



Review of monitoring techniques for biological contaminants

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techniques for biological contaminants**

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Abstract:

Water quality is a critical factor for public health worldwide. Accidents in the past and present have illustrated the vulnerability of our water supply chain.

Therefore fast, reliable, sensitive and cheap water-monitoring systems are needed, working independently at a low level of maintenance and hands-on time. Today there are only limited technologies to monitor pathogenic agents available on the market.

The following review should give an overview of the major technologies being developed and evaluated today which could have potential as monitoring systems in the near future.

1. Introduction

In modern communities environmental monitoring has become a matter of high importance for public health. Water is involved in numerous life support/sustaining systems and disruption of these infrastructures would seriously jeopardise public health and safety and thereby lead to considerable economic losses. Thus, water systems like drinking-, recycling-, industrial- and waste-water have been designated as a critical infrastructure where quality and quantity have to be maintained. In the past and present, outbreaks of waterborne disease have unmistakably shown the sensitivity of the water supply chain to biological contamination. Situations of that kind arising either from unintentional system failure (e.g. waste-water backflow, system leakage, maintenance work) or from deliberate biological attack have to be avoided. Therefore a lot of special regulations for monitoring drinking water supplies exist in different countries worldwide.

Today, systems for continuous or discontinuous real-time online measurement of physical (temperature, pH, turbidity, conductivity) and chemical parameters (ions, chlorine, oxygen, total organic compounds) are available in the market but specific, sensitive, reliable and rapid detection methods for specific poisonous compounds (ricin, dioxin) or highly pathogenic agents (viruses, bacteria, protozoa) are not really in place/insufficient and are in urgent need of improvements. Of course we have systems of high specificity and sensitivity that are reliable and even cheap but they involve time-consuming steps like sampling, transportation (of samples) to laboratories, preanalytical procedures (e.g. enrichment, purification and modification of analytes), analysis (processes leading to measurable signals like PCR or hybridisation), interpretation of primary/raw-results using complex algorithms and therefore are not viable for online monitoring. Most of these systems started as integral parts of classical laboratory workflows involving expensive, heavy and large/immobile devices, and after changing (miniaturising and simplifying) the whole conceptual set-up, they began to move from the laboratory to the point of use. Critical steps in this evolution to smaller, faster and cheaper monitoring systems in the field are driven by innovations in molecular recognition mechanisms, optimising biochemical and molecular biological processes, new and simplified fabrication technologies for a better fusion of sampling, transport of analytes and reagents within the devices (microfluidics), detection units (optics and electronics) and improved algorithms in data generation and processing. Triggering higher levels of system-automation, flexibility and independency this development gave rise to a variety of relatively expensive but commercially available monitoring systems for specific applications in medical diagnostics, environmental testing, and food safety.

The following gives an overview of what is possible now and what is feasible in the near future.

2. Common considerations: issues/problems to be solved

The definition of a detection limit is quite divergent: While high numbers of pathogenic agents are needed for some infections, only a few (less than 10) bacteria of *Francisella tularensis* are sufficient for infection. The theoretical detection limit of polymerase chain reaction (PCR) for example is one copy of target DNA but this depends on the quality of DNA and on statistics (this proposed/calculated copy of DNA might not be present in the sample aliquot under investigation). On the other hand, usually there are several copies of identical genetic information in one bacterial cell or even more than one copy of a gene in the same genome equivalent (e.g. ribosomal DNA), which makes it an ideal target sequence for sensitive detection by PCR. However, toxicants and poisons instead need substantial amounts to become life threatening and to be detected.

Currently, systems in use can be considered as surrogate techniques as they are indirect ones: Monitoring of behaviour of higher organisms (fish, daphnia) in separated/parallel compartments give a hint about the water quality but are delayed in time, unspecific and need a high level of maintenance (qualified personnel 24 hours). Once an alarm is triggered by this system the real search for the threat just begins.

Measurement of turbidity, temperature and free chlorine is an easy approach as sensors are available in the market and commonly in use. If turbidity and temperature increase and free chlorine drops down conditions for bacterial growth become better. This method is not sensitive enough to warn in time when a biological threat is just at the beginning.

The simplest set-up for monitoring analytes of interest is a sensor exposed to the fluid/flowing water, however there are some critical/technical limitations:

First, the system has to be installed, maintained and replaced without interrupting the water support chain. Pipes in parallel can give access to the water supply grid when one branch is taken off the system. However, development of systems with a lower maintenance rate and long-living components are appreciated.

Second, reaction partners for biochemical/molecular biological processes and electrical power for temperature control, switches and pumps in microfluidics, light sources and sensors for fluorophore excitation and detection have to be supplied more or less continuously depending on measuring-cycle regime. A general trend in reduction of reagents by involving microfluidic systems and decreasing power consumption by modern technology (e.g. light emitting diodes instead of lasers) supports these needs.

Third, collected data from all points of the monitoring grid have to be transferred in real-time to a data collection centre where evaluation and, in case of emergency, alert initiation takes place. It can be expected that the amount of data will be huge, when detection systems for all needed parameters are installed.

And last but not least, harmful and corroding substances like free chlorine for disinfection and biofilm formation will limit the lifespan of sensitive system components like biosensor surfaces (e.g. electrodes with immobilised recognition molecules like oligonucleotides or antibodies). Fouling or biofilm formation is a common problem in water supply as it protects bacteria against disinfection.

Considerations about the handling of the whole systems have to be made: physical (e.g. flow rate, volume, temperature and viscosity) and chemical sample characteristics (e.g. complexity of the analyte, interfering/inhibiting components, range of target concentration, component stability), need for technically competent personnel, assay time and frequency, size and weight of equipment, total costs, the environmental situation and complexity of maintenance have to be made.

Many of the described monitoring methods are not able to distinguish between living and dead pathogens. DNA derived from pathogenic organisms will still circulate in water when the origin is long extinguished by counteracting measures like chlorination.

To complete the workflow, reporting, investigations (localisation, causative agent) and management of counter measures need to follow well-established procedures after an outbreak is detected.

3. Technologies in evaluation/development, under investigation

The focus of detectible agents here will be put on organic compounds/biomolecules (DNA/RNA, proteins, peptides, hormones, metabolites, drugs, pesticides ...) and whole pathogens (viruses, bacteria, protozoa ...).

A summary of technologies from different fields useful for online monitoring of water:

3.1. Flow cytometry

Cultivation of bacteria, still the standard detection method for numerous pathogenic species, has a long assay time (e.g. 10 days for *Legionella pneumophila*) and a detection restricted to cultivable cells. A fast, quantitative flow cytometric workflow for *Legionella pneumophila* in water samples consists of four steps, involving a filtration/concentrating step followed by double-staining with FITC- and Alexa-conjugated *Legionella*-specific antibodies, immunomagnetic separation and finally, flow cytometric detection and quantification. This cultivation-independent method reduces assay time to 3 hours at a detection limit of around 500 cells/l and a recovery rate of *Legionella* cells of about 50 % [1].

3.2. Flow-through microscopy

Flow-through microscopy could be applicable as a tool for automated detection and classification of living organisms in drinking water. Usually all methods involving complex optical systems are relatively expensive and need specialised personnel. In recent years the area of algorithms for automated image/structure recognition has been an improving field. One possible/promising set-up is digital holographic microscope utilising coherent spatial illumination in combination with sophisticated algorithms to calculate the exact position of *Giardia lamblia* cysts even in a mixture with other similar organisms [2].

3.3. Living organisms as indicators

The effect of an agent on a surrogate or taster organism is an alternative to searching for innumerable possible toxins and pathogens in quality control of drinking water. Drinking water is continuously taken from the main water supply and passes through containments with living highly sensitive microorganisms or organisms. Their reaction is observed by specialists or is monitored by cameras in combination with sophisticated analysis software to distinguish between normal and abnormal behaviour (e.g. movement, colour) in comparison to the same organisms in pure water.

To increase reliability and decrease the rate of false alarms, a combination of this system with other sensors yet available is recommendable for online diagnosis. This concept of a broadband sensor can be employed in the detection of contamination with harmful substances as well as for monitoring water quality in supply facilities in general.

At the German Fraunhofer Institute of Optronics a system for continuous, real-time monitoring of drinking water, AquaBioTox [3], was developed, that can trigger an alarm on occurrence of hazardous substances like cyanide, ricin, or toxic metabolites from bacteria at low concentrations (nanograms per litre) within minutes. The sensor-system containing two different modified strains of bacteria and mammalian cells is fed with drinking water from the water supply system. Due to genetic modifications these sensitive microorganisms/cells are able to produce red fluorescent protein upon contact with toxic substances, which can be detected and analysed by monitoring the red fluorescent light by a highly sensitive camera. The software of this system is able to learn from historical data-sets in which fluctuations in the physical, chemical, and biological parameters are normal, therefore only an unusual signal-pattern will trigger an alarm.

To improve this system a daphnia taximeter from project partner bbe Moldaenke, an environmental manufacturing company at Kiel in Germany [4], was added, when they

noticed that these water fleas are especially sensitive to nerve poisons. In parallel a second system monitors and regulates parameters like temperature and nutrients for optimal life conditions for the surrogate organisms.

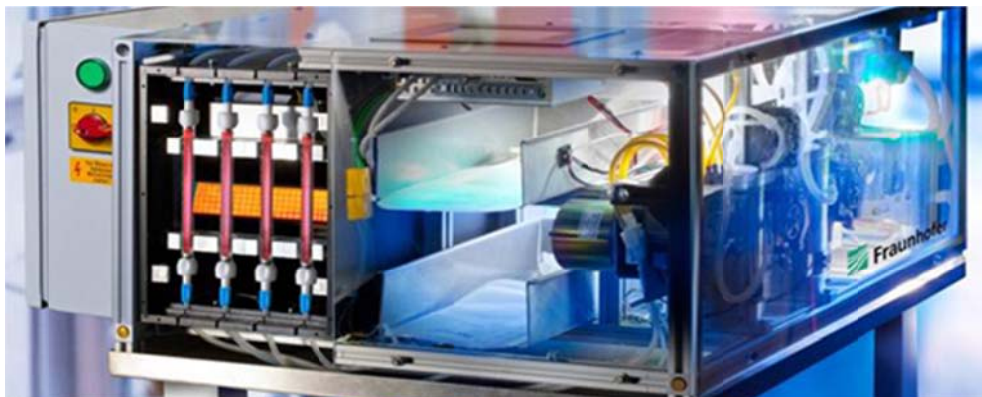


Figure 1: AquaBioTox [3]

3.4. Mass spectrometry

Mass spectrometry (MS) is a very powerful detection method as it is fast, accurate and the workflow is easy and cheap. The disadvantages are instrumentation price, size and weight and the need for pure and substantial amounts of analytes. Therefore pre-analytical steps coupled to MS like gas chromatography (GC) or PCR prolong assay times and complicate the workflow. Nevertheless, MALDI-TOF is well established for pathogen identification from single colonies in bacteriology. New generations of algorithms able to identify bacterial targets even in mixtures of low complexity are under development.

A combination of biothreat group-specific PCR with electrospray ionisation mass spectrometry (PCR/ESI-MS) is sufficient to distinguish the closely related but less harmful near neighbour organisms with a very low false positive rate [5].

3.5. Raman spectroscopy

Raman spectroscopy is a powerful analysis technique for non-destructive rapid identification of single bacterial cells at micron-scale resolution without use of fluorescent dyes. A variety of different instrument concepts and complex multivariate statistical analysis tools helped to improve speed and sensitivity of analysis even of single cells [6].

For example, microspectroscopy using multifocus confocal Raman was tested successfully for rapid real-time monitoring of individual bacteria and even germination dynamics of bacteria [7].

Confocal Raman spectroscopy in combination with laser scanning confocal reflectance imaging allows production of handheld devices for high-resolution imaging and spectroscopy for a variety of applications outside the laboratory [8].

3.6. Infrared spectroscopy

Infrared spectroscopy is a widely used powerful technology in organic chemistry but had only limited potential in applications like direct water monitoring due to the water molecules themselves. Contaminants therefore have to be extracted from water samples by various time-consuming methods as a prerequisite for infrared spectroscopy. Only when infrared quantum cascade laser technology — up to 1 000-fold stronger compared to conventional silicon carbide thermal emitters used in the laboratory to date — was developed, were light intensities sufficient to visualise and characterise organic molecules directly in water.

The Fraunhofer Institute (IAF) tested infrared quantum cascade laser technology successfully in a pilot project taking samples every few minutes directly from the water supply system and, without any further pretreatment, detection of spiked contaminant/impurity (sweetener) was carried out. This prototype will be developed and turned into a finished product in cooperation with Bruker Optik [9].

3.7. Amplification of DNA

3.7.1. Real-time PCR

PCR reaction has to be carried out under special conditions (exact temperature setting and cycling, buffer with exact pH, sensitive reaction partners like enzymes, labelled oligonucleotides and nucleotide triphosphates at certain concentrations) and therefore has to be separated from the water network in distinct reaction containments. Thus the samples have to be transferred from the drinking water supply system to the compartments where sample processing will take place. This separation process could be managed by samplers and microfluidic devices, simultaneously concentrating target molecules and organisms by absorption, centrifugation or filtering followed by DNA extraction. To accomplish these steps selective membranes carrying receptors, oligonucleotides or antibodies together with pore

forming proteins are imaginable but biofilm formation and ageing (e.g. due to exposure to chlorine) are destructive and thus counteracting processes to such sensitive components.

3.7.2. Isothermal amplification of nucleic acids

Analysis of trace amounts of nucleic acids is one of the most powerful — both selective and sensitive — methods to detect organisms. To date, the majority of miniaturised systems for nucleic acid analysis have used conventional polymerase chain reaction (PCR) for amplification but an increasing number of techniques without need for thermal cycling are emerging as alternatives: Isothermal microsystems need a shorter reaction time and constant temperatures throughout amplification and are therefore less energy consuming with lower complexity of electronics. This is an ideal technology for portable, battery-operated detection systems in the field and is, in combination with microfluidics and integrated optical detection elements, suitable for online monitoring devices. Further advantages over PCR-based techniques are shorter assay times at comparable sensitivity and specificity and robustness against inhibitors, which allows detection of target sequences in raw sample matrices even without time-consuming sample preparation. Isothermal PCR technology solves the problem of exact temperature cycling but still needs power supply for exact temperature setting and detection system (e.g. fluorescence).

Examples of isothermal methods [10]:

nucleic acid sequence-based amplification (NASBA)

loop-mediated isothermal amplification (LAMP)

helicase-dependent amplification (HDA)

rolling circle amplification (RCA)

strand displacement amplification (SDA)

exponential amplification reaction (EXPAR)

isothermal and chimeric primer-initiated amplification of nucleic acids (ICANs)

signal-mediated amplification of RNA technology (SMART)

3.7.3. Loop-mediated isothermal amplification (LAMP)

LAMP technology is based on amplification with four primers, where, after an initial phase, a modified sequence containing the target sequence is generated. The modification allows intramolecular loop-formation and circular amplification follows. This technology is employed in various fields like food testing and other applications for bacterial pathogens, toxicants and fungal contaminants [11].

A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for detecting Japanese encephalitis virus was shown to be more simple but as sensitive as a real-time RT-PCR and 10-fold more sensitive than conventional RT-PCR. It was less time-consuming even compared to real-time RT-PCR (1 h at 63 °C) and highly specific with a detection limit of 24 copies/ μ l [12].

3.8. Nucleic acid (RNA/DNA) hybridisation on microarrays

Hybridisation of nucleic acids allows simultaneous detection of hundreds or even thousands of different target molecules or discrimination of similar sequences by mutation analysis. To accomplish this method a pattern of different oligonucleotides is spotted and immobilised onto a glass slide or similar material by as different methods as spotting, spraying or in situ synthesis. Whenever complementary sequences to one or more of these catching oligonucleotides are present in the sample, they bind to it. If DNA is present at low level in a sample, amplification by PCR is needed before hybridisation to the microarray. Therefore all considerations concerning workflow are similar to those for PCR (e.g. temperature and microfluidics for washing steps). During PCR the target DNA amplicons are labelled with fluorescent dyes coupled to the primers. As these steps are critical for online monitoring, microarray-systems working at low DNA-concentrations and label-free methods are absolutely required. The resulting pattern of detected spots is analysed by suitable computing software (statistical algorithms) by localising and deciphering the corresponding oligonucleotides.

Microbial detection on the basis of DNA microarrays became very useful for monitoring of pathogenic organisms in clinical, environmental and food samples or even in biodefence. They fill the gap between narrow-range PCR and broad-range next generation sequencing.

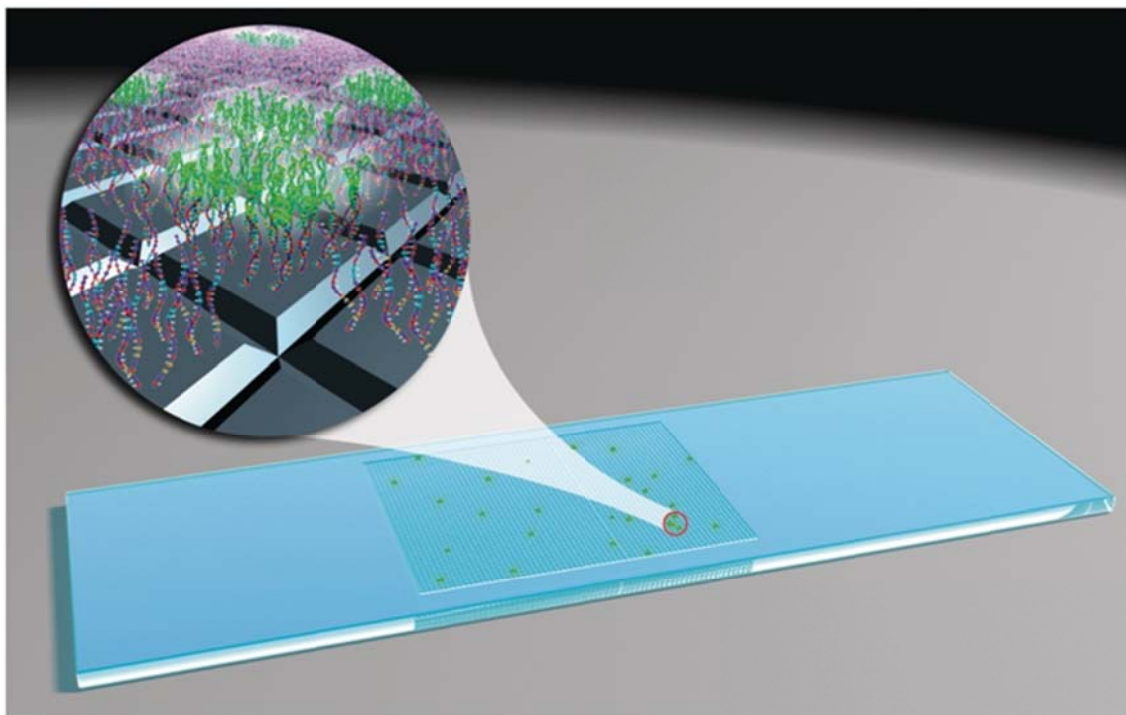


Figure 2: Microarray [13]

To avoid PCR amplification as a prerequisite these assays have to perform well at low attomol concentrations (1aM, 10^{-18} mol/L). Silver nanoparticle aggregate technology combined with electrochemical detection was shown to detect approximately 120 molecules of DNA in 40 μ L (5aM) [14].

A highly sensitive and selective DNA-detection chip can be prepared by copolymerisation of polypyrrole with ferrocene on a gold surface. Hybridisation of target DNA to the immobilised catching oligonucleotides on the polymer becomes visible by the resulting redox signal of the ferrocenyl group within the copolymer. This electrochemical sensor technology allows the production of high-density arrays with individual microelectrodes [15].

3.9. Immunological techniques

Instead of oligonucleotides for the detection of DNA/RNA, antibodies immobilised onto microarrays can be used as biosensing molecules for the analysis of antigens.

CombiMatrix array chips consist of thousands of individually addressable microelectrodes where an electrochemical signal originating from an enzyme-enhanced reaction is

transduced by complementary metal oxide semiconductor (CMOS) technology. The trigger for the first reaction is target-molecule binding to the corresponding recognition element which can be an oligonucleotide as well as an antibody immobilised on the surface of the chip adjacent to the CMOS. Antibody chips can be applied on recognition of a variety of biological pathogens like saxitoxin and bacterial spores [16], ricin, M13 phage, and α -1 acid glycoprotein [17], *Yersinia pestis* with a detection limit of 10e6 CFU/mL, *Bacillus anthracis*, and *staphylococcal enterotoxin B* down to a concentration of 5pg/mL [18, 19].

Antibody-captured bacteria can be detected and typed with sets of biorecognition elements (e.g. antibodies or aptamers) in a fast high throughput platform based on microarray/multiwell plate substrates and laser-induced fluorescence of a nucleic acid intercalating dye/stain [20].

A chemiluminescent system for the detection of food-borne pathogens was developed by combining cross-flow immuno-chromatography with a lens-free CMOS image sensor (CIS). A cellulose membrane-based immunosensing ELISA-on-a-chip (EOC) was aligned with the CIS module to transfer the chemiluminescent signals directly from the EOC into the sensors. This cheap and rapid small-sized immunosensor device showed a detection limit of about 4 000 CFU/mL of the food-borne pathogen, *Salmonella typhimurium*, and about 65 CFU/mL when it was coupled to an immuno-magnetic separation unit [21].

3.10. Detection of metabolites

Living or dead microorganisms release a variety of biomolecules which can serve as targets for detection systems. Detection of adenosine triphosphate (ATP) using bioluminescence is an example for monitoring coliforms and *enterococci* in drinking water supply on the basis of a very sensitive system [22].

3.11. Biosensors

Generally biosensors consist of a biological sensing element (bioreceptor) and a physicochemical transducer (electrical interface) associated within a compact device. The electronic system first amplifies the weak signal generated upon binding of target molecules to the sensor (signal amplifier) and then software algorithms process sets of signals to readable results.

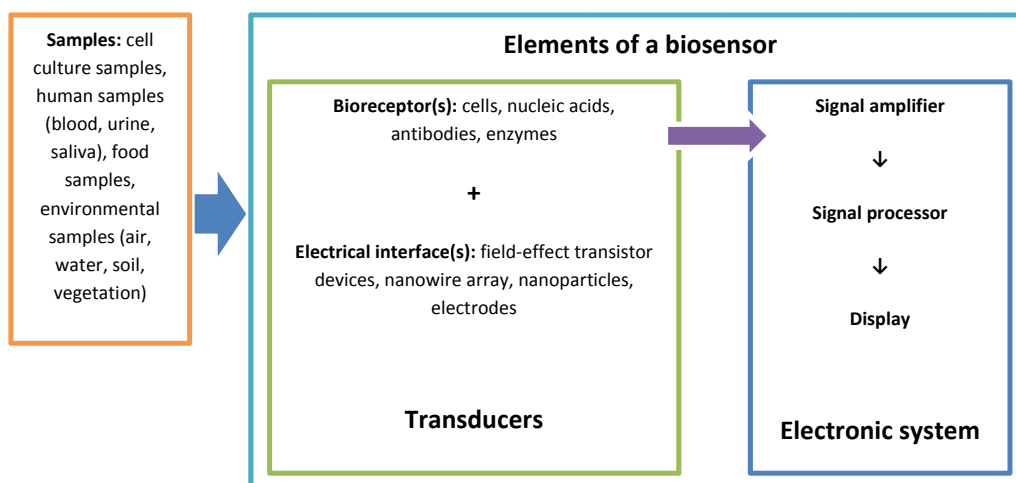


Figure 3: Concept of a biosensor [23, modified]

Biosensors mainly arose through environmental concerns for development of on-site monitoring systems to reduce the response time and cost of pollution control in comparison to shipping samples to a central laboratory. In this context biosensors are being tested for monitoring of environmental pollution and toxicity in air, water and soil, with the primary interest coming from environmental regulatory agencies.

3.11.1. Optical sensors with integrated biological recognition molecules (optical biosensors)

Since the early days of optical biosensors in the 1980s lots of discoveries in optics, fluidics, electronics, and biochemistry were made and this helped in accelerating the invention of smaller, cheaper and better systems [24, 25].

New genetically engineered recognition molecules and improved immobilisation chemistries helped to generate components of higher stability and activity. Specially designed antibodies can now be synthesised *in vitro* and selected for unique specificity. Molecular modifications (mutations) help to increase stability after exposure to heat or solvents for storage and use in extreme environments. For continuous monitoring applications, the development of small, single-chain antibodies with high capacity to denature and renature repeatedly and rapidly is an important milestone [26–28].

Free DNA originating from pathogenic organisms can be bound and detected by specific complementary capture-oligonucleotides. Under less stringent conditions these

oligonucleotides are even capable of binding to closely related DNA-sequences, thus detection of mutated or closely related strains of pathogens becomes possible with only one or few group specific capture molecules. If more overlapping nucleotides are placed on a single chip hundreds of pathogen strains can be detected by sequencing small characteristic DNA-portions of potential pathogens (resequencing arrays technology) [29].

But these short nucleotide chains (aptamers) are able to bind and detect other molecules than DNA specifically and furthermore generate signals upon complex formation with target molecules (e.g. proteins) [30–34].

To increase sensitivity a weak signal can be amplified by a self-replicating cascade catalysed by a replicating enzyme after target-binding to a DNA construct (molecular machine in a homogeneous system, in combination with simple optical components) [35].

In a classical way, both oligonucleotides and antibodies bind specifically to predicted, well-characterised targets. In this case at least one unique or better a group of sensor molecules recognise each single target of interest. To cover almost all possible/probable harmful agents a huge number of recognition molecules immobilised on microarrays would be needed but even then without the proof of total coverage of existing and newly arriving agents. Generic recognition approaches for the detection of unknown agents without exact target identification are needed urgently. In this context different approaches were made using whole cells [36], cell receptors [37], carbohydrates [38–40], anti-microbial peptides [41–42] and siderophores [43].

A huge variety of different oligonucleotides, antibodies, carbohydrates and peptides, immobilised on high density arrays specific for large numbers of target molecules and even whole organisms have been used in combination with optical readout systems for simultaneous detection.

Development of new solid-state optical devices, enzymes for signal amplification and long-lifetime fluorophores counteract problems in measurement of the relatively weak fluorescent signals and dye-fading/ageing.

3.11.2. Electrochemical nucleic-acid-hybridisation biosensors (RNA/DNA sensors, genosensors)

The specific and sensitive interaction between nucleic acid molecules (hybridisation) can be employed for the detection of target-DNA/RNA and even for the selective monitoring of more complex structures like target proteins, pollutants or drugs bound as ligands to DNA or RNA as receptors. This genosensor technology with oligonucleotides of known sequence

immobilised on sensor electrodes/surfaces can be used in lab-on-a-chip-devices for environmental pollution monitoring [44].

To further improve sensitivity a so-called template-enhanced hybridisation strategy was developed: two probes that do not hybridise to each other can form a ternary complex ('Y' junction structure) in the presence of the target-molecule (down to concentration in the pM-range) with increased discrimination of single-base mismatches [45].

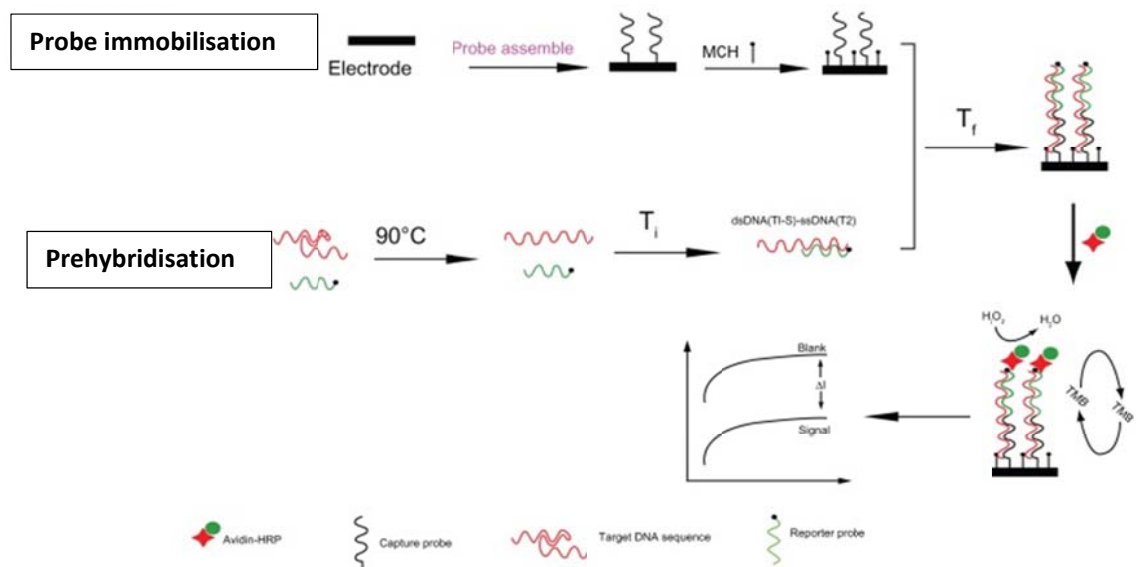


Figure 4: DNA electrochemical biosensor [46, modified]; MCH, Mercaptohexanol; T_i , initial hybrid temperature; T_f , optimal lower temperature; HRP, horseradish peroxidase; TMB, tetramethylbenzidine

Figure 4 shows the working scheme of a multistep temperature-regulated hybridisation device for the detection of target sequences of the *Mycobacterium tuberculosis* by generating ternary DNA hybrids on an electrochemical DNA sensor [46].

The production and handling of these stable and reusable devices seems easy and the results show good reproducibility, but the need for labelling dyes is still an issue for continuous monitoring.

3.11.3. Biosensors using fluorogenic DNAszymes (deoxyribozymes, DNA enzymes)

When organisms are growing they will release some specific products into their environment. Detection of these products indicates the presence of the corresponding organisms. Target substances like metabolites, degradation products or trace amounts of components of culture media like antibiotics can be seen as a hint for unintentional or even intentional growth of pathogens.

DNAszymes-technology is based on secondary structure of single-stranded DNA molecules and their specific reactions catalysed by this species of molecules. A prerequisite for this method is a specific selection procedure for isolating DNAszymes from a random-sequence DNA pool to isolate those enzymes that are able to catalyse a certain chemical reaction of interest: for example, the cleavage of a DNA–RNA chimeric substrate at a single ribonucleotide junction (R) that is flanked by a fluorophore (F) and a quencher (Q). Once F and Q are separated after cleavage, quenching of the fluorescent light is not possible any more and fluorescence intensity will increase (Figure 5). If the selection procedure is carried out in the presence of target molecules derived from a bacterial species, the interaction between the target molecules and the potential labelled DNAszyme species will trigger a change in secondary structure and cleavage will take place. These candidate-DNAszymes can be selected and enriched for the production of an assay system, where the potential pathogen-derived target molecules are detected only in case of contamination [47].

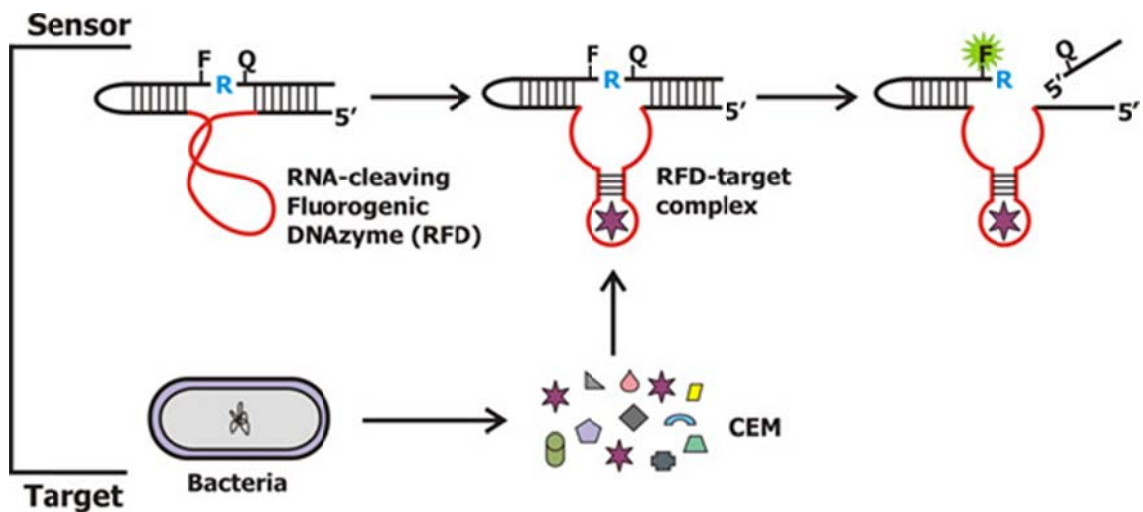


Figure 5: Detection of bacteria using fluorogenic DNAszymes [47]

3.11.4. Surface plasmon resonance biosensors (SPR)

To solve the problem of mobile labelled oligonucleotides in genosensors, label-free optical biosensing technologies based on surface plasmon resonance (SPR) have been developed. When analyte molecules in a sample bind to biorecognition molecules immobilised on the SPR sensor the properties of the biomolecular layer change with hybridisation and the refractive index on the sensor surface changes [48].

A special type of label-free optical biosensors involves localised surface plasmon resonance (LSPR) with immobilised peptide nucleic acids (PNAs) on gold-capped nanoparticles. While hybridisation of target molecules on the surface is proceeding, the light-absorbance strength changes with increasing thickness of the surface layer. Even better results were obtained with immobilised oligonucleotides and PCR-amplified samples, with a detection limit of less than one pM of target DNA together with increased selective discrimination against single-base mismatches [49].

SPR technology has been successfully used in immunosensors when coupled with specific antigen-antibody reactions instead of nucleic acid hybridisation. This seems to be a useful tool for the detection of analytes like proteins, drugs, DNA, and microorganisms as well as for quantification of bacteria [50].

Biosensors can be used in parallel on microarray format for multiplexed analysis of bacteria, viruses and soluble macromolecules. The surface of the gold-coated sensor chip covered with hundreds of different immobilised capture molecules (antibodies or nucleic acids) is scanned using grating-coupled surface plasmon resonance imaging, an optical diffraction technique where the angle at which coupling occurs is dependent on the capture of target molecules [51].

Biomolecules can lose their biological activity during immobilisation processes and ageing. For the enhancement and stabilisation of sensing performance in biochip devices biomolecules can be embedded in polymer thin films with metal nanoparticles to keep conformation and orientation [52].

3.11.5. Nanoparticles

Once designed an array is capable of detecting target molecules according to the recognition molecules immobilised on the array surface. Whenever new biological agents have to be detected, the whole array has to be completely redesigned or reformatted. An alternate approach to overcome this problem of low flexibility in planar microarrays is nanoparticle technology.

In coded-particle assays the detection element for each potential target consists of a set of specific nanobeads with discrete recognition surfaces, which are mixed and processed in a single batch for multiplexed target analysis. Whenever further recognition molecules are demanded, additional sets of beads with distinguishable surfaces can be added to the mixture of particle sets [53, 54]. Use of tagged magnetic and non-magnetic nanoparticles supports reagent and sample processing within microfluidic devices by speeding up diffusion-dependent reactions and further facilitates the concentration and purification of trapped target in microfluidic systems. Together this will improve automation and sensitivity compared to planar microarrays.

Nanoparticles (e.g. gold or silver) of different but uniform size and shape can influence optical excitation and signal intensity generated on planar surfaces in biosensors and they are able to enhance surface plasmon resonance (SPR) and hence avoid labelling [55, 56].

Electrowetting of surfaces, a new approach of nanotechnology under investigation, has the potential to counteract unspecific binding when switching/cycling the surface from superhydrophilic to superhydrophobic and back. This control of wettability can be used as a self-cleaning process and thus can reduce fouling [57–59].

3.11.6. Automation by microfluidics

Microfluidic technology started in the early 1980s and was employed in inkjet printheads, DNA chips, lab-on-a-chip technology, micro-propulsion, and micro-thermal technologies. In microfluidic systems small volumes of fluids move through different process compartments and over sensing surfaces and therefore reduce both costs by decreasing requirements for expensive reagents and processing/assay times that are proportional to liquid volumes. Further reduction results from improved fabrication techniques and integration of processes like target preconcentration, target separation, sample homogenisation, mixing of samples with reagent, separation by using solid-phase materials and on-chip temperature control to maintain reaction temperature.

As new technologies and materials for production of microfluidic subsystems are more and more implemented the devices are shifted from planar to three-dimensional structures/shapes. Plastics are a very flexible and cheap basic material compared to glass and silicon and can be designed for special applications with properties like resistance to temperature or organic solvents, or to serve as wave guide. Binding of biomolecules on plastic surfaces to integrate sensor chambers within fluidic systems needs better understanding of interactions and improved immobilisation techniques, but optical biosensors of this kind are starting to be marketed.

3.11.7. System integration to generate one device

Online monitoring needs continuous or periodic automated sampling. Samples are then handed over to the processing unit, where biochemistry within microfluidics takes place. After these pre-analysis steps, when the analyte is prepared, detection by an optical system is performed. If for example biosensors are integrated in the sampler/concentrator further reduction of complexity and costs would be achieved.

Today biosensors are generally not developed as a whole system, but are assembled from particular components such as sampler, concentrator, processor and detector. When this concept changes the vision of a whole system, including biochemistry, fluidics, optics, electronics, altogether it will be more than the sum of the parts [60, 61]. Improved software will help to design all-in-one microfluidic systems in the future [62, 63].

Integration and energy supply of small pumps and valves into the system and their exact regulation/control to fulfil the needs of the device is a great challenge today. Many of the on-chip pumps and valves are relatively sensitive to inhomogeneities in fluids, which of course is not a significant issue in drinking water. On the other side grid-independent energy supply is still a critical problem and techniques reducing energy requirements and surrogate solutions for batteries have to be developed: microfluidic fuel cells or biomimetic photocentres are discussed [64, 65].

A next step in simplifying the system automation is integration of microfluidic components with the optical components [66, 67]. Capillaries directing the flow of samples and reagents can be used as surface for the immobilisation of recognition molecules, and simultaneously serve as waveguides for excitation of bound fluorophores by embedded laser-emitting diodes and acquisition of fluorescence emission by avalanche photodiodes [68, 69]. Fabrication processes like photo-patterning of glass for the production of these all-in-one systems combining optical components integrated with the sensing surface within micro-channels are not yet available and under investigation [70].

A variety of disposable cartridge systems, but mainly for diagnostic purposes, is being developed for the market, like Philips' Magnotech technology [71] for the measurement of picomole concentrations of target molecules in blood or saliva. A single droplet of blood applied to the cartridge disperses automatically preloaded magnetic nanoparticles covered with catching molecules that bind target proteins present in the sample. An electromagnet moves all these nanoparticles to the biosensor's active surface, which is covered with catching molecules able to bind to a second site on the target protein. During this process the surface concentration of the target protein increases and the binding process accelerates. With activation of a second electromagnet on the opposite side of the reaction

chamber only unbound magnetic nanoparticles are pulled away from the active surface and finish this fast and well controlled separation procedure (Figure 6). The target molecules are trapped now in a sandwich between the active surface and attached nanoparticles (Figure 7). In the absence of target molecules, total internal reflection occurs because a beam of LED-light entering at a specific angle from outside, is reflected without any loss. However, when the active surface of the sensor inside the reaction chamber is occupied by magnetic nanoparticles, intensity of the reflected light is reduced by scattering and absorption. A CMOS image sensor monitors these intensity fluctuations in the reflected light, which are proportional to the amount of attached nanoparticles (see Figure 8).

Catching molecules for several different proteins can be immobilised to different sections of the active surface within the sensor-cartridge to perform multiplex assays in one unit.

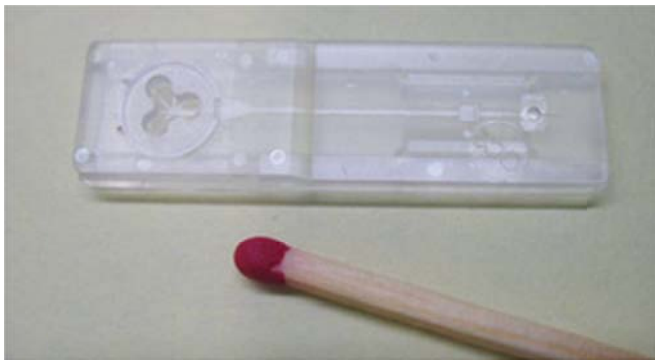


Figure 6: Philips' Magnotech: Concept for a biosensor with magnetic sample processing unit enclosed in a disposable cartridge [71]

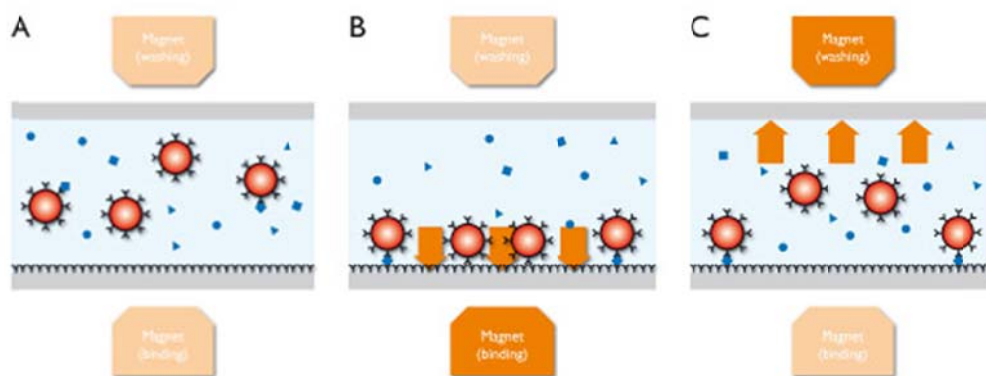


Figure 7: Magnetic sample processing with opposite electromagnets [71]

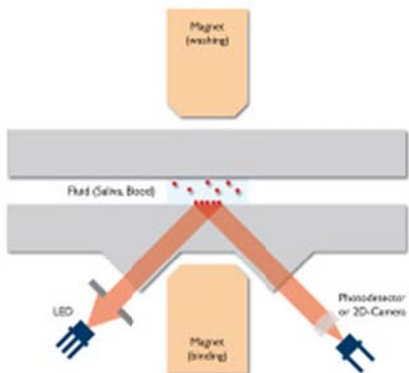


Figure 8: Detection by frustrated total internal reflection within the biosensor [71]

The full-plastics disposable cartridge itself has no moving or electronic parts and plugs into a handheld device containing all the expensive components, the electromagnets, optical detection system, control electronics, software and the read-out display.

Currently, optical elements like CCD and CMOS detectors, avalanche photodiodes, and light emitting diodes will be substituted by integrated and miniaturised flexible organic filters [72], adjustable organic micro-lenses [73], organic light emitting diodes (OLEDs) [74] and organic photodiodes (OPDs) [75].

Finally, automated microfluidic systems have the capacity to combine different analytical technologies. For instance, on-chip high-pressure liquid chromatography and capillary electrophoresis can be coupled to multi-spectral imaging and optical spectrometry on-chip, and capillary electrophoresis of on-chip amplified DNA has been combined with fluorescence detection [76, 77].

3.11.8. Nanopore technology — Nanochannels as biosensors

Selective transport in nanochannels (protein-based ion channels) is a widely used biological mechanism in organisms for divergent processes like electrical signalling or selective uptake/separation of substances. By mimicking this principle for new processes, nanochannels or arrays thereof can be developed as specific biosensors [78] or for purification and/or preconcentration of target molecules by transport through specific nanochannels.

3.11.9. Oxford Nanopore Technologies [79]

Oxford Nanopore was founded in 2005 around 10 years after the first concepts of nanopore-biosensors were proposed. Nanopores are nano-scale holes in barrier-membranes fabricated in different ways with various components: first, pore-forming proteins in lipid bilayer membranes as biological nanopores or second, synthetic materials such as silicon nitride, SiO_2 or graphene with holes prepared by focused ion or electron beams as solid state nanopores or third, to improve specificity, pore-forming protein complexes in synthetic materials as hybrid nanopores. Protein nanopores are usually very stable, reproducible and easy to modify.

When setting a voltage across this membrane an ionic current is generated through the nanopore. Measurement of characteristic current disruptions created by larger molecules passing through the pore or near its aperture makes it possible to identify the molecule in question. Thus this system can be used to identify target proteins, small molecules, or even distinguish between the four bases of DNA.

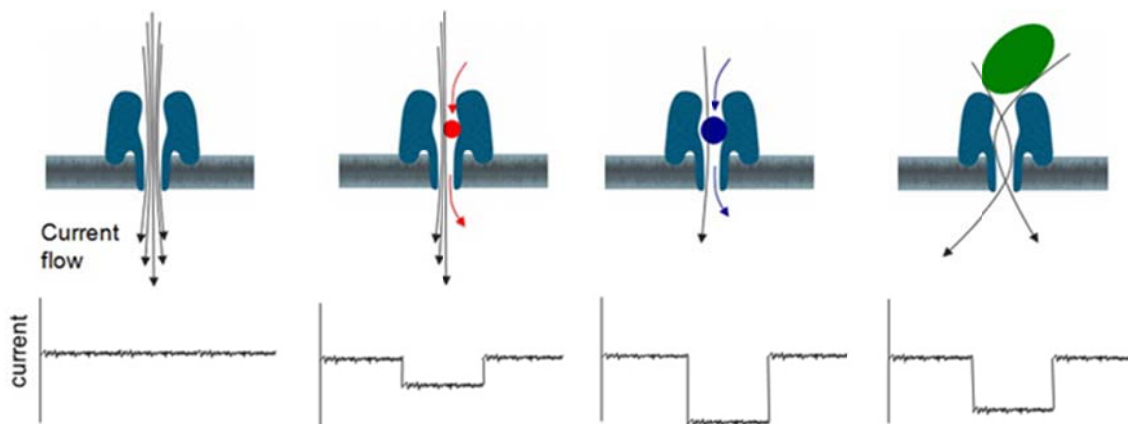


Figure 9: Oxford Nanopore technology [79]

Oxford Nanopore sequencing ('strand sequencing method') is a 3rd generation, single molecule sequencing concept generating long reads without deterioration of accuracy during sequencing.

A protein nanopore of a few nanometers in diameter is formed by insertion/embedding of pore-proteins into a high electronic resistance membrane created by synthetic polymers. Measurable disruption in electronic resistance occurs while molecules are passing through. When a complex of DNA with enzyme attaches to the outer side of the pore the enzyme unzips the double stranded DNA and one strand enters the aperture of the pore one base at

a time, changing conductivity base by base. Particular combinations of base pairs give characteristic electronic fingerprints by passing through and during deciphering these signals the sequence data are generated in real time.

As suitable candidate for protein nanopore assembly is α -hemolysin forming robust pores with an aperture of 1 nm, permeable for many single molecules, including DNA. Techniques to adapt the pore properties to specific needs include internal structural changes, incorporation of specific binding sites or DNA probes or attachment of enzymes or ligands to interact with target proteins outside the pore.

Future generations of nanopore sensing devices could consist of synthetic material like silicon nitride (SiNx) or silicon dioxide (SiO₂) with pores of variable size inserted by focused ion or electron beams. Solid-state nanopore technology is cheap and scalable to highly parallel processing but needs to reach chemical specificity of protein nanopores. This could be achieved by the integration of a protein pore component into the solid-state membrane generating hybrid pores.

Nanopores with integrated sensors like tunnelling electrode-based detectors, capacitive detectors and graphene-based nano-gap or edge state detectors are promising methods for replacement of ionic current measurement at much higher level of integration density and speed.

The electrical potential on a nanopore in a thin membrane of graphene (a single atomic layer of graphite) separating two compartments with ionic solutions can be measured while a DNA-strand is passing through the pore [80]. A trans-electrode of the thickness of one-atom-layers could be sufficient to distinguish between the four bases of the DNA-molecule passing by one at a time resolving the exact sequence by generating typical signatures at high resolution.

Graphene is a strong and chemically inert substance with excellent electrochemical properties and therefore well suited as sensor material. The technique of membrane fabrication and pore formation with precise structure and edge chemistry needs further development before applicability in label free sequencing.

Reverse transcription is needed as a prerequisite in most standard procedures for molecular characterisation of RNA (PCR, sequencing, cloning). Nanopore sequencing technology described here would be applicable as a future tool to analyse and characterise RNA strands in a direct manner.

Cell debris, particular proteins or fragments thereof released during cell degradation could serve as biomarkers for contaminations by pathogenic bacteria.

For the detection of proteins Oxford Nanopore is developing techniques for electronic analysis of proteins by combining nanopores with aptamers, short oligonucleotides binding specifically to proteins. These complexes attach to nanopores, but only aptamers pass through apertures and are detected, unbound aptamers passing through give distinguishable signals. Thus mixtures of different aptamers for different proteins are feasible for simultaneous detection of proteins by sequence analysis of their specific aptamers.

Even molecules like metabolites or small cell degradation products derived from pathogenic bacteria, envelope proteins from viral origin, toxic products from industry, drugs, pesticides, poisons could be detected by the same technology.

The modular instrumentation platforms, GridION™ and Min ION™, consists of array chips — combinations of nanopore membranes with standard semiconductor material within cartridges — and compatible proprietary electronics that enable multiple parallel experiments including data collection and analysis in real time.

Taken together nanopore sensing by GridION™ Technology can serve as a direct electronic method of nucleic acid-, protein- and small molecule-analysis and therefore can provide an early warning system for pathogenic bacteria by detection of cell degradation products (DNA, RNA, proteins, metabolites) with high specificity and sensitivity in the future.

3.11.10. Electronic tongues (ET)

Electronic tongues started with commercial applications in the food industry as fast, automatic systems for quality control and are capable of reliable discrimination between different brands of various beverages like mineral waters, tea, milk, beer and juices without any pretreatment in many applications [81–84].

They usually consist of sensor arrays with different electrodes (e.g. potentiometric ion-selective electrodes are widely used or electrochemical microsensors) and pattern recognition tools (e.g. principal component analysis and/or linear discriminant analysis) for data extraction and analysis of large amounts of data by various numerical techniques [85–91].

ETs can be designed in various architectures like electrodes or flow through cells and sensor types: electrochemical-voltammetric [92–95], electrochemical-potentiometric, the most widely used ET-sensors [96], electrochemical-impedimetric [97, 98], optical [99] or enzymatic sensors — also known as biosensors [100]. ETs consist of a series of polymer membranes, each coated with a different lipid that can be easily manufactured by application of membrane solutions on the transducer-core-surfaces or fitted onto a plastic

tube. When this ET is in contact with a sample, the electrical potential of the membranes changes in a characteristic way and the resulting signal pattern corresponds to taste.

Some examples for commercially available systems are the Taste Sensing System from Anritsu Corp., Atsugi (Japan) and Intelligent Sensor Technology of Kanagawa (Japan), ASTREE from Alpha MOS (France) and the Multiarray chemical sensor from McScience (Korea) [85, 101].

Advantages of ion selective electronic tongue, the most popular type of potentiometric sensors, are inexpensiveness, easy fabrication processes, simple set-up, knowledge of working principle, a high level of flexibility in modification of selectivity for variable species of molecules and miniaturisation potential.

Disadvantages are the dependence on temperature and matrix changes, alterations of membrane surface properties by adsorption of solution components and fouling, changing signal baseline (baseline instability/drift in long-time measurement) which can be counteracted by constant temperature, rinsing the electrodes with solvents or applying anti-fouling layers or correlation to the signal of a reference solution.

For monitoring of water quality calibrated flow-through analysis with miniaturised transducers is preferable because of reducing the sample volume (and consumption of reagents) and the response time. On the basis of modular flow-cell systems these units can be coupled sequentially with different types of sensors and/or with various pretreatment systems (e.g. homogenisation, filtering, dilution, etc.) [102, 103].

As a next step integrated sensor arrays were developed and successfully tested as electronic tongues in various applications (e.g. food production and cell culture monitoring). Usually they consist of core-bodies (e.g. epoxy-glass laminate or low temperature cofired ceramics) covered by membranes of polyvinylchloride or polyurethane including various chemosensitive components to form potentiometric sensor layers [104].

Originally developed mainly for automatic online quality control in the production of foodstuff, many new applications were found in environmental (e.g. monitoring of water contamination) and industrial process monitoring (pharmaceutical industry) and non-invasive medical diagnostics (analysis of human body fluids). Miniaturisation together with modern fabrication technologies and materials will allow the production of integrated arrays as components in mobile electronic tongue devices able to analyse small volume samples. Nevertheless, improvement concerning detection limits is needed to be sensitive enough for the analysis of trace amounts of substances dissolved in drinking water.

3.11.11. Electronic noses (EN)

Electronic noses consist of three components: sample processing system, detection system and data computing system. The EN-concept aims to mimic the mammalian olfactory system in a way that non-specific sensors generate signals upon interaction with odorants. These data-sets are then analysed by multivariate statistics algorithms to determine patterns which are compared to each other and show if one sample is similar to or different from another. Currently e-nose sensors are made of materials like organic polymers, metal oxides, and involve techniques like quartz crystal microbalance, gas-chromatography or mass spectroscopy [105].

Applications for EN technology in the food industry are focused on monitoring of production, shelf-life, freshness and authenticity [106].

A new generation of EN, the bioelectronic noses consist of olfactory receptor proteins and carbon nanotube transistors and are able to recognise specific odorants below a concentration range of one pM. This sensitivity can even be improved to a detection limit of one fM when nanovesicles derived from real cells involve human olfactory receptors and calcium ion signal pathways [107].

3.12. Next generation sequencing (NGS) [108–110]

The first system in the market, the 454 Genome Sequencer 20 from Roche, started in 2005 with 20 million bases (20 megabases or 20 Mb) per run. Unlike with classical sequencing, in this new technology every single sequence is visible and not covered by background signals. In ‘ultra deep’ sequencing approaches DNA-sections present in all bacteria are amplified from a sample and thousands or even millions of these amplicons are sequenced and analysed in a highly parallel way. After alignment of these more or less similar sequences — the differences are specific for each species — qualitative and quantitative measures can be deduced from databases and the number of hits. The result is knowledge about the different species and their relative amount in the investigated sample.

Unfortunately these NGS techniques from different suppliers do not allow real-time online monitoring yet. They are quite expensive and laborious with regard to preanalysis steps but the last 8 years showed a dramatic decrease in price for a megabase, which dropped from more than EUR 500 down to much less than EUR 0.1, and hands-on-time/time-to-result with a reduction from several days to a few hours. On the other side, output was greatly increased and comes close to one Tb per run (terabase or trillion of bases).

Next-next generation sequencing or better to say ‘third generation sequencing’ looks very promising as a candidate for online sequencing. Advantageous is the reduction of

preanalytical steps but DNA still has to be purified and concentrated. Development of small elements like USB sticks harbouring all system components like microfluidics, chips with nanopores and integrated semiconductor detection and data acquisition units is on the way. Online computing algorithms connected to these sensors could search online for sequences of highly pathogenic agents by comparing with public or special database collections like BLAST [111].

One available single molecule real-time sequencing (SMRT) technology developed by Pacific Biosciences [112] employs the 'zero mode waveguide' (ZMW) principle with a single observation volume of 20 zeptoliter ($1\text{zL} = 10\text{e-}21\text{L}$). Sequencing by synthesis is carried out by adding nucleotides from a pool/mixture of four phospholinked hexaphosphate nucleotides labelled with four dyes corresponding to the four different types of nucleotides (Figure 10). This technique allows the detection of very weak fluorescent signals as background is minimised by ZMW and sequencing is carried out very close to the detection surface on a single DNA-polymerase molecule immobilised on the bottom of the hole. Only one molecule of DNA is sufficient for sequencing and therefore obviates the need for preanalytic PCR-amplification procedures and reduces time to result dramatically. First introduced to the market in 2010/11, SMRT now generates average and maximum read-lengths of 8 500 and 30 000 bases, respectively, in 150 000 ZMW-holes on a sequencing chip in parallel. At present, disadvantages of this system are instrument costs, size/weight and a relatively low accuracy for single reads, which can be improved by increasing the coverage (e.g. 20-fold sequencing will increase accuracy to 99.999 %).

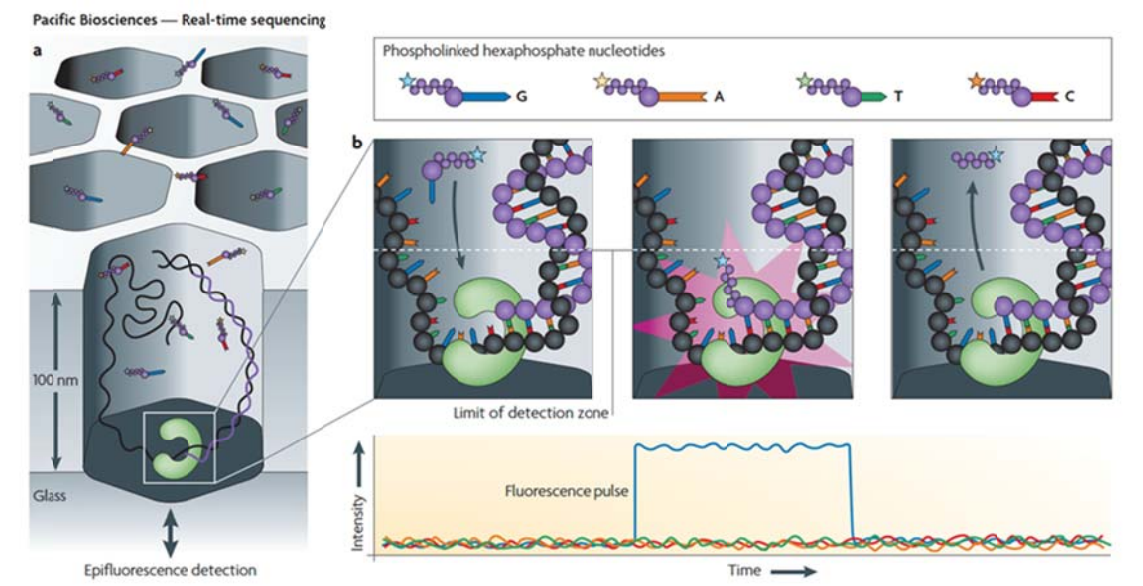


Figure 10: Single molecule real-time sequencing (SMRT) by Pacific Biosciences [113]

3.13. Epidemiology and 'human sensors'

Analyses of outbreaks of waterborne diseases from the last decades in different countries have shown the importance of complete epidemiological and environmental data-sets including immediate reporting of incidents by the operator, rapid case-reporting from hospitals, health insurance data on drug-reimbursement, visual (turbidity) and olfactorial (off-flavour) sensations/observations from the population [114, 115].

4. Summary/discussion

Our water supply networks are exposed to deliberate or accidental contamination and therefore need continuous examination. Yet, numerous systems to measure physical (temperature or turbidity) and chemical parameters like ions and organic compounds are available and well established for online monitoring in flow-through systems. They respond in a rapid and reliable way, eliminate false alarms, are easy to handle, operate economically and their specificity and sensitivity is sufficient for their application. But at present, analytical techniques for agents like strong biological poisons or pathogens like viruses, bacteria and protozoa are time-consuming. Parameters like turbidity or free chlorine are the only commercially available and widely used online tools to indicate threats but ultimately they are just surrogate-indicators that give unspecific and vague hints for the growth potential of any pathogen. Early identification of contaminants in drinking water requires an online-capable broadband sensor system. At present there are a number of important water quality parameters that cannot be monitored in real-time.

Most of the summarised technologies shown here are technically not mature yet but on the way to becoming more and more viable in fulfilling the needs for online monitoring devices in water quality control (Table 1). Biosensors, for example, a rapid, sensitive and specific detection technology of chemical and biological analytes can be used in medical diagnostics, environmental monitoring, food safety and security. Nevertheless, single-use, disposable cartridges need automated exchange devices or have to be cleaned/rinsed to be reused several times. Electronic tongues, especially for monitoring toxicity, and electronic noses are quite promising and are rapidly developing by improving capabilities, reducing production and reagent costs, becoming smaller and easier to handle. They could be applied for

monitoring of pesticides, endocrine disrupting compounds, pharmaceuticals, trihalomethanes, nitrosamines and volatile organic compounds. Although biosensors and electronic tongues develop rapidly, there are no commercially available sensors for pathogen detection performing comparably to chemical sensors (e.g. pH sensors).

Method	Analytes	Sensitivity	Costs	Market	Remarks
Real-time-PCR	DNA, (RNA)	high	low	available (a)	microfluidics, (multiplexing)
Hybridisation	DNA, RNA	medium	low	available (a)	microfluidics, multiplexing
Immunology	proteins	medium	low	available (a)	microfluidics, multiplexing
Enzymatic	metabolites	medium	low	available (a)	microfluidics
Electronic tongue/nose	soluble/volatile substances	medium	low	available	potential for miniaturisation
Nanoparticles coated with DNA, antibodies	DNA, RNA, proteins, molecules	medium	low	available	magnetic, purification, enrichment, transportation, microfluidics
Nanopores	DNA, RNA, proteins, molecules	medium–high	low	in development	potential for 3rd generation sequencing (single molecule sequencing)

Table 1: Techniques used in biosensors; (a) available mainly for medical diagnosis

Other systems (Table 2) like mass spectrometry or next generation sequencing seem to be far away from being suitable for online monitoring in the field, but NGS especially, and third generation even more, are evolving extremely rapidly and are therefore very promising technologies.

Method	Analyte	Sensitivity	Costs	Market	Remarks
Next generation sequencing	DNA, (RNA)	high	high	no flow through	miniaturisation in 3rd generation sequencing?
Flow through microscopy	cells, particles	medium–high	high	in development	smaller devices possible
Surrogate organisms	toxins	medium	medium	available	small devices
Mass spectroscopy	(bio-) molecules	medium–high	high	combination with other techniques	miniaturisation difficult/not possible?
Raman spectroscopy	cells	medium–high	medium	in development	small devices possible
Infrared spectroscopy	organic compounds	medium	medium	in development	small devices possible
Flow cytometry	cells, particles	medium–high	medium–high	in development	microfluidics, miniaturisation possible

Table 2: Other techniques

In the meantime, as long as specific broadband sensors for waterborne pathogens are absent in the market, surrogate technology is highly appreciated as an indicator for deviations from normal water quality to compensate this lack in specific early warning systems.

It can be concluded that more effort in research has to be undertaken to identify and characterise waterborne threats in real-time and additional funds have to be made available to increase water security.

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Abstract

Water quality is a critical factor to public health worldwide. Accidents in past and present documented the vulnerability of our water supply chain. Therefore fast, reliable, sensitive and cheap water-monitoring systems are needed, working independently at a low level of maintenance and hands on time. Today there are only limited technologies to monitor pathogenic agents available on the market. The following review should give an overview about the major technologies being developed and evaluated today and could have potential as monitoring systems in the near future.

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