test batteries and to expand the concept to other toxicity mechanisms. Differentiated ecotoxicological risk assessments will only become feasible, however, when we succeed in construction of the complete chain of "cause and effect" from the primary interaction at the molecular level to the observable effects on the population or ecosystem level.

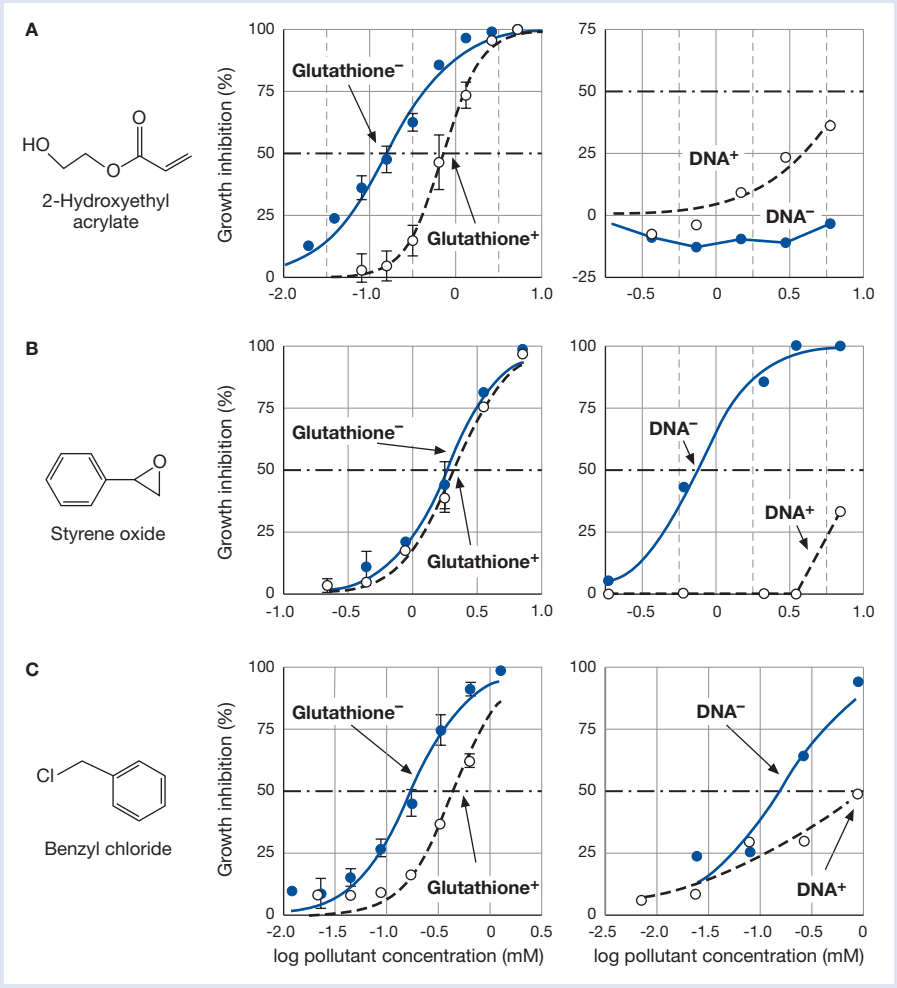


Fig. 3: Growth of the two *E. coli* strain pairs glutathione$^+$/glutathione$^-$ and DNA$^+$/DNA$^-$ in the presence of different pollutant concentrations.
A: 2-Hydroxyethyl acrylate as an example of a toxin causing protein damage.
B: Styrene oxide as an example of a toxin causing DNA damage.
C: Benzyl chloride as an example of a nonspecific reactive chemical, reacting with proteins and DNA.

Beate Escher, chemist and leader of the work group "Mode-of-action Based Risk Assessment of Chemicals" in the department "Environmental Microbiology and Molecular Ecotoxicology". Lecturer for environmental chemistry and ecotoxicology at ETH Zurich.
Research interests: Uptake and distribution of pollutants by organisms, mechanisms of toxicity, methods for hazard and risk assessment.

Coauthors: Angela Harder, Paolo Landini, Christian Niederer, Nicole Tobler

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[2] Harder A. (2002): Assessment of the risk potential of reactive chemicals with multiple modes of toxic action. Dissertation ETH Zurich, 78 p.

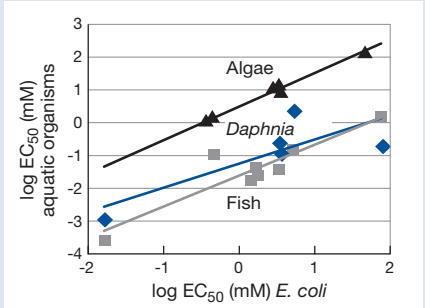


Fig. 4: Toxicity data (EC_{50} values) for pollutants examined in this study. Comparison between EC_{50} values for *E. coli* and EC_{50} values for algae, *Daphnia* and fish.

Bacterial Biosensors to Measure Arsenic in Potable Water

Worldwide, arsenic is one of the most important inorganic pollutants in drinking water. Particularly alarming is the situation in Bangladesh where more than one million people are already suffering from arsenic poisoning. In order to test each one of the roughly 9 million private drinking water wells, an inexpensive, reliable and sensitive field method is needed. For this reason, an EAWAG team has developed a new biosensor for arsenic. The paper strip test uses genetically modified bacteria that produce blue coloration even at low arsenic concentrations. EAWAG has applied for a patent for this sensor.

Inorganic arsenic is a common contaminant of potable water throughout the world [1–3]. Being usually of geochemical origin, arsenate and arsenite can occur in ground waters in concentrations up to 1 or 2 mg per liter. The safety limit for arsenic in drinking water in most countries is 10 µg or 50 µg per liter. Chronic exposure to arsenic, even at low concentrations around 50 µg per liter, leads to an increased risk for arsenosis and arsenic-mediated cancers. Therefore, it is important that arsenic-containing waters are not used as a drinking water source. Rather unfortunately, current regions in the world with the highest exposure to arsenic in potable water are those with the lowest scientific infrastructure, such as Bangladesh and Vietnam [1, 2]. Furthermore, the drinking water supply in both countries is organized very locally, with individual households each having their own tube well. Since it has now become clear that the accurate prediction of the contamination level of potable water in individual tube wells is very difficult because of strong local and seasonal variations in arsenite concentrations, accurate analyses of the water quality do form an important strategy in arsenic mitigation, as long as no effective treatment methods for arsenic removal are available.

Arsenic Measurements

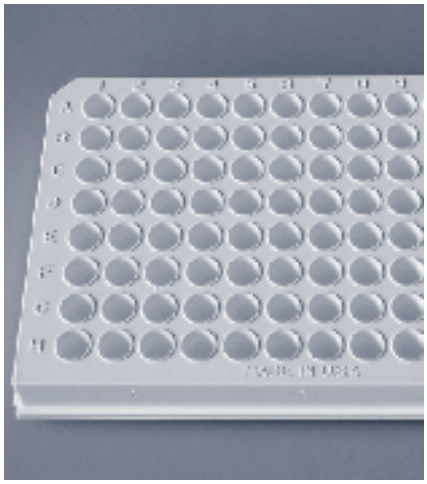
Traditionally, arsenic is measured with colorimetric tests, like the mercuric bromide stain method. However, this method, which is the basis of several commercial field test kits, has been shown to be insufficiently accurate at the level around 70 µg arsenic per liter and below and, moreover, gives rise to arsine gas and heavy metal (Zn, Hg, Sn) contamination. In contrast, arsenic measurement by atomic absorption spectrophotometry or atomic fluorescence spectroscopy is very accurate and reliable, but requires substantial financial investment. Therefore, an easy, accurate and inexpensive arsenic test system using genetically modified bacteria as biosensors has been developed at EAWAG. How was this made possible?

Exploit the Bacterial Defense Mechanisms Against Arsenic

Arsenic is not only toxic for humans and animals; arsenic toxicity is even found in simple organisms like bacteria. They have a few relatively effective biochemical mechanisms to deal with arsenic ions which have entered the cell (Fig. 1). Two well known bacterial proteins deal with the most common forms of arsenic: arsenite and arsenate. One protein is a pump which is integrated into the bacterial cell wall and removes any arsenite from the interior of the cell to the outside, where it can do no harm. The

Photographs: J.R. van der Meer, EAWAG

Bacterial cells of the light producing biosensor must always be kept on ice.



For the assay, the cells are incubated with different water samples in a 96-well plate.

The intesity of light emission as a measure of arsenic pollution is analysed by a luminometer.

other protein is called “arsenate reductase” and reduces arsenate to arsenite.

A further cellular protein is needed in order to elicit the defense reponse whenever arsenic is present in the cell’s interior. This protein, called ArsR, is an arsenite sensing protein. It has two binding capacities: In the absence of arsenite, it binds to a specific element on the DNA and thereby prevents the arsenic defense genes from becoming transcribed by RNA polymerase (Fig. 1). Repression, however, is not complete and small amounts of ArsR, the arsenate reductase and the arsenite pump are always present. When arsenite enters the cell, ArsR changes its habits; it will immediately bind to the arsenic compound and lose affinity for the DNA binding site with the result that the protein “falls off” the DNA. As a consequence, ArsR no longer represses the defense mechanism so that the arsenic pump, and the arsenate reductase are produced by the cell in larger amounts.

For the development of the arsenic biosensor, we took advantage of the biochemical capacities of ArsR. However, our interest was not to have the defense mechanism produced when the cell experiences arsenite, but some other protein or enzyme which can easily be measured. This is where gene technology comes into play. By genetic engineering, the bacterial cells can be altered in a way that they produce a light response, or a fluorescent signal, or a colorimetric reaction when they come into contact with arsenite [4]. Therefore, we linked together the gene coding for the ArsR protein, the DNA binding site for ArsR, and a reporter gene coding for a light producing enzyme, the luciferase (Fig. 1). After transfecting *Escherichia coli* bacteria with this DNA construction, the biosensor in principle was ready [5].

An Accurate Light Producing Liquid Biosensor

How to use the new bacterial biosensor? In their most simple form, the biosensor cells are precultured in a liquid medium until a cell density of about 2×10^8 cells per ml is reached. The bacteria are harvested and resuspended in a salt solution with glycerol, in which the cells can be aliquoted and frozen at –80 °C. Such aliquots stay viable for periods longer than 5 years. For an assay, the cells are thawed, diluted with fresh culture medium (to revive them) and mixed with the aqueous sample to be measured. At the same time, a standard calibration assay is performed with known arsenite concentrations between 0 and 0.5 µM (0 and 40 µg arsenic per liter). The volume of the assay mixture can be as low as 200 µl and many assays can be carried out simultaneously in 96-well plates. For the assay, the cells are incubated at 30 °C for a time period of at least 30 minutes. During this time, the bacterial luciferase is produced. After incubation, one drop of substrate for the bacterial luciferase (n-decanal) is added, mixed

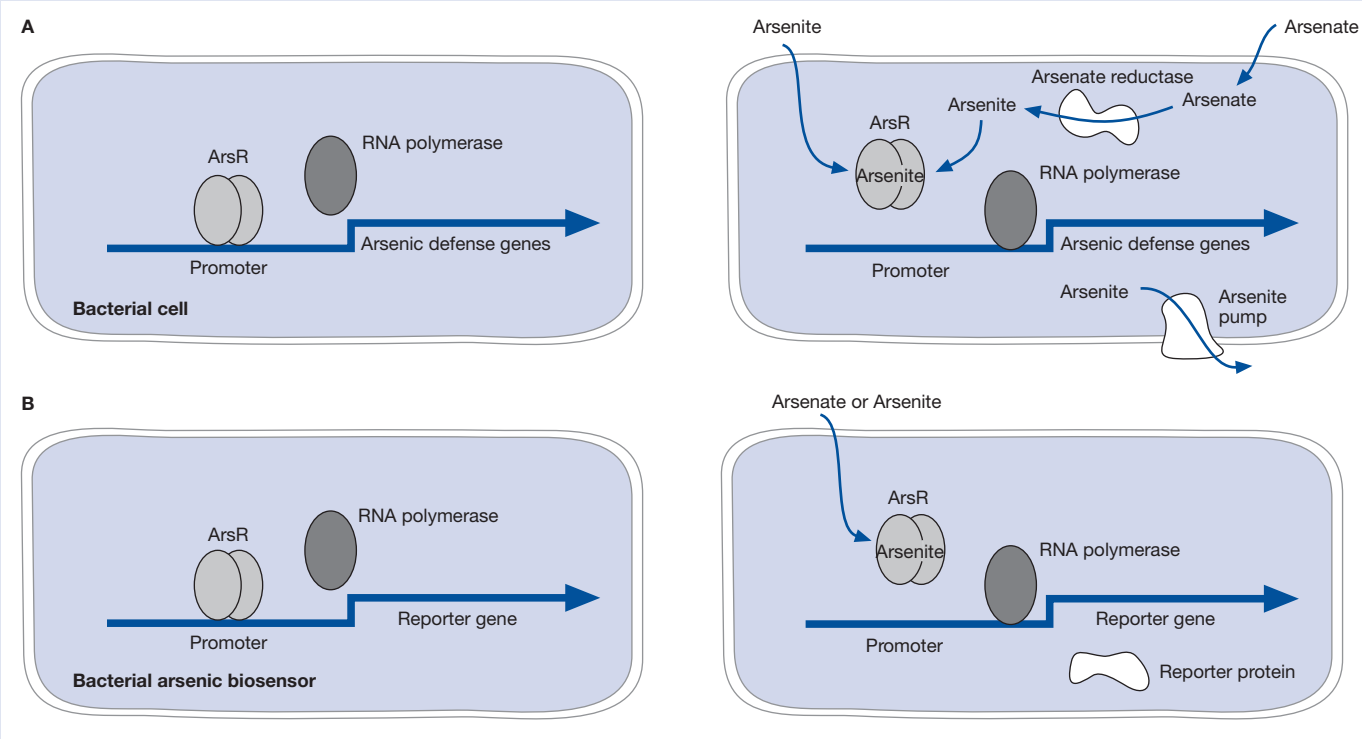


Fig. 1: Principle of the arsenic biosensors. A) The ArsR regulatory protein binds to a specific DNA element and thus inhibits the expression of the genes coding for the arsenic defense mechanism. When arsenite enters the cell, it binds to ArsR. ArsR dissociates from the DNA and RNA polymerase can access. The arsenic defense genes (the ArsR protein itself, the arsenite pump and the arsenite reductase) are expressed in high amounts. B) In the biosensor cells, the ArsR control DNA element is coupled to a reporter gene. Expression of the reporter gene is inhibited by ArsR, but when arsenite enters the cell, it again dissociates and the reporter gene product is synthesized. These products are either luciferase, green fluorescent protein or β-galactosidase.

and the light emission measured in a luminometer. The calibration curve with these biosensor cells is usually linear within the range of 0 to 0.5 µM arsenite (Fig. 2). At high concentrations or for unknown samples, different dilutions have to be made in order to perform accurate measurements.

A Simple Paper Strip Biosensor

For two main reasons, the use of the above described biosensor is not easy enough and remains restricted to the laboratory: a rather expensive luminometer has to be installed and the handling of liquid bacterial cultures is too critical in the field. We therefore tried to develop another biosensor system where the genetically modified bacterial cells are immobilized on small paper strips [5]. Instead of the luciferase reporter gene, this second system contains a gene for the enzyme β-galactosidase that produces a color reaction in the presence of arsenic. These biosensor cells are also grown in culture broth, but mixed after harvesting with a solution containing various sugars, amino acids and gelatine. Small amounts of this mixture are pipetted on paper strips (Fig. 3) and carefully dried at controlled temperature and partial vacuum. The cells on the paper strips remain active for about one month storage at temperatures between –20 and 30 °C. For an assay, a paper strip is placed in a vial with 1 ml of aqueous sample, incubated for 30 minutes at 30 °C and taken out. A drop of substrate for the β-galactosidase enzyme is added to the paper and – depending on the amount of β-galactosidase – converted into a blue product. The color intensity is a qualitative measure for the amount of arsenite which the cells have been exposed to. When compared to a standard solution with 10 µg or 50 µg arsenite per liter, one can judge if the arsenic level is above or below the drinking water limit (Fig. 3). Thus, the paper strip biosensor is less accurate and has a shorter storage life than the liquid biosensor but seems to be more appropriate for utilization in the field.

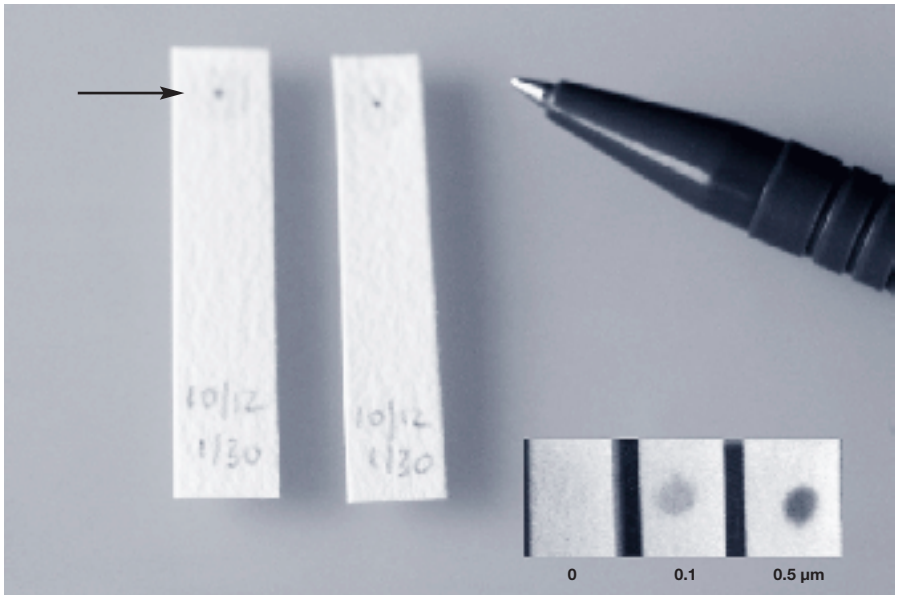


Fig. 3: The arsenic paper strip test: paper strips (4 x 0.5 cm) with spots of dried bacterial cells (arrow). After incubation with arsenite containing water samples, the cells produce β-galactosidase. The activity of this enzyme can be visualized by conversion of a substrate into a blue molecule (inset). Color intensity depends on the concentration of arsenite.

Unsolved Questions

So far laboratory practice. Many important questions and problems remain before we can think of using the biosensors routinely outside the laboratory. For example, how can the quality of the biosensor cells (i.e., their immediate activation potential) be guaranteed? How good are biosensor measurements when compared to chemical analyses? Does the chemical composition of the aqueous sample influence the biosensor response? Would local authorities in developing countries be sufficiently skilled to carry out the biosensor test reliably? What happens with the genetically modified biosensor bacteria after the test? Solutions to these questions may only be achieved by proceeding step-by-step. EAWAG has applied for a patent for the arsenic biosensor at the European patent office. At the moment, potential industrial partners are being traced who might be interested in licensing the arsenic biosensor technology and funding additional developments. Further improvement and testing of the biosensors will hopefully result in a realistic comparison of the biosensor test with chemical methods and its usefulness.

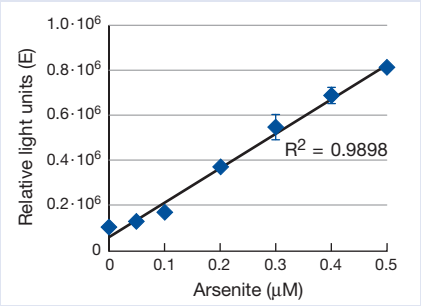


Fig. 2: Example of a calibration curve with the luciferase biosensor. Increasing amounts of arsenite (in a range of 0.05 to 0.5 µM) result in a linear increase in light production by the sensor cells.

Jan Roelof van der Meer, portrait see page 8.

Coauthor: Judith Stocker

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Defense Genes as Indicators of Toxicity

From Basic Research to Practical Application

As a result of exposure to certain pollutants, various toxic oxygen derivatives form in the cells of plants and animals, including the so-called "singlet oxygen". Fortunately, most cells have specific defense mechanisms against the toxic effects of these derivatives. Currently, research at EAWAG is examining in detail how the unicellular green alga *Chlamydomonas reinhardtii* reacts to the presence of singlet oxygen. The long-term goal of the work is to develop a biosensor for the detection of singlet oxygen, which would give us an indirect indicator of pollutant levels.

Since aerobic respiration provides the energy required for growth and metabolism in plants and animals, molecular oxygen (O_2) is required by all higher life forms. Oxygen may, however, be life-threatening; namely when reactive, strongly-oxidizing oxygen species are formed inside the cells (see box and Fig. 1) [1]. If the cell cannot successfully defend against this oxidative stress, vital components of the cell, such as lipids, proteins and DNA, can be damaged, and the cell will die. The formation of reactive oxygen species is enhanced when organisms are exposed to heavy metals or certain organic contaminants, such as herbicides or halogenated organic compounds. This toxic effect is enhanced by intense solar radiation. Most cells, however, have developed specific defense mechanisms against reactive oxygen species [2].

Molecular Defense Scenarios

Briefly, the molecular defense mechanism works as follows [3]: the cell possesses sensors that allow it to detect stress situations, such as the intracellular formation of reactive oxygen species. These sensors, in turn, activate so-called "stress genes" that are read by the transcription apparatus, leading to the production of gene copies (messenger RNA) in quantities proportional to the strength of the activating stress. Finally, the corresponding stress proteins are synthesized. These proteins have one of two functions: (1) they remove the source of the stress itself (i.e., they convert reactive oxygen species into non-toxic species), or (2) they repair cell components that have already been damaged. The cell produces general stress proteins that are formed regardless of the stress source, as well as specific stress proteins that are able to target and efficiently remove specific stress factors [4].

The genetic activation pattern may provide valuable clues about the nature and intensity of the stress within the cell. Expression (activation levels) of specific genes has already been used successfully to detect and quantify environmental pollutants, such as in the biosensor for arsenic that was developed at EAWAG (see article by J.R. van der Meer, p. 12). The goal of the project described here is to develop a biosensor for the detection of water contaminants that cause the formation of reactive oxygen species inside cells. Since very little is known about the defense mechanisms against singlet oxygen (see box and Fig. 1), we have decided to focus our work on this reactive species.

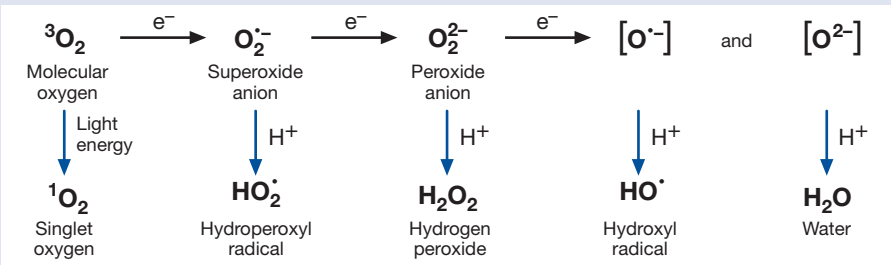


Fig. 1: Formation of reactive oxygen species from molecular oxygen by incomplete reduction or transfer of light energy. Oxygen species in parentheses are not stable and immediately transform to their protonated forms.

Reactive Oxygen Species

Due to its electron configuration, naturally occurring molecular oxygen is relatively inert and not harmful to living organisms. It can, however, be activated physically by energy transfer or chemically by electron transfer. Such activated oxygen molecules are called reactive oxygen species. They are highly reactive and can be formed in anaerobic organisms even under normal physiological conditions. Transfer of electrons, e.g., from the respiratory chain in the mitochondria, can lead to the formation of the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$) (Fig. 1) [1]. If light is the energy source causing the electron transfer, singlet oxygen is formed (Fig. 1). Singlet oxygen is chemically identical to molecular oxygen; only the electronic configuration has changed. It is, however more reactive, and reacts rapidly with cellular components forming organic hydroperoxides. Unsaturated fatty acids in cell membranes react particularly rapidly with singlet oxygen, which results in the formation of lipidperoxides and may cause damage to the membrane. It is, therefore, extremely important for all organisms to have a specific defense mechanism against singlet oxygen and the damage it causes.

Cellular Stress	Contaminant	Induction Factor
Hydrogen peroxide	Hydrogen peroxide (2 mM)	2.8
Superoxide anion	Menadione (5 µM)	6.4
Organic hydroperoxides	Tert-butylhydroperoxide (0,1 mM)	4.7
Singlet oxygen	Rose bengal in light (5 µM)	78.9
Rose bengal (control)	Rose bengal in the dark (5 µM)	3.1
Photosynthetic inhibition	DBMIB (herbicide) (1 µM)	9.5
Heat shock	25 °C → 40 °C shift	1.3
Salt/osmotic stress	NaCl (200 mM)	1.5

Tab. 1: Activation of the *Gpxh* gene in *Chlamydomonas* under various stress conditions. *Chlamydomonas* cultures were exposed to various stress factors for 60 minutes. Induction factor = *Gpxh* expression in stressed culture divided by *Gpxh* expression in control cultures. *Gpxh* expression was measured as the amount of mRNA produced by the transcription process.

DNA Chips Yield First Clues

Photosynthetic organisms are particularly affected by oxidative stress [2], since the photosynthetic apparatus itself is an important source of reactive oxygen species. For this reason, we have selected a photosynthetic model organism for our studies: the unicellular, flagellated green alga *Chlamydomonas reinhardtii.* This organism has the same cellular structure as higher algae and plants, but is easier to cultivate and very suitable for the use of molecular techniques [5]. A particular advantage is the fact that DNA chips with the genetic information for *C. reinhardtii* have very recently become available. These devices allow us to examine expression patterns for the majority of the genes of a given organism [3, 6]. In our case, this will enable us to detect specific defense genes that are as yet unknown.

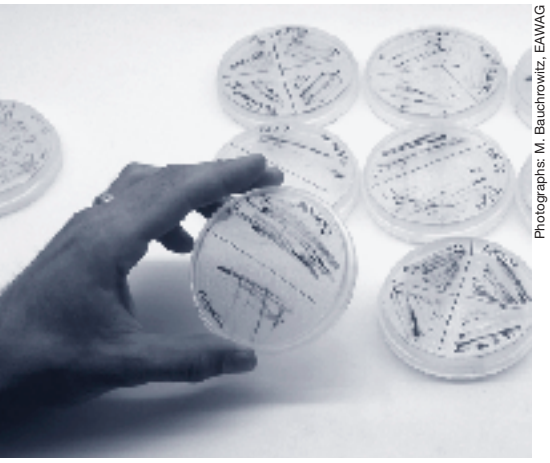
The DNA chips we are using contain a total of 2792 genes of *C. reinhardtii.* In order to differentiate between defense genes specifically responding to singlet oxygen and nonspecific genes responding to oxidative stress, the DNA chip experiments were carried out either with singlet oxygen or hydrogen peroxide (H_2O_2), another commonly occurring reactive oxygen species. A comparison of the two expression patterns indicated distinct differences. There were several genes that were activated by singlet oxygen, but not by H_2O_2 (see image of DNA chip on the front cover page; each point represents a *Chlamydomonas* gene; induced genes are red, suppressed genes are green, and genes with unaltered expression are yellow).

Specific Induction by Singlet Oxygen

The genes that were most strongly and specifically induced by the presence of singlet oxygen were examined further for their possible expression and function in other stress situations. One gene was found to be particularly interesting. The gene in question is a homologue of glutathione-peroxidase *(Gpxh)*. Related gene products in other organisms are responsible for the degradation of organic hydroperoxides [7]. Although the *Gpxh* gene is also induced by other oxygen species, expression is strongest when exposed to singlet oxygen (Tab. 1) [8], indicating that in the case of *Chlamydomonas,* singlet oxygen is detected by a specific sensor which then activates specific defense genes against it.

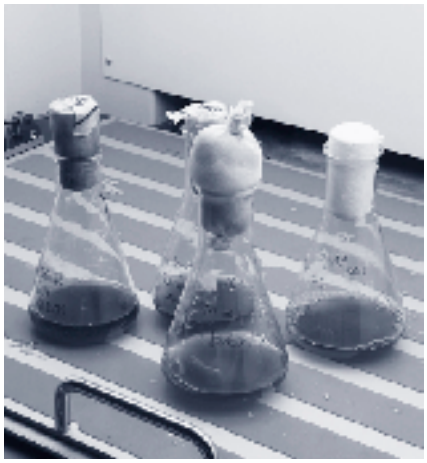
The *Gpxh* Promoter

In order to gain a better understanding of the induction mechanism of the *Gpxh* gene, we are currently examining the promoter region of the gene in more detail. A promoter region is a section of DNA in front of the actual coding sequence that controls the expression of that particular gene. It contains special regulatory elements that allow the binding of specific transcription factors which would lead to an activation of the gene. These regulatory elements have a specific base sequence that is recognized only by the associated transcription factor (Fig. 2). Through comparison with other genes, we have been able to identify such a regulatory element in the promoter region of the *Gpxh* gene. We think that this element plays an important role in the induction by singlet oxygen, since our experiments showed that after removal of the *Gpxh* regulatory element from the promoter region, the gene was no longer activated by singlet oxygen [8]. In a next step, the *Gpxh* regulatory element was introduced into the pro-

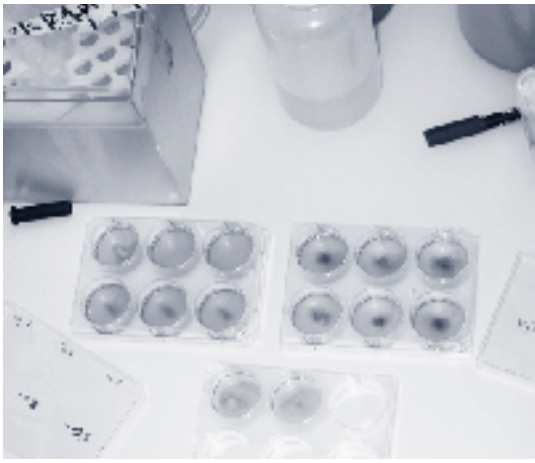


Photographs: M. Bauchrowitz, EAWAG

Growth of Chlamydomonas on solid medium in Petri dishes …



… or in liquid medium unter constant temperature and light conditions.



Induction experiments with algal cells will be carried out in multiwell cell culture plates.

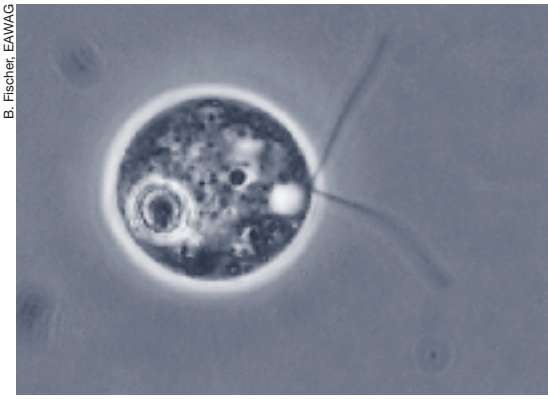
moter region of a gene that is not normally induced by singlet oxygen. We used the β-tubulin gene of *Chlamydomonas,* a gene that encodes the structural protein tubulin which occurs in the flagella of a number of microalgae, including *Chlamydomonas.* Results confirmed that the transgenic β-tubulin gene becomes weakly induced by singlet oxygen after the *Gpxh* regulatory element has been inserted into the promoter region of the gene. So far, these experiments suggest that the *Gpxh* regulatory element plays a significant role in gene activation by singlet oxygen. The regulatory element found in *Chlamydomonas* has two closely related and well described homologues in mammals. Despite intensive efforts, it has not yet been correlated with one of these two mammalian regulatory elements. It is possible that the sequence was not completely conserved between mammals and algae or that the *Gpxh* regulation element in *Chlamydomonas* may be a new, as yet undescribed, regulatory element. Once the corresponding transcription factor has been isolated and characterized, it should be possible to answer these questions.

Additional research is also needed in order to determine whether or not there is a specific mechanism for the induction of *Gpxh* by singlet oxygen in *Chlamydomonas.* If this were the case, the *Gpxh* regulatory element could be used as an element in a molecular contamination sensor. Such a biosensor would allow us to detect contaminants that cause the formation of singlet oxygen in cells.

Beat Fischer, molecular biologist and doctoral student in the department "Environmental Microbiology and Molecular Ecotoxicology".

Coauthor: Rik Eggen

B. Fischer, EAWAG



The biflagellated alga *Chlamydomonas reinhardtii* under the microscope (100-times magnified).

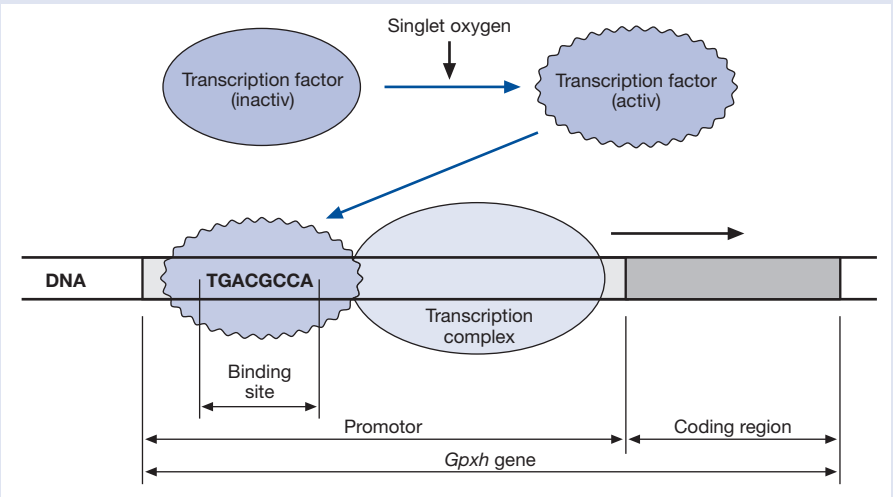


Fig. 2: Hypothetical activation path of the *Gpxh* gene by singlet oxygen. In the presence of singlet oxygen in the cell, the inactive form of the *Gpxh* transcription factor changes to the active form and binds to the *Gpxh* regulation element. Only then can the transcription complex bind to the *Gpxh* gene and read it.

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New Paths in the Analysis of Drinking Water Quality

The Tedious Search for a Fast Alternative Method

Drinking water is routinely tested for the presence of bacteria by culturing methods which have the disadvantage of being very time consuming. It takes at least one day before results are available. For this reason, EAWAG is currently developing a faster method. The application of new molecular techniques is promising, although the development of an actual method is proving to be rather difficult.

The hygienic quality of drinking water has been routinely tested for many decades now by determining several bacterial parameters. These tests typically screen for so-called indicator organisms, such as the intestinal bacterium *Escherichia coli* [1]. The assumption is that these harmless bacteria are secreted along with potential pathogens and could be introduced into drinking water supplies. A simple and inexpensive culturing method for the detection of *E. coli* has been used for many decades. However, this method is rather time consuming: it takes at least 24 hours before results are available. There are situations in which the water supplier would like to be able to determine more rapidly whether or not the water delivered to customers is hygienically acceptable. It is possible, for example, that after several days of intense precipitation, one or more of the sources of drinking water receives poorly filtered water. In a worst-case scenario, this could lead to fecal matter-contaminated drinking water. Were this the case, the drinking water supplier would have to immediately take measures to decontaminate the drinking water supply (disinfection, flushing) and would also have to advise the population to boil water before use. When it takes 24 hours before test results are available, it may be too late for the appropriate response. For this reason, EAWAG is developing a faster detection method that is based on newer molecular methods of analysis.

The *E. coli* Reference Method

Drinking water is considered a "food" and is, therefore, subject to the Swiss Ordinance on Hygiene [2]. The ordinance specifies that drinking water quality must be assessed using the reference methods defined in the Swiss Food Manual [3]. The use of other methods is permissible if "they were proven to lead to the same interpretation as the reference method" [2]. The reference method for the detection of *E. coli* in drinking water is the culturing method (Fig. 1, top). The Ordinance on Hygiene sets the tolerance for *E. coli* at "not detectable in 100 ml water sample". Therefore for the culturing method, 100 ml of water are passed through a filter, on which *E. coli* and other organisms are retained. The filter is then laid on an agar plate where individual bacterial cells grow to colonies that can be enumerated. Whether a particular colony is, in fact, a colony of *E. coli*, is confirmed by an enzymatic reaction whereby *E. coli* colonies turn blue in color.

The Newly Developed Alternative Method

In the search for faster alternative methods, techniques from molecular biology have received much interest. In our opinion, the use of the "Polymerase Chain Reaction" (PCR) is particularly promising (Fig. 1, bottom). In this method, short DNA segments charac-

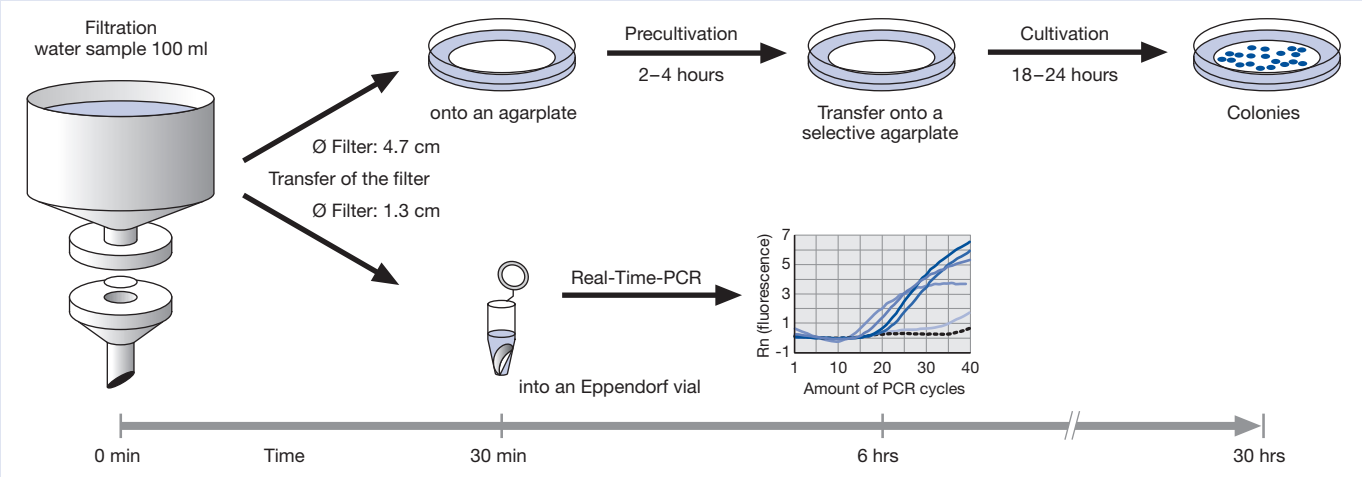

Fig. 1: Individual steps and time requirements for the culturing method (top) and the newly developed PCR method (bottom).

teristic for *E. coli* are replicated in a temperature cycling process. A fluorescent dye which stains double stranded DNA makes the synthesized DNA fragments visible. In the "real-time PCR method" that we use, fluorescence is measured in real time and recorded by a computer. The result is the number of thermal cycles required to reach a detectable fluorescence signal. With respect to a particular water sample, this means that the more *E. coli* cells (i.e., the more *E. coli* DNA) were initially present in the sample, the fewer thermal cycles are needed before the fluorescence reaches the detection level. A crucial element of our PCR method is the pretreatment of the sample with the enzyme DNAse, which removes any free DNA that might be present due to dead bacteria, thus preventing false positive results.

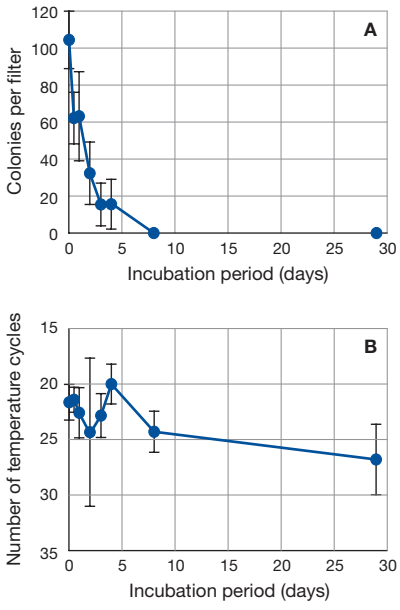

Fig. 2: Results of *E. coli* determinations by the culturing method (A) and the PCR method (B) in artificially contaminated drinking water.
All tests included determinations in non-contaminated water; culturing method: 0 colonies; PCR method: detection level of fluorescence not reached after 40 temperature cycles.

The Difference becomes Apparent

In order to test the agreement between the new PCR method and the traditional culturing method, we inoculated drinking water in the laboratory with *E. coli* cells and stored the water at 4 °C. Over the period of one month, we withdrew samples and analyzed them using the two methods. At the beginning of the experiment (t = 0), both methods detected bacteria (Fig. 2A + B). After that, the number of bacteria detected by the culturing method decreased rapidly. We expected therefore that the number of temperature cycles observed in our PCR method would increase. However, this was not the case. Even after one month, the PCR method still detected the presence of *E. coli*. Apparently, the cells were no longer able to grow on the culture media plates.

Cells that can no longer be cultured but can still be detected by other methods are often called "viable but not culturable" (VBNC). There is a controversy among microbiologists as to whether the detection of such VBNC stages is relevant in the case of the *E. coli* indicator organism or pathogenic organisms. Some studies suggest that such cells can be reactivated or that they can become infectious, respectively [4]; other reports indicate the opposite [5, 6]. Based on our results, we suggest that the cells die continuously, and that the rate at which the cells die depends on the "preconditioning" of the *E. coli* culture.

A Proven Method is Difficult to Replace

This simple experiment shows that the two methods do not lead to the same interpretation and that the PCR method used here cannot replace the culturing method. The principles on which the two methods are based, i.e., the ability to replicate in the culturing method and the detection of specific DNA fragments in the PCR method, must be too different from one another. This case illustrates how strongly the thresholds set for microbial parameters depend on the analytical methods employed.

There have been proposals in the literature to perform a short culturing period of a few hours before using the PCR method, which does improve the agreement between the PCR method and the reference method [7]. In general, there are discussions ongoing and proposals for standards published or in progress in order to normalize the validation of alternative methods in the future [8]. The PCR method presented here could probably be established as a "new" method. This would require, however, that we are able to determine what specific physiological stages of *E. coli* are being detected. For the time being, our new PCR method appears to be applicable rather to research than to the routine monitoring of drinking water.

Acknowledgements
We would like to thank the Bachema Institute in Schlieren for initiating and supporting this research project.

Annette Rust developed the method presented here as part of her doctoral research in the department "Environmental Microbiology and Molecular Ecotoxicology".

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The Anammox Process for Nitrogen Removal from Waste Water

The Fruitful Collaboration Between Microbiologists and Process Engineers

As a country bordering the Rhine, Switzerland shares in the responsibility to reduce nitrogen export to the North Sea. In wastewater treatment plants, this is currently accomplished through a costly expansion of the main activated sludge system. During the 1990s, however, there were several reports of nitrogen being eliminated rather unexpectedly under certain operating conditions. It was found that this is due to a recently discovered group of bacteria, which were also found in Swiss wastewater treatment plants. Based on this discovery, process engineers have developed a new process that reliably removes nitrogen.

Nitrogen elimination is commonly regarded as the conversion of biologically available nitrogen compounds, such as ammonium (NH_4^+), nitrite (NO_2^-), or nitrate (NO_3^-), to elemental nitrogen (N_2), which is released into the atmosphere as a harmless product. Today, wastewater treatment plants almost always use a biological nitrification/denitrification process for the elimination of nitrogen (see box). Swiss wastewater treatment plants often omit the denitrification step, which means that the discharge water still contains relatively high nitrogen loads, mostly in the form of nitrate. As a country that borders the Rhine, Switzerland has committed itself in the Water Protection Act of 1998 to reduce nitrogen input to the Rhine by 2000 tons by the year 2005. Solutions must, therefore, be found in the very near future. The upgrading of all Swiss wastewater treatment plants with an adequate nitrification/denitrification stage will be expensive. In addition, the operation of this process is energy and resource intensive [1]. Therefore, a new, low-impact process is needed.

Nitrification

= Aerobic oxidation of ammonia by nitrifying bacteria.
Ammonium (NH_4^+) is oxidized by oxygen (O_2) via the intermediate nitrite (NO_2^-) to nitrate (NO_3^-):

$$NH_4^+ + 1.5\ O_2 \Rightarrow NO_2^- + H_2O + 2\ H^+$$
$$NO_2^- + 0.5\ O_2 \Rightarrow NO_3^-$$

Denitrification

= Nitrate respiration by denitrifying bacteria.
Nitrate is reduced under anaerobic conditions to elemental nitrogen (N_2) with the addition of organic carbon (e.g., methanol):

$$NO_3^- + \text{org. carbon} \Rightarrow N_2 + 2\ CO_2$$

Anammox

= Anaerobic oxidation of ammonium by anammox bacteria.
Ammonium is oxidized to elemental nitrogen by nitrite under anaerobic conditions:

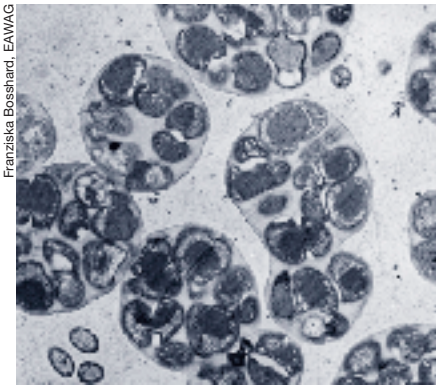
$$NH_4^+ + NO_2^- \Rightarrow N_2 + 2\ H_2O$$

Tracking Down Unknown Microorganisms

In the 1980s and 1990s, there were several indications that nitrification/denitrification are not the only processes that can remove ammonium, but that there is an organism that can oxidize ammonium to nitrogen gas using nitrite instead of oxygen as its terminal electron acceptor. Dutch and German scientists first identified these organisms. They belong to the order of Planctomycetales: *Brocadia anammoxidans* and *Kuenenia stuttgartiensis* [2, 3]. The phenomenon of anaerobic ammonium oxidation was also observed in the Swiss wastewater treatment plant of Kölliken. Our investigations revealed that a multilayered system of biofilms had formed in this treatment plant. Such biofilms create pronounced oxygen gradients, where the upper-most layer can be oxygen-rich, while the layer closest to the carrier material can be anaerobic [4]. We assumed that the microorganisms we were looking for were located in the lowest layers of the biofilms. Using specific gene probes and the FISH technique (fluorescence *in situ* hybridization) [5], we were able to confirm the presence of a large population of bacteria belonging to the order Planctomycetes (Fig. 1). Until now, it was not possible to isolate a pure culture of these new bacteria using classic methods of microbiology. We were able, however, to enrich a sample of the biofilm, where approximately 90 % of the bacteria were Planctomycetes [6]. The organism present in the Kölliken wastewater treatment plant is *Kuenenia stuttgartiensis.* Using techniques of molecular biology and other physiological experiments, we could show conclusively that *K. stuttgartiensis* oxidizes ammonia to nitrogen under anaerobic conditions [3, 6]; this process is, therefore, called “anaerobic ammonium oxidation” (anammox).

Anaerobic Ammonium Oxidation – a Sustainable Process for Nitrogen Removal

Process engineers were able to build on this knowledge. Wastewater treatment plants with anaerobic sludge digestion produce a particularly ammonium-rich effluent, which is nowadays recombined with the influent to the treatment plant. Consequently the nitrogen load to be treated is increased by approximately 15–20 %. Instead of “recy-



Franziska Bosshard, EAWAG

Fig. 1: Anammox bacteria form dense, globular clusters. Individual bacteria are visible as small, open circles within the clusters. The cell clusters have a diameter of approximately 15 µm. Magnification: 1000x.

cling" this effluent, it could be treated separately using the new anammox process, requiring minimal input of energy and other resources. What is required, however, is nitrite, which is not present in the effluent but is formed as an intermediate product in the nitrification process (see box). The following two-step process (Fig. 2) is a possible solution: in a first, aerated reactor, ammonium is partially oxidized to nitrite *(partial nitritation),* which is then reduced to elemental nitrogen in a second oxygen-free reactor using the remaining ammonium *(anammox).* The overall process is, therefore, called "partial nitritation/anammox", or simply the anammox process. So far, it can only be used for effluents rich in ammonium. Thanks to genetic methods, anammox bacteria can be detected easily and rapidly, which is important during the startup phase and in determining process failure. Compared to traditional nitrification/denitrification, the anammox process has several advantages (Fig. 3):

- Oxygen additions can be reduced by 60 %. This also reduces the energy required to introduce the oxygen.
- Anammox bacteria do not require organic carbon. By contrast, organic carbon, such as methanol, has to be added in the nitrification/denitrification process.
- Anammox bacteria produce very little biomass, reducing the amount of sludge that has to be disposed of.

The new anammox process uses not only less energy and fewer resources, but it is also less expensive than conventional nitrification/denitrification.

Testing the Anammox Process

In order to test the feasibility of this two-stage process in practice, EAWAG has built and operated a pilot plant (4 m^3) in cooperation with the wastewater treatment plant Werdhölzli (Zurich) and other partners [7]. This pilot project has yielded new insights into the process and has confirmed its applicability. In addition, the pilot plant has been used to develop guidelines for the scale-up and operation of full-scale plants. Unfortunately, the new process is still met with caution and hesitation, mostly because of the slow growth rates of the anammox bacteria and the lack of experience in operating the process. Due to the many advantages of the process, however, we anticipate the construction of the first large-scale anammox reactors over the next few years.

Christian Fux, process engineer, completed his doctoral research on the anammox process in the department "Environmental Engineering" at EAWAG. He is currently a postdoctoral researcher at the "Advanced Wastewater Management Centre", University of Queensland, Australia.

Coauthors: Konrad Egli, Jan Roelof van der Meer, Hansruedi Siegrist

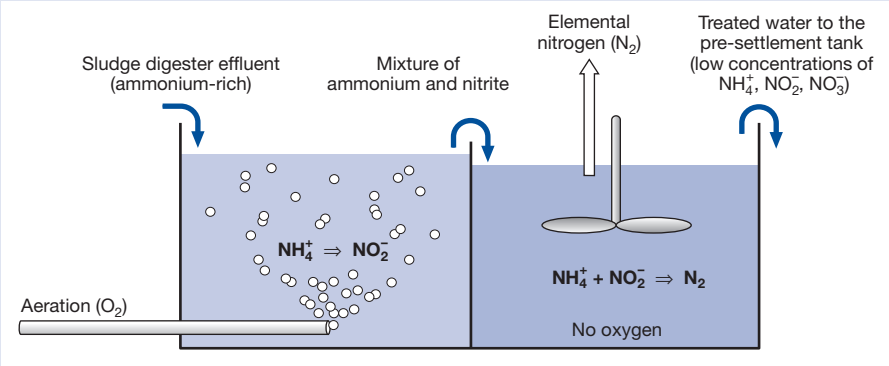


Fig. 2: The anammox process.

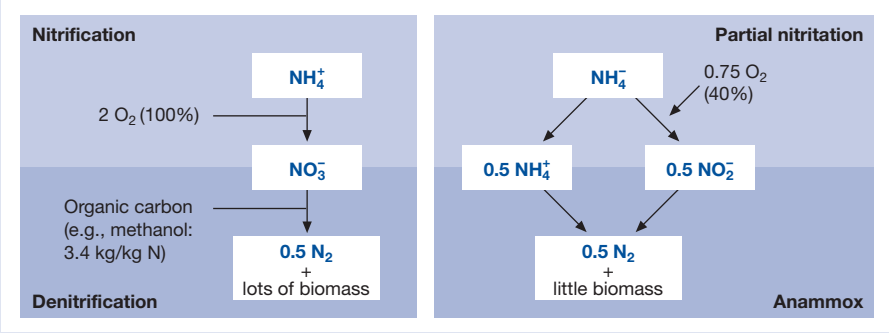


Fig. 3: Comparison between the anammox process and conventional nitrogen removal by the nitrification/denitrification process.

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Genetic Diversity of *Daphnia* in Alpine Lakes

The diversity of zoo- and phytoplankton in alpine lakes has been studied intensively for over 100 years. It is now generally accepted that the species diversity of plankton communities decreases with increasing altitude. This study addresses the question as to whether or not this general pattern also applies at the population level. *Daphnia*, a typical member of zooplankton communities, was chosen as the study organism. Genetic diversity was determined for 11 *Daphnia* populations in mountain lakes at varying elevations. We found that the genetic diversity was very heterogeneous.

The extreme environmental conditions in alpine regions result in a small selection of flora and fauna that can adapt to survival in these regions; therefore, biodiversity (or more precisely species diversity), decreases with increasing altitude. It is unclear, however, whether or not this is also reflected in another aspect of biodiversity; namely, genetic diversity. In order to answer this question, we examined *Daphnia* populations, a typical member of zooplankton communities, in a number of Swiss mountain lakes. These organisms are particularly interesting for such a study since they can reproduce sexually or asexually by parthenogenesis [1].

Parthenogenesis and Resting Eggs

Under favorable environmental conditions, parthenogenesis is the normal reproductive method in *Daphnia*; mother organisms form genetically identical daughters (so-called clones). This allows *Daphnia* to multiply extremely rapidly, thereby making optimal use of short growth periods in alpine ecosystems. When environmental conditions worsen, e.g. during cold periods or when food is sparse, *Daphnia* produce males and sexual females. Sexually fertilized eggs are encased in a resistant shell, the so-called ephippium, which resides in the breeding cavity and is sloughed with the next shedding of the skin. Ephippia can survive for years in the lake sediments. Under favorable conditions, these resting eggs can produce parthenogenetic females, and the cycle begins again. If the conditions are favorable all year, no resting eggs are formed, so that one parthenogenetic generation follows another.

Environment, Reproduction and Genetic Diversity

Our project investigated the relationship between environmental conditions, reproduction method, and genetic diversity in alpine lakes. We examined 11 mountain lakes in the Swiss Alps. The lakes varied in elevation, where environmental conditions become harsher as elevation increases. *Daphnia* populations in these lakes were sampled in four consecutive years (1997–2000) during late summer or early fall.

Our experiments were based on the following hypotheses:

- Alpine lakes should harbor a high proportion of sexual individuals, since *Daphnia* can survive cold and food-deprived winters only in the form of resting eggs.
- In *Daphnia*, genetic diversity depends primarily on the sexual exchange of genetic material. We postulated that populations in lakes, where *Daphnia* reproduce mostly sexually, would exhibit higher genetic diversity.
- Since environmental conditions in alpine lakes become harsher with increasing elevation, we expected that the genetic diversity in *Daphnia* populations would increase with increasing altitude and not decrease as is the case for species diversity. Alpine lakes should, therefore, show higher genetic diversity than lakes in the lowlands.

Sexual Individuals in the Majority

The relative fraction of asexual and sexual *Daphnia* in our study lakes varied dramatically and ranged from 2 to 90% (Fig. 1 lower panel). In 8 of the 11 mountain lakes examined, sexual *Daphnia* outnumbered asexual individuals. In the two lowest alpine lakes, the Upper and the Lower Arosasee, we found almost exclusively asexual females, while the highest lake, Riffelsee II, contained almost exclusively sexual individuals. The distribution between sexual and asexual *Daphnia* for lakes at intermediate elevations, however, was heterogeneous; we could not decipher any clear patterns.

There was, however, one surprising finding: while males often made up less than 1% of the *Daphnia* populations in lowland lakes, they made up as much as 30–40% of the

The Genetic Examination Method

Allozyme electrophoresis is a proven method for the determination of genetic diversity of populations [2]. It is based on the following principle: the genome of an organism may contain the genetic information for a certain enzyme once or several times. If the gene is present several times, it is likely that the individual genes have changed over time, usually due to mutations. As a result, the cell will synthesize different enzymes, so-called allozymes, which have slight differences in their amino acid sequences, but will exhibit the same functionality as the original enzyme. In the simplest case, there are two allozymes which differ by only one amino acid. If the new amino acid carries a different charge, the two allozymes will have different migration velocities in an electric field and can be separated by gel electrophoresis. Subsequently, the enzyme can be detected through the reaction with its substrate. If the substrate reaction is coupled with a color reaction, the enzyme can be made directly visible. This method indicates how many enzyme variations are present within a population and, therefore, provides an index of genetic diversity.

populations in the mountain lakes. In addition, the fraction of sexual male individuals in alpine lakes was always higher than that of sexual females.

Genetic Diversity

In order to assess the genetic diversity of different *Daphnia* populations, we examined up to 80 individuals per lake by the allozyme method (see box). The clonal diversity, i.e., the number of different clones within a population, is a first indication of overall diversity. In the 11 mountain lakes examined in this study, we found between 2 and 42 different clones (Fig. 1 upper panel). Melchsee and Lago Cadagno showed the highest clonal diversity.

A better description of genetic diversity is obtained when the number of individuals for each of the clones is taken into account in addition to the number of different clones. A population with 10 different clones, each making up 10% of the population, is more diverse than a population with 10 different clones, but one single clone accounting for 99% of all the individuals. Therefore, we calculated the Simpson index ($D_{Sim} = 1-\sum p_i^2$, p_i = fraction of a clone i in the overall population) for each of the 11 populations that were examined (Fig. 1 upper panel). This index describes the probability that two randomly chosen individuals have a different genetic structure. High D_{Sim} values (near 1) indicate that all clones occur with similar frequency, which translates to high diversity. Low D_{Sim} values (near 0) show that one clone dominates the population, resulting in low diversity. It was found that the diversity of *Daphnia* populations was relatively high in a number of lakes under examination, whereas it was low in the Upper Arosasee, Leisee and Riffelsee II.

The Spatial Genetic Structure of *Daphnia* Populations is Complex

The high proportion of sexual individuals in alpine lakes was surprising and is an indication that *Daphnia* survive the harsh winters only in the form of resting eggs; however, the fraction of sexual individuals did not correlate with genetic diversity, neither if expressed as clonal diversity nor in the Simpson index. Furthermore, we were not able to establish a correlation between the genetic diversity of *Daphnia* populations and the elevation of the alpine lakes examined in this study. We compared our data to the genetic diversity of *Daphnia* populations in two lowland lakes, namely Greifensee ($D_{Sim} = 0.96$) and Lake Lucerne ($D_{Sim} = 0.48$). We found that the genetic diversity in mountain lakes can be as low as that of Lake Lucerne, or almost as high as that of Greifensee.

This study [3] represents a first step in examining the genetic diversity of a major zooplankton species in alpine lakes and in determining those factors that influence genetic diversity. Apparently, sexual reproduction does not necessarily lead to higher genetic diversity in *Daphnia* populations. It can, therefore, be assumed that other factors, such as the presence of certain *Daphnia* species or *Daphnia* hybrids, have a stronger influence or that only certain clones are adapted to the extreme environmental conditions in alpine lakes. High genetic diversity does not appear to be a prerequisite for survival of *Daphnia* in alpine lakes. Other strategies, such as their flexible behavior and life cycle, may be more important in coping with the harsh conditions in the alpine environment.

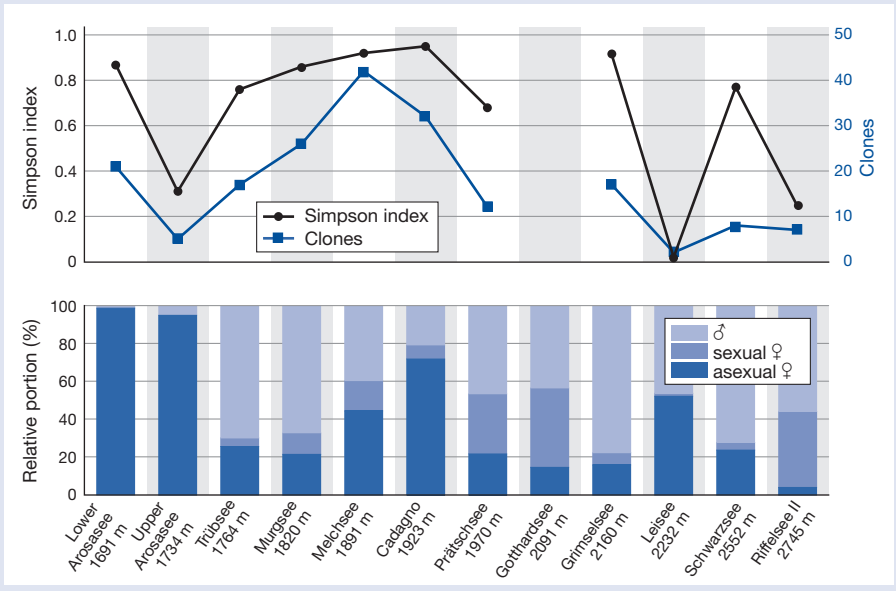

Fig. 1: Investigation of *Daphnia* populations in 11 Swiss alpine lakes at varying elevations. Lower panel: relative proportions of sexual and asexual individuals; upper panel: genetic diversity, expressed as number of clones and by the Simpson index.

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Conclusion of Round Table "Water"

In January 2003, the pilot project "Round Table – Science et Cité" on the topic water came to a close, with a final event at the EAWAG and the publication of the final report. For three years, citizens and researchers from EAWAG have taken part in dialogues, have found ways of mutual understanding and formulated several deductive principles.

In the panel discussion on the question "Is the dialogue between society and science necessary?", they discussed with Charles Kleiber, State Secretary for Science and Research. As president, he represented the interests of the foundation Science et Cité. EAWAG-director Alexander Zehnder, member of the Round Table, and Cité-representant Alfred Meier-Jucker reported on their experiences with the dialogue. Christine Burgener, community president of Thalwil ZH, explained why politicians are interested in such platforms.

The final report contains several principles showing how similar platforms for a dialogue between society and science can be designed in a constructive way. It is very important to choose topics which are closely related both to the scientific work at the research institution and the problems and interests of the society. Furthermore, such a dialogue requires time, allowing the vital climate of mutual tolerance and understanding to develop.

At the EAWAG first steps towards translating the dialogue into action have already been taken: The citizens of the "Round Table" took part in the EAWAG budget planning for the period 2004–2007.

The final report (in german only) can be downloaded from: www.eawag.ch/news/science_et_cite/schlussbericht.pdf

Students in Practice: Case Study Thur

Modern environmental research cannot be learned in a classroom. This is why students of the environmental sciences at the ETH Zurich worked on a real environmental problem between October 2002 and February 2003, exercising interdisciplinary teamwork. Subject of this case study was the Thur which had been forced into a tight corset during 140 years of regulations and is now being revitalised in an extensive programme started in September 2001. Experiences made there are not only valuable as landmarks for major projects in the future but also for the specialists to-be.

Forming six teams, the students analysed – together with scientists from the ETH Zurich and the EAWAG – a diversity of aspects connected with the revitalisation of the Thur and, for example, thought about how to evaluate the success of revitalisation projects, how landowners involved can be convinced of the benefits of the revitalisation projects, how to align ecology and flood protection, and whether the climatic change affects the flood situation along the Thur more than a change of land use does.

The results gained in the teams were presented to the public at a river inspection in February. They are supposed to be included in the revitalisation project, which is why the authority for water supply in Thurgau is now examining the "Case Study Thur" in detail.

Further information: www.eawag.ch/thur

First Graduates from PEAK-Course "Ecotoxicology"

The aim of ecotoxicology is to evaluate toxic effects of chemicals and prevent them wherever it is possible. Specialists in industry, administration and science are responsible for the careful handling of these substances. They need good basic knowledge of ecotoxicology and have to know the latest state-of-the-art. The ecotoxicology course "coetox" (collaboration en écotoxicologie) – initiated by the EAWAG and the EPF Lausanne in 1994 – serves the education and further training of these specialists and intends to promote the exchange of experience. In June 2003, the first graduates were able to receive their certificates. Presenting their seminar papers was the final step of their comprehensive training.

The whole course takes three years and contains five modules presented in German or French. There is a new coetox-course starting in March 2004.

Further information: www.eawag.ch/events/peak