

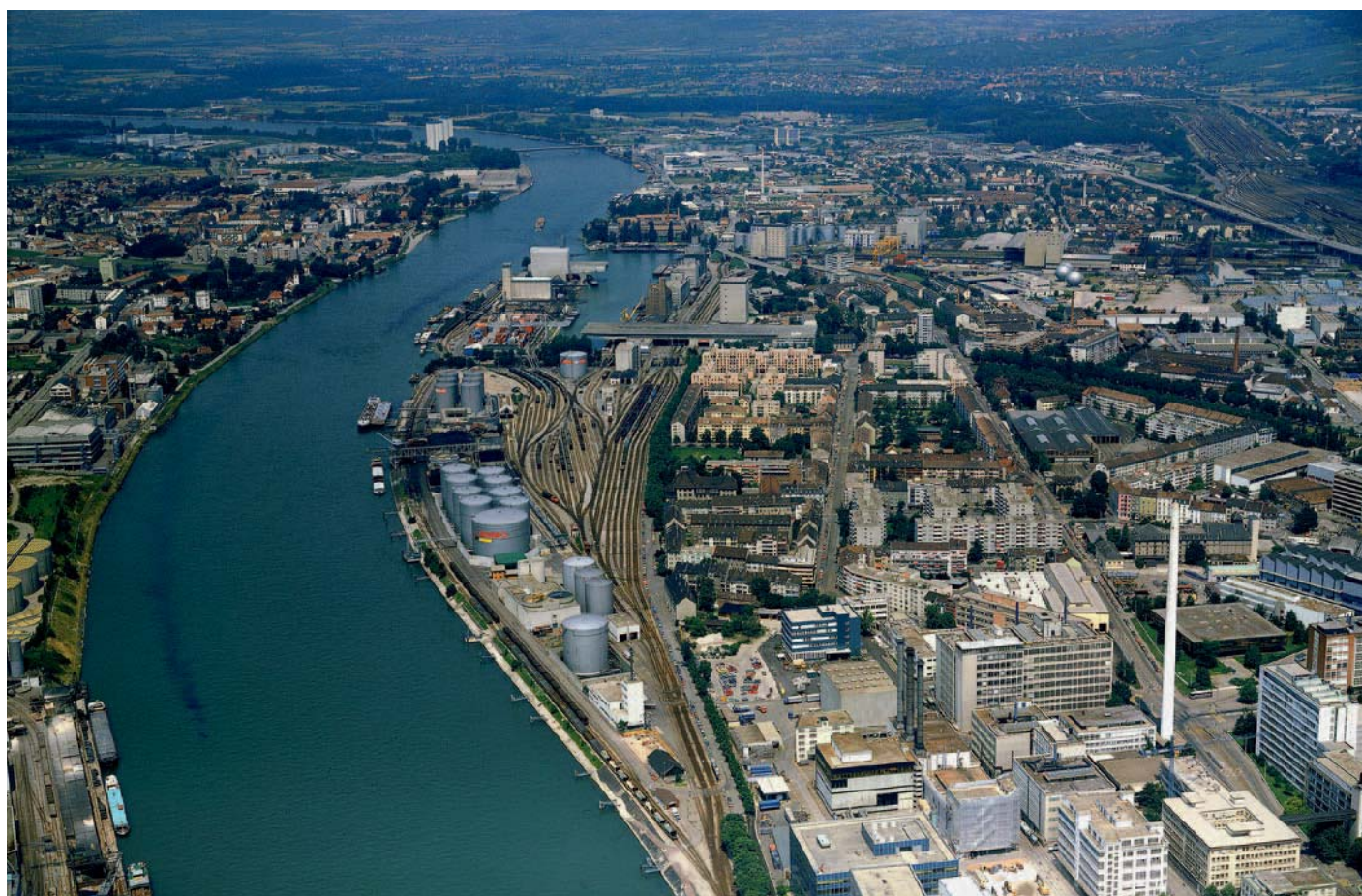
# Searching for unknown substances

Even at very low concentrations, organic micropollutants in natural waters can be harmful to aquatic organisms. Owing to technical constraints, analytical methods have so far been confined to the detection of a relatively small number of known compounds. With new methods, previously undetected substances can also be identified.



Heinz Singer, environmental chemist, studies the occurrence of micropollutants in natural waters.

Co-authors: Matthias Ruff, Juliane Hollender



Numerous micropollutants from household, industrial or agricultural sources enter the Rhine (pictured here is the Rhine port at Basel).

More than 65 million substances are currently listed in SciFinder, the world's largest chemical database, with a large proportion being synthetic organic compounds. Around 15,000 substances are added each day, and this rapid growth is likely to continue. In fact, this is not surprising, when one considers that a decil-

lion (= 1 followed by 60 zeros) different chemical structures are theoretically possible. It is difficult to estimate what percentage of the compounds known today are used in significant quantities. An indication is provided by the number of substances which have been notified to the EU since the Regulation on the

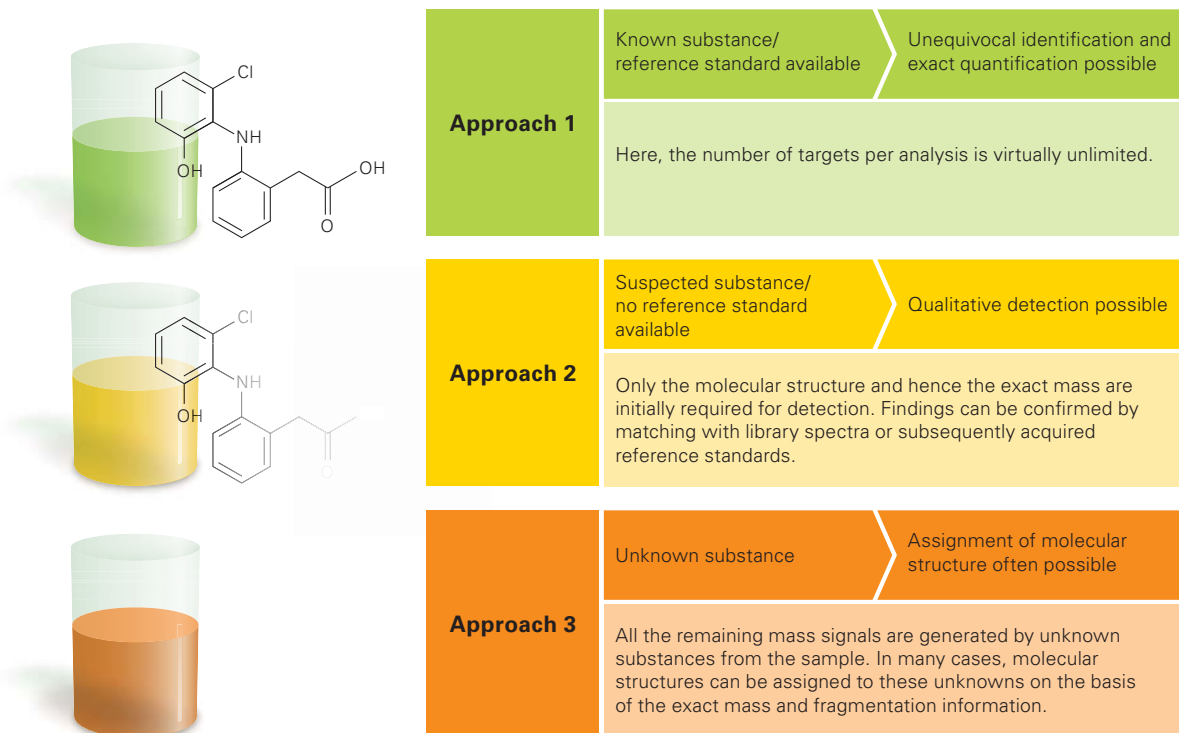


Fig. 1: Analytical and data evaluation approaches for comprehensive screening, using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) [1].

Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) came into force in 2007: by this reckoning, Europe produces or imports around 140,000 substances.

To what extent – and by what pathways – these compounds enter natural waters can be estimated by modelling. However, reliable predictions can rarely be made, as the necessary information on volumes used, sites of use and substance properties is frequently incomplete. Given the enormous variety of chemicals available, the range of substances which have been evaluated to date and ultimately also detected in waterbodies seems extremely limited, even though it already encompasses several thousand compounds.

### More comprehensive analysis possible

In recent decades, research institutes and authorities monitoring wastewater, surface waters and groundwater have detected, in particular, household, agricultural and industrial chemicals, such as pharmaceuticals, pesticides, surfactants, plasticizers and steroid hormones. As more sensitive detection methods have been developed, analyses have become increasingly accurate.

Since the 1990s, gas chromatography coupled to mass spectrometry (see Box, p. 11) has become the method of choice for many non-polar or volatile substances. The coupling of liquid chromatography with tandem mass spectrometry permits the detection of polar and highly hydrophilic compounds. With these

methods, however, only a few dozen substances can be studied per analytical run. In addition, the substances have to be selected in advance, and the sensitivity of the detector adjusted accordingly. Under these conditions, one can barely begin to identify the whole range of substances present in a sample.

The situation has been fundamentally changed by the introduction of high-resolution mass spectrometry. With this method, the mass of a molecule can be measured extremely accurately. In addition, it is possible to discriminate between molecules differing in mass by only 0.001 atomic mass units ( $1.66 \times 10^{-30}$  kilograms). This means that virtually every substance detected yields a specific signal. The method is also suitable for the entire mass range across which environmental chemicals occur. When combined with liquid chromatography, many of the substances detected can be identified with a high degree of sensitivity. In contrast to previous methods, identification does not require reference standards. These are only needed for purposes of quantification. Essentially, three analytical approaches can be adopted (Fig. 1).

In view of these advantages, liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) could well become established as a standard method for routine surface water monitoring in particular. Apart from the possibility of detecting an almost unlimited number of substances with optimal sensitivity, the highly resolved data can also be used to identify further substances retrospectively. Thus, for example,



Fig. 2: After the chemical spill at Schweizerhalle in 1986, a number of monitoring and sampling stations were set up by the Rhine riparian states.

Pharmaceuticals and transformation products (39.6%)



Fig. 3: At the Weil am Rhein monitoring station near Basel, daily samples are currently analysed for 318 known micro-pollutants. The aim is to track long-term developments and also to detect problematic discharges as rapidly as possible.

chemicals newly considered relevant to water quality can be extracted from earlier data so as to reconstruct historical water pollution patterns.

### Lessons learned from Schweizerhalle

The international monitoring station at Weil am Rhein near Basel has already been using the new method for several years. The Rhine is a source of drinking water for more than 20 million people. Its catchment extends from the Swiss Alps to densely populated regions of Germany and the Netherlands. As well as treated municipal and industrial wastewater, the river receives run-off from intensively used agricultural land and extensive urban areas. The monitoring station began operating in 1992, in the aftermath of the chemical spill which occurred at a Sandoz warehouse at Schweizerhalle in 1986 (Fig. 2). After a major fire, large quantities of toxic chemicals had entered the Rhine with quenching water, causing substantial ecological damage and forcing the temporary closure of downstream waterworks.

Since then, water samples have been analysed daily at the Rhine monitoring station in order to study long-term trends in water quality and also to detect inappropriate or accidental discharges in a timely manner. For this purpose, a multicomponent screening method – based on the LC-HRMS technique – was developed by Eawag in collaboration with the Federal Office for

the Environment and the Environment and Energy Office of Canton Basel-Stadt (AUE) [2]. Within the time frame of a day, samples are analysed step by step for known (target), suspected and unknown substances. The list of known substances currently comprises 318 compounds and transformation products from groups including pesticides, biocides, pharmaceuticals, narcotics, industrial chemicals, corrosion inhibitors and sweeteners (Fig. 3). The list is continuously updated with new findings from literature studies and with data from national and international monitoring programmes.

The analyses show that 84 of the 318 compounds from households, industry and agriculture can be regularly detected in the Rhine (Fig. 4). About half of the substances found – with concentrations of less than 10 nanograms per litre – cause barely detectable contamination of the Rhine. A small number of substances exceed the limit of 100 nanograms per litre specified for pesticides in the Water Protection Ordinance. But at this concentration and with a mean discharge of approx. 1000 cubic metres per second, the river is already carrying a daily load of 10 kilograms of each of these substances towards Germany.

### Identifying unknown substances

Although the analysis is extremely comprehensive compared with previous methods, it remains unclear what proportion of



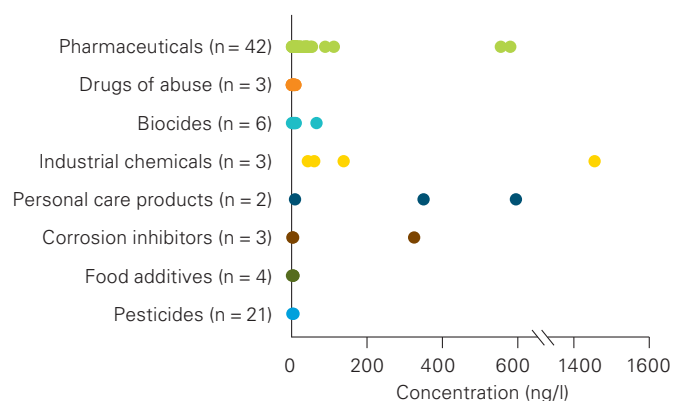
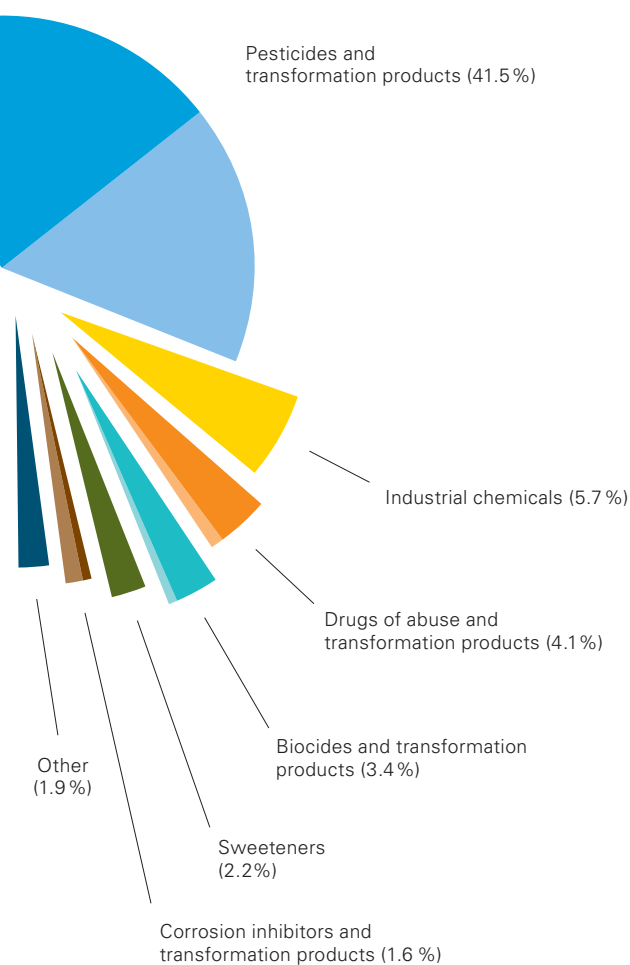


Fig. 4: 84 household, industrial and agricultural chemicals can be regularly detected in the Rhine.

the substances present in the Rhine is actually detected. In order to screen for unknown substances, Eawag has developed software which can extract all the substance signals from the mass spectrometry data, sort them by intensity and eliminate background signals. Evaluation of the data reveals that, as well as the quantified micropollutants, a sample contains on average between 2000 and 3000 other compounds. Of these, 100–150 show signal intensities which suggest a concentration of more than 10 nanograms per litre. Thus, numerous organic compounds have previously gone undetected in the Rhine. Whether these are synthetic or naturally occurring substances remains to be established.

For unknown substances of this kind, no information is available apart from the analytical data, such as the precise molecular mass or isotope pattern. The identification of suspected substances therefore has to proceed in accordance with Approach 2 (Fig. 1). In the absence of reference standards, one searches within the water-relevant group of pesticides, biocides and pharmaceuticals for registered active substances with an appropriate molecular mass. If necessary, hits can be confirmed with the aid of spectral libraries.

In most cases, however, unknown substance masses have to undergo elaborate evaluation (Approach 3). On the basis of the

precise mass and the isotope pattern, an unequivocal molecular formula can first be assigned to each substance. Matching structures can then be sought in specialized databases. Finally, comparison of the fragmentation patterns leads to the most likely structure. Using this approach, we were able, for example, to successfully detect the antidepressant moclobemide. Between 12 and 28 January 2011, a chemical suddenly appeared in the Rhine samples in the form of a strong mass signal, which was assigned the structural formula  $C_{13}H_{17}Cl_1N_2O_2$  (Fig. 5). A measurement campaign conducted along the entire length of the Rhine during a second discharge period from 9 to 15 March 2011 clearly indicated a point source lying between Rheinfelden and Basel. Assessments showed that about 14 kilograms of moclobemide entered the river during this period, and around 40 kilograms in January. The substance could be tracked all the way to the river's mouth in the North Sea. It was calculated that the moclobemide concentrations detected did not pose a significant risk to the Rhine or to drinking water produced by bank filtration.

The LC-HRMS method can readily be used to detect accidental or inappropriate discharges. As the parties responsible often do not notice such accidents or are unaware of the precise composition of the effluents discharged, daily monitoring can detect contamination peaks in the Rhine in good time and ideally also

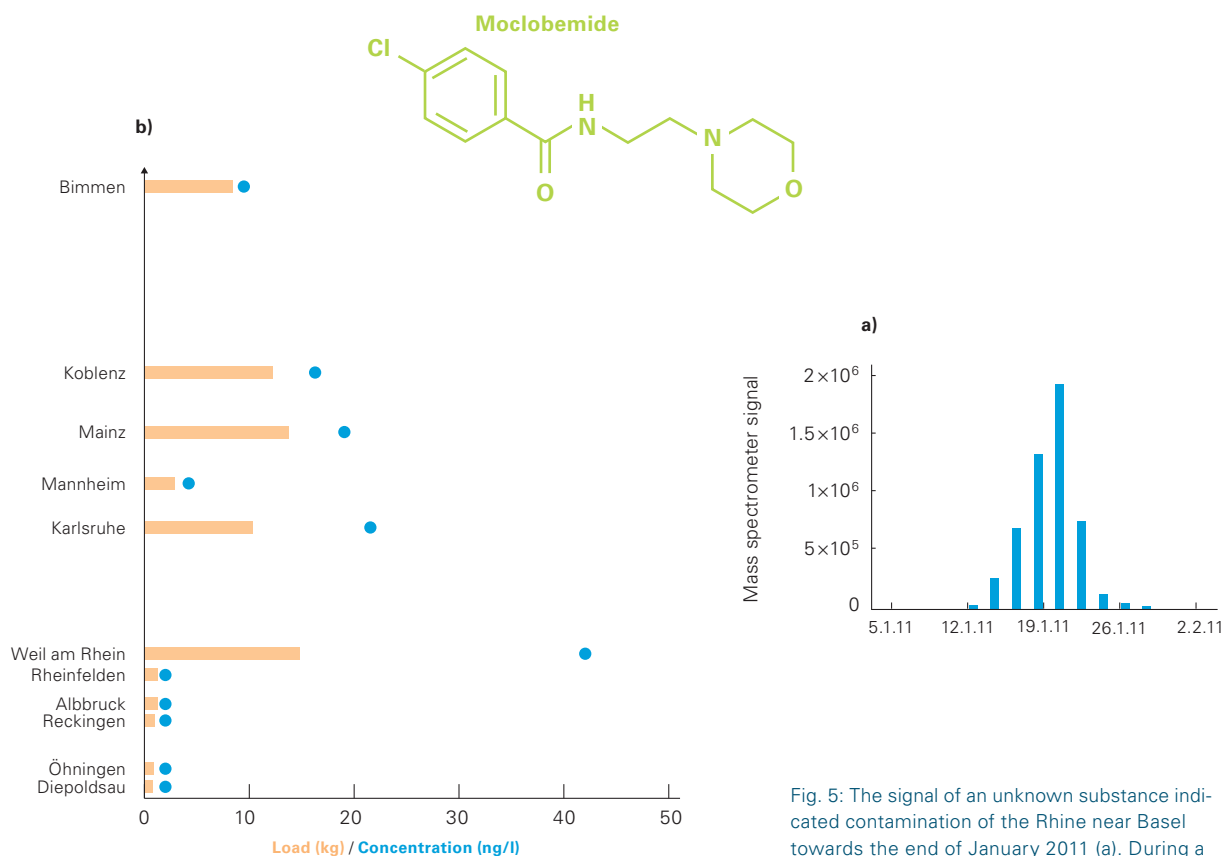


Fig. 5: The signal of an unknown substance indicated contamination of the Rhine near Basel towards the end of January 2011 (a). During a second discharge period, the substance was measured all the way from Switzerland to the Netherlands (b).

identify the substances in question. At present, however, the identification procedure is still too time-consuming for routine daily use. As the next step, therefore, the detection of substance masses appearing unexpectedly is to be integrated into the monitoring station's daily operations with the aid of statistical evaluation methods.

### Detecting unknown transformation products

Because of the costs involved, monitoring of organic micropollutants in large catchments and at the national level generally only covers selected priority substances. To review the selection of substances, Eawag carried out several pilot studies using the LC-HRMS method, in cooperation with the federal and cantonal authorities. Analyses were conducted within the national groundwater (NAQUA) and surface water (NAWA) quality monitoring programmes, and also in Lakes Constance and Lucerne. Measurements were combined with predictive exposure models. It was thus possible, for example, to predict exposure for the Lake Constance catchment using a material flux model and, in addition, to measure the catchment-specific substances at selected sites [3].

The fact that this approach can also be effective in extremely large catchments such as the Haihe river system in Northeastern China is demonstrated by the results of a joint international research project (cf. the article on p. 36). This river system ex-

tends over an area of 300,000 square kilometres. As more than 20 million people live and work in this region, it is extremely difficult to predict what organic micropollutants will be found. Using LC-HRMS analyses, the relevant substances were detected with only a small number of samples. These priority substances were then studied to determine input pathways and their fate in the catchment area. It was found that the wastewater treatment plants of Beijing are the main source of organic micropollutants. As over 90 per cent of the water downstream is used for irrigation, the organic micropollutants also find their way onto the agricultural fields [4].

Apart from its use in water quality monitoring, high-resolution mass spectrometry can also provide important additional information in laboratory tests. For example, in biodegradation experiments, Eawag scientists detected previously unreported transformation products of pharmaceuticals in wastewater [5]. Possible transformation products were first predicted with the aid of a computerized expert system. LC-HRMS was then used to screen for the suspected structures in wastewater. In some cases, it was possible to confirm the identity of the compounds by comparison with reference standards in samples from wastewater treatment plants [6].

The above examples from research and practice reveal the considerable potential of the new analytical technique. However,



Renouf Schaffner

this can only be fully exploited if continued efforts are made to develop efficient software routines suitable for automation. As in other data-intensive fields, success is largely dependent on advances in data analysis. The combination of mass spectrometry methods with appropriate computer-aided evaluation algorithms is essential if this technique is to attain its full potential in the future.

[heinz.singer@eawag.ch](mailto:heinz.singer@eawag.ch)

- [1] Krauss M., Singer H., Hollender J. (2010): LC-high resolution MS in environmental analysis: From target screening to the identification of unknowns. *Analytical Bioanalytical Chemistry* 397, 943–951.
- [2] Singer H., Huntscha S., Hollender J., Mazacek J. (2009): Multi-komponenten-Screening für den Rhein bei Basel. Abschlussbericht, Eawag, in Zusammenarbeit mit dem Bundesamt für Umwelt BAFU, S. 62.
- [3] F. Hoffmann-La Roche AG/Ltd Basel: Predicted no-effect concentration (PNEC) according to EU Technical Guidance Document (TGD) on Risk Assessment; derived no-effect level (DNEL) according to Straub and Flückiger, 2010, SETAC Europe.
- [4] Longrée P., Singer H., Moschet C., Götz C., Schärer M., Keusen M. (2011): Organische Mikroverunreinigungen im Bodensee. GWA 7.
- [5] Heeb F., Singer H., Pernet-Coudrier B., Qi W., Liu H., Longrée P., Müller B., Berg M. (2012): Organic micropollutants in rivers downstream of the megacity Beijing: Sources and mass fluxes in a large-scale wastewater irrigation system. *Environmental Science and Technology* 46, 8680–8688.
- [6] Helbling D., Hollender J., Kohler H.P., Singer H., Fenner K. (2009): High-throughput identification of microbial transformation products of organic micropollutants. *Environmental Science and Technology* 44, 6621–6627.
- [7] Kern S., Fenner K., Singer H., Schwarzenbach R., Hollender J. (2009): Identification of transformation products of organic contaminants in natural waters by computer-aided prediction and high-resolution mass spectrometry. *Environmental Science and Technology* 43, 7039–7046.

## Detection methods

Chromatography is used to separate a mixture of compounds into its individual components. In this technique, the mixture – transported in a mobile phase – is passed through a separation column with a stationary phase (modified silica gel particles or coated capillary). Depending on their physical properties, the individual components are more or less strongly retained on the stationary phase and are thus separated. The substances successively eluting from the column are measured as electrical signals (peaks) by a detector. They can then be identified by comparison with the curves for reference standards (calibration curves). The substances can be quantified on the basis of the peak intensity or area. In **gas chromatography**, gas (generally helium or hydrogen) is used as the mobile phase; in **liquid chromatography**, liquids (mixtures of organic solvents or water) are used for this purpose. Thus, with gas chromatography only vaporizable substances can be analysed, whereas with liquid chromatography all soluble compounds can be separated. Because of their selectivity and sensitivity, mass spectrometers have become the detectors of choice.

With **mass spectrometry** the mass of a molecule can be determined. After chromatographic separation, the substances to be analysed are ionized in an interface. In the mass spectrometer, the ions are first separated according to their mass-to-charge ratio in an electric or magnetic field and then detected. As well as intact molecules, fragments are generated whose size and abundance can be used for the identification of substances. In **tandem mass spectrometry**, two instruments are combined in sequence so as to enhance selectivity and sensitivity. With a **high-resolution mass spectrometer**, ion masses can be measured with high accuracy, making it possible to discriminate between ions with very similar mass-to-charge ratios. This is useful in elucidating the structure of unknown compounds.