Biotechnology II

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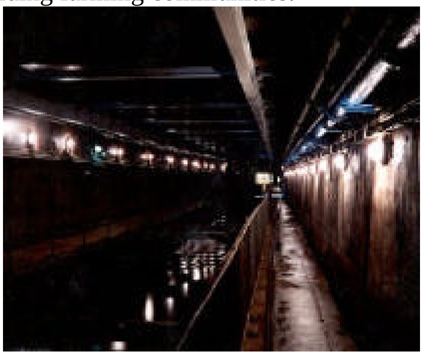
Part I - Introduction - Liquid Waste: As an applied science, microbiology deals with many important practical problems in medicine, agriculture, and industry. Some of the most important diseases of humans, animals, and plants are caused by microorganisms. Microorganisms play a major roles in soil fertility and animal production. Many large-scale industrial processes are microbially based, which has led to the development of a whole new discipline, **biotechnology** (BT). In the broad sense BT entails the use of microorganisms in large-scale industrial applications of genetic procedures, generally to create novel microorganisms capable of synthesizing specific products of high commercial value. BT is highly dependant on **genetic engineering** and **recombinant DNA**, the disciplines that concern the artificial manipulation of genes and their products. This second part of the Biotech-lesson will penetrate deeper into specialized BT applications focusing on physical and engineering aspects.

Potential applications of BT in communal effluents: Because of the enormous metabolic diversity of Bacteria, a large gene pool exists in bacteria from natural habitats. In some cases these genes code for proteins that degrade environmental pollutants. Genetic engineering is beginning to tap these resources for the purpose of environmental cleanup. In many cases the gene donors are bacterial strains isolated from contaminated sites. Some examples include genes for the biodegradation of chlorinated pesticides, chlorophenolics, naphtalene, toluene, anilines, and various hydrocarbons. The desired genes are isolated from species of *Pseudomonas*, *Alcaligens*, and a few other bacteria and then cloned into plasmids (see BT-I).

I. 1) **Historical flashback in wastewater treatment** (traditional methods - in reference to the underground sewage system of **Paris**). Till the turn of the 19th century, sewage was generally dumped in household bunkers. The very simple construction made is possible that the waste matter gradually drained off its liquid fraction into the ground water. In many other cases, where bunkers were not available, sewage was transported out of town and used to fertilize the fields of the surrounding farming communities.

¬ Mairie de Paris (FRA)

The sewer museum (Paris - FRA) ®



- In about 1200, P.August made the decision to pave the streets of Paris and lay a drainage ditch down the center. In 1370, H.Aubriot, provost of Paris had a vaulted, stone sewer built in the rue Montmartre, which joined up with the Ménilmontant stream. The network then developed slowly through the centuries and it was not until **1850**, with the arrival of Baron Haussmann, prefect of the Seine, and the engineer E. Belgrand, that today's Parisian sewers and water supply network was developed. The dual network, comprising water distribution (potable and non-potable) and sewers reached 600 km in 1878.
- In **1907**, Paris decided to introduce the water closet (water entered from one side of the bowl and through the top at the same time, causing the bowl to flush); as a consequence, many sewage bunkers were spilled over and worsened the already tight situation. In addition, sewage was just flushed down the road (rainwater drainage canal) into the Seine river.

¬ Pneumatic closet by H. Huber & Co. in 1884.

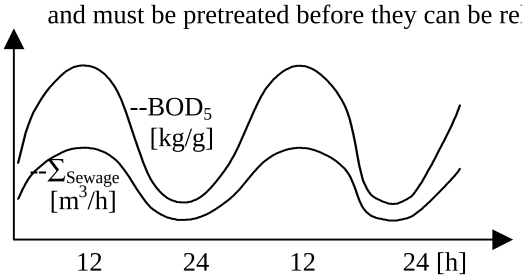
- In order to tackle the problem, the authorities started to build an underground canal system. Though, the implementation of such an underground drainage system not only required profound expertise in constructional knowledge (3rd man movie - shot in Vienna) but it did not really solve the problem. Sewage was just relocated from above to underground with no treatment what so ever; the classical “out of sight out of mind” mentality. Inevitably, sewage went on to pollute most European waterways.
- Only since the **1950**s, the postwar industrial boom aggravated this trend, as communal sewage and industrial waste waters started to become a major issue of concern. In the States, oil polluted rivers (overload of hydrocarbons) literally burned as they self-combusting reactions triggered the reaction. Since the 1960s, waste water treatment plants have been constructed to face this hygienical and environmental emergency. Only then, the end of pipe (EOP) attitude started to give place to a cause and effect oriented thinking as more and more sewage collection pipes delivered its cargo to specially adapted processing plants (e.g. in the early 1980s Austria established a network of ring canals to overcome the problem of eutrophicating lakes). By now, it has been shown that the EOP-approach is not only expensive but an unnecessary liability for future generations.

The attitude of “dissolution is the problem to pollution” could no longer be maintained as the quantity and toxicity of liquid wastes became a pressing challenge. Consequently, modern day sewage plants have to deal with a mixed pollutant composition (industrial and communal). Although, production of a valuable commercial product is not the goal of sewage and wastewater treatment, the process itself is clearly a large-scale use of microorganisms and can be considered a type of bio-conversion. In addition, the clean-up industry created a extensive spectrum of professionals that involve engineers, physicists, geneticist, and biotechnicians.

Action and Reaction	Dumping of sewage	®	Collection pipe system	®	Mechanical purification	®	Biotech. treatment
Time scale	“1890”		“1920”		“1960”		2000
	------------ sewage dilution ----------				----------- sewage processing ----------		
Occupational profile	craftsmen		engineer		public health specialist, environm. technician		Biotechnician

I. 2) Sewage and **Wastewater**: These are materials derived from domestic sewage or industrial effluents, which for reasons of public health and for recreational, economic, and aesthetic considerations cannot be disposed of merely by discarding them untreated into lakes or streams. Wastewater plants are usually constructed to handle both domestic and industrial wastes.

- **Communal wastewater**: As it originates predominantly from individual households, kindergartens, laundries, hospitals, restaurants, etc. is made up of sewage, "gray water", and wastewater from food processing it is the most heterogeneous among all.
- **Industrial wastewaters** include those from petrochemical, pesticide, food, plastics, paper, pharmaceutical, and from metallurgical industries. Many industrial wastes contain toxic substances and must be pretreated before they can be released for wastewater treatment.



Sewage plant capacity vs. time

BOD = O_2 needed to breakdown organic material [mg O_2/L] - it is an indicator to determine the extent of pollution in a body of water.
High BOD: Water with sewage pollution - eutrophic lakes (100-1000mg/L)
Low BOD: Oligotrophic lakes - clean mountain streams (2mg/L)

The scan on the left depicts the cyclical pattern of wastewater generated in a city with about $200 \cdot E^3$ inhabitants (e.g. Salzburg - AUT):

The **biochemical oxygen demand** (BOD) refers to the amount of oxygen that would be consumed if all the organics in 1L of water were oxidized by bacteria and protozoa. An O_2-meter is used to determine the concentration of oxygen within the vial containing the water sample. Another vial of sample is sealed and placed in darkness and tested five days later. BOD is then determined by subtracting the second meter reading from the first. The range of possible readings can vary considerably: water from an exceptionally clear lake might show a BOD of less than 2mg/L of water. Raw sewage may give readings in the 100s and food processing wastes may be in the 1000mg/L!

Any sewage treatment plant must be able to handle both peak loads of waste water as well as the average load without turning into tilt (e.g. Y2K problem in an LA-sewage treatment plant; by ignoring the compatibility to Y2K, the plant spilled over when the date-shifting took place).
Therefore, the plant must be capable to accommodate daily, weekly, monthly, and seasonal fluctuations in waste water inputs (depends also largely on industrial process applications, that may discharge $100 \cdot E^3$ liters of waste water after a production cycle came to an end).

Composition of raw sewage

Category	M	O	∑		[inhabtant/m^3]
solid fraction (settles down)	20	30	50	} BOD_5	M....inorganic
Solid fraction (not settling)	5	10	15	$60g \cdot inhab.^{-1} \cdot day^{-1}$	O.....organic
Dissolved fraction	75	50	125		T.....total
Σ	100	90	**190**		190 g/(inhabitant·day)
Amount of sewage	M	O	∑		[g/(inhabitant·day)]
solid fraction (settles down)	100	150	250	} BOD_5	
Solid fraction (not settling)	25	50	75	300 g/m^3	.... generally factor 5
Dissolved fraction (dominates)	375	250	625		
Σ	500	450	**950**		950 g/(inhabitant·m^3)

Case study (26^{th} of April 1986): After the explosion of the Chernobyl nuclear power plant, a radioactive cloud moved over W-Europe. The washout-effect enriched many alpine areas with ^{137}Cs. As a result , the milk of grazing cows was highly radioactive; the radioactivity accumulating property of whey, which is left over after cheese production was even more contaminated than the raw product itself. Treating whey in sewage plants is difficult, but radioactive contamination turned it into toxic waste (toxic **"whey train"** that moved across Europe and finally was parked in Rosenheim - Bavaria; the cargo was later on incinerated, while the radioactive ash was discharged in appropriate landfills.

Industrial Sewage and Population Equivalent: As mentioned before, wastewater from commercial applications (not related to service industry; i.e. Cafeterias, white collar offices, etc.) alter significantly in their composition from communal sewage. Treatment of industrial wastewater basically depends upon the:

- Method of production
- Amount
- Composition
- Used raw materials

} .... determines the way sewage is treated

Process water of vegetable or animal refinement from the food, paper, wool, tanneries, liquor burning industry, etc.) can be treated with the biological means (theoretically, also sewage with a high organic-chemical load could be treated in this way). Heavily polluted waters undergo first a pre-cleaning phase, in order to obtain a 50-70% reduction of the most problematic toxins, while the reminder, moderately polluted outflow, is feed into the communal waste water treatment plant.

- Reminder: The relation b/w the waste water and the community / industrial facility is expressed by the **Population equivalent** (PE); i.e. as it would equal to the equivalent of 10, 100, $100 \cdot E^3$ people. PE is a measurement of organic biodegradable load, and a population equivalent of 1 (1p.e.) is the organic biodegradable load having a five-day biochemical oxygen demand (BOD_5) of 60g of oxygen per day (the load shall be calculated on the basis of the maximum average weekly load entering the treatment plant during the year, excluding unusual situations such as those due to heavy rain);

Industrial application	**Processing quantity**	**PE** [60g $B5B_5$ / $(I \cdot d)$]
Brewery	1000L of beer	150-250
Paper-mill	1t of paper	200-900
Dairy (w/o cheese production)	1000L of milk	25-70
Wool-cleansing	1t of wool	2000-4500

According to the PE and the presence of an industrial facility, the communal waste-water treatment plant experiences an increase in the overall handling capacity as if the community would experienced a boost in its allover population.

According to the definition given above, sewage is not a homogenous substrate. Despite its a complex mixture, four major criteria can be established to characterize sewage:

1. Neither qualitative nor quantitative constant substrata are present over the day, week, month, or year.
2. Uncontrollable, not foreseen events (local downpours, dry periods, etc.) may lead to a more diluted / concentrated sewage may result in peak-values that exceed average values, leading to a temporary overload.
3. Substrate that is supplied in such huge quantities does not allow implementation of biotechnological means under sterile conditions (except inoculation).
4. The average substrate concentration are usually so low, that any inoculated organism is usually washed out when kept in a “flow-through” mode rather than grow to maintain biological degradation; i.e.
   - holding back biomass in order to avoid wash-out effect;
   - washed out biomass has to be re-fed into the system;

Accordingly, wastewater treatment is considered as a "living organisms" that works under the open sky (at a temperature range of sometimes –20 - +20°C).

Stages in wastewater treatment: Sewage treatment is generally a multistep process employing both physical and biological treatment steps. **Primary treatment** of sewage consists of physical separations. Sewage entering the treatment plant is passed through a series of grates and screens that remove large floating objects, and then the effluent is left to settle for a number of hours to allow suspended solids to sediment (see also BT-I). The **secondary treatment** is aimed to reduce the high nutrient loads that remain in sewage effluent following primary treatment. **Tertiary treatment** is the most complete method of treating sewage. It implies a physico-chemical process (ion exchanger, absorber, etc.) employing precipitation, filtration, and chlorination to sharply reduce the levels of inorganic nutrients, especially phosphate and nitrate, from the final effluent (Wastewater receiving proper tertiary treatment is unable to support extensive microbial growth). All together the plant has to focus on two essential aspects:

i) Microbial removal of these components require $\mathbf{O_2}$;
i) removal of toxins require a gradual sequence of microbial activity (**Biocoenosis**).

I. 3) Parameters in wastewater treatment: As mentioned before, waste water does not have distinct characteristics; i.e. can't be described by a s single parameter or compound but rather as a complex assemblage of key constituents and parameters:

- **Biological Oxygen Demand** (BOD_5): It is a measure of the quantity of oxygen used in the biochemical oxidation of carbonaceous and nitrogenous compounds in a specified time, at a specified temperature and under specified conditions. The standard measurement is made for five days at 20°C and is termed BOD_5. BOD is an indicator of the presence of organic material in the water - see also p.3.
  The efficiency of such a plant is referred by the fastness of the BOB_5-removal over time.
- **Chemical Oxygen Demand** (COD): It is a measure of the quantity of oxygen used in the chemical oxidation of compounds in a specified time, at a specified temperature and under specified conditions; e.g. how many mg/d of a chemical oxidizer (as $KMnO_4$) is required.
  None of these parameters characterize sewage properly; ultimately, it depends upon the processes applied. As waste water introduced untreated into the environment deprives fresh and salt-water organisms in the wild of their oxygen, and triggers major die-offs, sewage has to be cleared from the O_2 consuming agents before it can be discharged. Biotechnological sewage treatment tries to achieve just that; it is able to achieve a temporal gradient; i.e. a change of COD over time ($\Delta c/\Delta t$).
- **Phosphate** (P) and **Nitrogen** (N) **components**: It is essential to reduce the organic nutrient level within the sewage effluent; failure to do so will ultimately result in algal blooms and eutrophication of affected water-bodies (N is the limiting nutrient in *limnic* systems, as P is for *marine* environments).Fortunately, clapping of chemicals and other refuse into the ocean is no longer allowed within the EU. And still, insufficient sewage treatment in the industrial belt of northern Italy still trigger major marine snow outbreaks, which appear in the northern Adriatic Sea off the shores of the Po-delta as algal blobs or even as huge algal carpets that block off sunlight.

  N: n + components have 4 major impulse (determinant).
  i) Consumption of Oxygen during nitrification of ammonium
  i) Fish poisoning (NH_3-amonium)
  i) Promotes plant growth (eutrophication)
  P: Another eutrophicating agent → reduction & removal of inorganic nutrients.
  Removal of both nutrients are achieved in a coagulation basis, where chemicals are added to mediate this process:
  Removal of N in the biologic pathway implies the conversion of ammonium to nitrite:
  Nitrification: $2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$
  Conversion of nitrite to nitrate: $2NO_2^- + O_2 \rightarrow 2NO_3^-$
  Denitrification: $2NO_3^- + 10H \rightarrow N_2 + 2OH^- + 4H_2O$
  Removal of P is achieved by precipitating it via chemical agents; such agents contain aluminum and iron.
  As most phosphate originates from washing detergents, the meta-phosphate $Na(PO_3)_n$ is enzymatically converted to ortho-phosophate PO_4^{3-}; orthophosphate can be precipitated by adding aluminum-sulfate $Al_2(SO_4)_3$ as trivalent aliminum-phosphate:
  $2PO_4^{3-} + Al_2(SO_4)_3 \rightarrow 2Al_2PO_4 + 3SO_4^{3-}$
  Alternatively, phosphate can be extrcted with trivalent iron-ions:
  $4Fe^{3+} + 4PO_4^{3-} + 4H^+ + O^2 \rightarrow 4FePO_4 + 4H_2O$
  a truck accident in the UK, which was loaded with phosphate fertilizer threatened to pollute a ground-water reservoir; treatment with Al-compounds trapped the phosphate ions and cause them to flock out as an insoluble precipitate.
  Biological removal of P can be obtained by introducing P-consuming microorganisms into the sludge – usually the so called 3rd stage in sludge treatment includes P- and N-trapping devices.
  Threshold levels in drinking water- waste management treatment are not removable by standard means ; today's applied "end of the pipe" (currently, cleaning of polluted water doesn't yet involve the prevention of pollution!).
- **Chlorination**: It is the most common method of ensuring microbiological safety in a water supply. In sufficient doses it causes the death of most microorganisms within 30mins. In addition, chlorine reacts with organic compounds, oxidizing and effectively neutralizing them. Chlorine is added to water either from a concentrated solution of sodium or calcium hyperchlorite or as a gas from pressurized tanks. Chlorination of water is the final step in sewage processing and only required if the water is provided as potable water to the community.

II) Biotech applications: BT offers alternative means. In the following clean-up technologies will be described:

- Biotechnological waste water treatment is one of the most successful applications of biological processes for keeping the environment clean; sewage treatment is thus a new specific application of biological wastewater treatment. Large-scale technical application of biological processes to detoxify, treat and upgrade wastes that are to a large extent of organic origin.
- Land and soil bio-remediation is another "mild" technology used to clean up contaminated soil and other contaminated sites (filling stations, industrial installations, landfills, etc.).
- Biotechnological waste gas treatment may in certain conditions represent an effective alternative to physico-chemical technologies.
- Bio-hydrometallurgy: a promising technology for the treatment of waste and waste water containing metals and - from an international point of view - for the enrichment of ores, or as mineral leaching.

Objective: Process control will focus on monitoring, description and control of a biological process. The original objective was formulated on the basis of an experimental system for anaerobic wastewater treatment. During this process anaerobic bacteria decompose organic matter, which is gradually degraded to biogas (a mixture of methane and carbon dioxide). Depending on the nature of that substrate material, biological activity is capable to remove up to 99% of the organic matter. So far most industrial applications relied on anaerobic processes. They have the big advantage in that the process elements and dynamics are very typical for most biotechnological applications which are predominantly anaerobic batch processes.

It should not be forgotten that **biological processes** are among the most challenging to predict and to control. Bacteria are not that simple as widely believed:

- Under a biochemical aspect, they are complex organisms who can adjust their behavior to a changing environment.
- Bacteria grow and multiply; therefore the response of a biological reaction changes irreversible over time.
- When non-pure cultures are used (as is the case of a biological treatment process), many different species are involved. Each of the different species have different physiological requirements, which means that they catalyze different (bio)chemical conversion processes, often related to the gradual degradation of fats, proteins and carbohydrates.
- Different species make up an ecosystem. Many species are interrelated because they are in their "feeding patterns" interdependent, often similar to a food chain. Other interactions may occur as well such as symbiosis, inhibition by toxicity, interactions with viruses and predation (to name a few).
- Any change in the environment can change the ecosystem, its components, how they behave and eventually the biocenosis of a reaction chain.

The result of these complexities is that bacterial systems are often difficult to predict (in particular mixed cultures that house a variety of many different species - it is quite possible to carry out three identical experiments and obtain three different results!).
Such unpredictable system behavior requires an advanced, intelligent control system which learns from the observations of the process dynamics and takes appropriate control action.

Another problem with biologically controlled processes is that on-line sensors which detect the essential parameters do not exist, and if they exist they are prohibitively expensive. Several parameters such as pH and ORP (Oxidation Reduction Potential) can be measured, but their significance in relation to the process is often not fully understood. It is also common for sensors (and process variables) to behave differently during different stages in the process. For instance, the dynamics of gas production in an anaerobic process are much slower in a stressed system than in an unstressed, well adapted system.

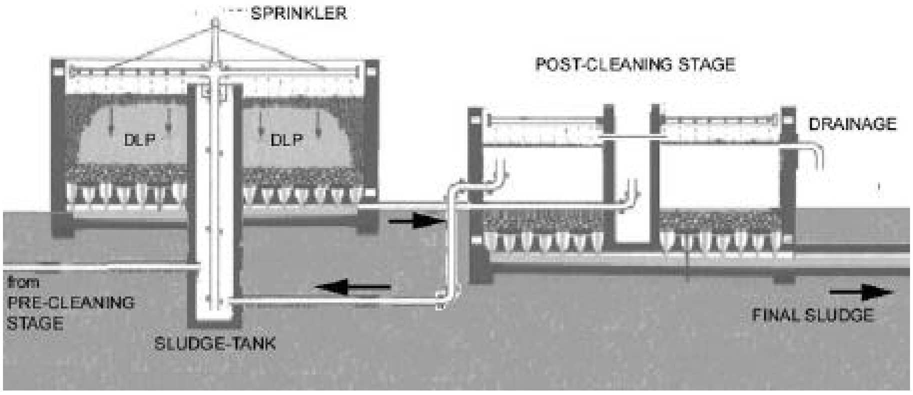
II. 1) DRIP procedure (DLP, also known as a **trickling filter**): In early 1894, Corbett developed an optimized procedure to reduce the volumetric requirements of waste simply by accumulating biologic material. He found out (upon exposure to light) that a biocenossis of bacteria, algae, worms, larvae, etc. has developed in his tiny grid as sewage effluent gradually dripped across it; i.e.

- a vertical gradient from top to bottom (reduced nutrient content the further down the solution drips)
- dissolution of contaminant (colloidal) as bacteria and protozoa consume them, whereas larval and worms predominantly account for the mechanical disintegration of the substrate;

He had to face one problem though: the clogging of the drip grid down the mat and a severe temperature dependence. He was able to tackle clogging simply by allowing insects to control the invertebrate population, while it was not so easy to overcome lower cleaning efficiencies in winter (seasonal dependence).

Nonetheless, the setup had a clear advantage: O_2 as the limiting factor does not restrict reaction as sufficient ventilation is guaranteed (compact setups although, require artificial ventilation).

Working principle of DLP: A sprinkler equally distributes the wastewater onto the math until an equilibrium between addition of nutrients and removal of processed water (without biomass) is reached; a balanced in- and output prevents clogging of the grid block and later on mineralization of the biomass;



Technical realization of DLP: The wastewater is sprayed onto the trickling filter bed; the organic matter adsorb to the grid and microbial growth takes place.

Modern DLPs: the diameter of the cylinder is determined by the amount and the pollution level of waste water; the grid block is made of concrete that is loaded with granulates. Traditionally, slag of steel furnaces or coal power plants have been used to produce the grid of granulate (provided a surface area of 90-100 m^2/m^3; average $\varnothing$ = 4-8 cm); these specifications guaranteed a remnant pore volume of ≥ 50%. Using synthetic granulates with a surface area of 250 m^2/m^3 and a pore volume of ≥ 90% boosts bacterially mediated decomposition enormously. Synthetic granulates were designed for high load sewage and are capable of removing up to 50% of the load from the wastewater. Shifting from naturally occurring microorganisms to GM-strains seem to rise cleaning efficiencies even further.

Post-stage: the post stage is required to remove any sludge that might exit the DLP as run-off.

A low of BOD_5 does not automatically involve an involuntary removal of sludge in the run-off; it rather clogs the system rather than removing the O_2-starving BT-organisms sitting on the grid.

Generally, a low load facility processes very dry sludge; whereas, a high load facility tend to process a wetter sludge. In the latter, biomass tends to be washed out, depriving the drip-lawn of its essential microorganisms.

A major advantage, that currently is not used due to the controversy of the subject, is the potential utilization of the wet sludge as an agricultural fertilizer.

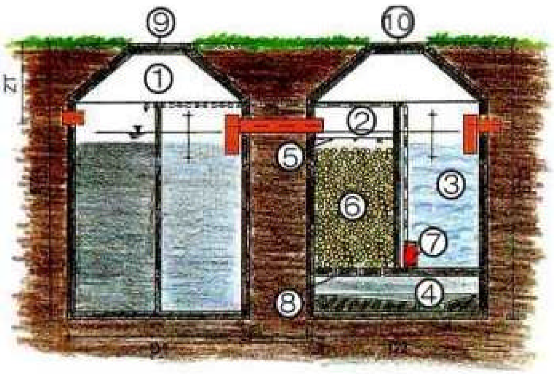
Ventilation of a DLP: Ventilation is achieved by aeration slits; i.e. takes place without any artificial means: The temperature gradient inside the gridblock versus ambient air-temperature facilitates an uplifting air-current through the granulate. Theoretically, non-ventilated DLPs experience two extreme working conditions:

- in summer, when outside temperatures are warmer than inside the DLP, the flow of air is directed downwards,
- while in winter, with ambient air temperatures a lot lower than inside the DLP, it is reversed. This implies that the ventilation flow changes twice a year; i.e. in spring and fall ventilation is very poor (due to the limited temperature gradient).

To maintain a constant cleansing result, artificial ventilation is applied. As the DLP has to be sealed off, it enables the use of charcoal filters to avoid downwind swells, while keeping the myriad of flies trapped within the reaction chamber.

New approaches involve a slowly rotating drip-lawn which enable floculation of bacteria in the waste water while increasing granular ventilation; doing so further improves cleansing results. Nevertheless, nitrate and phosphate-removal remain still very poor.
To overcome these limitations, Lockett & Akdern (1913) modified this principle by exposing the sludge to increased O_2-levels in an attached aeration-stage. Although N- and P-removal where still not very satisfactory, this method further improved the final cleansing results.

Traditional: Organic waste components are mineralized to CO_2, CH_4.... or are incorporated into the sludge. The latter requires aeration with compressed air to achieve a high turnover rate.
New: The **Bubble Reactors** works differently as with increasing depth, pO_2 rises within the wastewater column; higher sludge and waste concentration are achieved by ventilating it with pure O_2 (an expensive option). Bubble reactors are nowadays also made as cascades which generates a concentration gradient (higher contamination vs. lower ones). Splitting sludge processes into several steps also optimizes cleansing efficiency.



- 1. Pre-stage; 2. DLP; 3. Post-stage; 4. Settlement; 5. Stainless steel tubes; 6. Synthetic granulate 7. Pump to recycle sludge; 8. Perforation in DLP; 9. Lid w/o ventilation slits; 10. Lid w/ vents

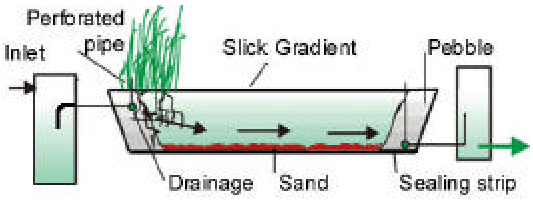
Efficiency: Again efficiency (η) is based on how much oxygen is required (determined via unit BOD_5 consumed versus dry sludge per day):

$$\eta = \frac{BOD_5\ [kg/d]}{DS\ [kg/d]}$$

the ratio of waste load compound vs. dry sludge produced automatically determines the size of the plant to obtain a desired efficiency level $[m^{-3} \cdot d^{-1}]$.

What to do with the sludge? The sludge as the harvested product consist mostly of floculated bacteria. The microorganisms contained within are usually not only of bacterial origin (mostly $Gram^{neg}$ and few $Gram^{pos}$; i.e. Micrococcocea), but include also fungi (saprophytes) and protozoa.
Sludge-Filtration: Filtration increases transparency of the final "waste" product; BT uses indicator organisms to determine the level of reaction. It has to be remembered that any GM-organisms used in this application is transferred from the lab to the open environment, thus leaving the controlled, sterile, and sealed lab-environment! Therefore, these modified microorganisms are not only exposed to fluctuating temperatures, pH-gradient, salt-fluctuations (winter road salting), but come into contact with the real world - once out there it can't be removed any more!
The **fermentation of sludge** usually occurs as bacterial formation attach in stream-like aggregations to the CH`s and sulfates of the organic matter; such aggregations disturb the process, the reason for such a behavior is not yet well understood; in any case, processed sludge has to be chlorinated in order to bring the process to a halt.

II.2) Biological waste water ponds (WWP), are becoming increasingly attractive as they are based on natural (look-alike), biotopes, using autotrophic (algae) as well as ragweed, and heterotrophic organism (zooplankton to fish, etc.); in addition, benthic chemical reactions due to sedimentation facilitate cleansing effect.



Accordingly, several types can be distinguished:

- Classical sedimentation WWP: for sold matter sedimentation only.
- Non-ventilated WWP: exposing body of water to air and sunlight (proliferation of algae).
- Ventilated WWP: aeration with O_2 increases pO_2, and turbulence (boosts turn-over rate).
- Plant WWP: artificially planted vegetation to increases cleansing efficiency.
  a) Artificial current waste water ponds: vertical, or better horizontal currents across the pond towards the plantation belt; suspended organic matter ultimately become trapped by the root system.
  b) Drip fields: soil with a high ratio of solid-air interface; as the sewage drips through the field, it traps suspended dissolved organic matter (DOM) and sold fraction.
  c) Irrigation flats: for a period of 10-20 days a particular area is sprinkled with sewage, stopped while a substitute area is treated.

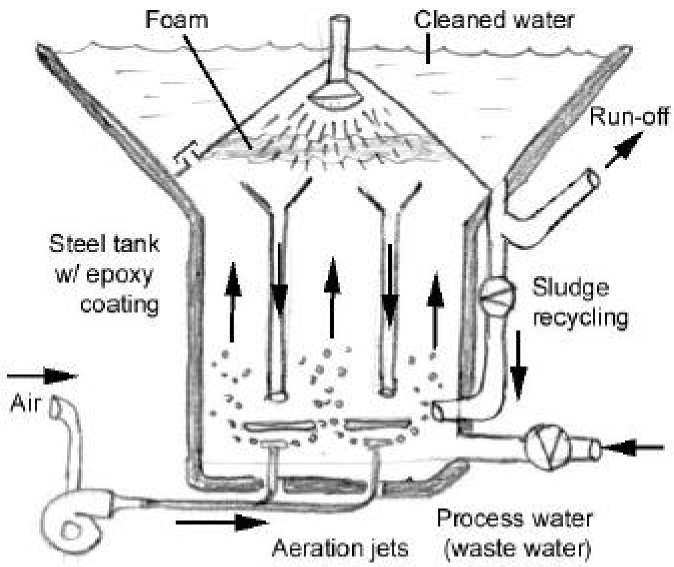
The disadvantage of such ponds is evident: the biotope is left on its own once the system is set up, it is somewhat "uncontrollable".

II. 3) Bio-Reactor Towers (BRT) for the treatment of Wastewater:

First trials in Europe started in the early 60`s; early BRTs consisted of a huge shallow concrete basins that covered an enormous amount of landspace were used to treat sewage biologically. Later trials, involving the Batch Process, worked with huge amounts of water that required aeration. For that purpose, large quantities of compressed air were used that consumed huge amounts of electricity. Besides being hungry for land, these attempts had other major disadvantages:

- Compressors generated a substantial amount of noise.
- Shallow but huge basins were kept under the open sky, generating downwind smell.
- Peak loads caused anoxic and toxic gradients that caused the system to go into tilt.
- Autotrophic organisms have only a limited lifetime; furthermore, huge basins are usually more unstable than smaller ones; micro-cracks enabled microorganisms to leak out of the confinement to pollute the groundwater-systems, leading to secondary contamination that resulted in an extra cost burden.

Out of these difficulties, the concept of a 20m tall bio-reactor tower was developed;



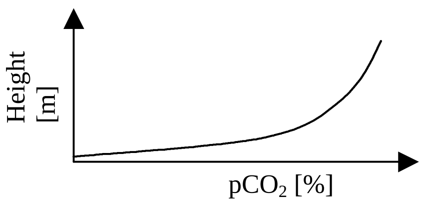
Here, the wastewater to be treated is mixed and aerated in a large tank. The activated sludge soon boosts growth of slime forming bacteria, primarily *Zoogloea* species. As they grow and form flocs, they themselves serve as substratum in which protozoa and small animals attach (ocassionally filamentous bacteria and fungi are also present). Similar as in a batch procedure, the cultivation will reach its end point once all organic matter is used up. The effluent containing the flocs is pumped into the holding tank where they can settle. Some of the floc material is then returned to the aerator to serve as inoculum, while the rest is sent to the sludge digestor. The residence time in the BRT is generally 5-10 hours, too short for complete oxidation of organic matter. The main process occurring during this short time is *adsorption of soluble organic matter* to floc and incorporation of some of the soluble material into microbial cell material. The BOD of the liquid is thus considerably reduced by this process (75-90%), but the overall BOD (liquid plus solid) is only slightly reduced as most of the adsorbed organic matter still resides in the floc.

Advantages of the BRT over conventional waste-water treatment facilities:

- Vertical concept means a reduction of surface area (away from the horizontal with depths of 0.5m to the vertical concept, with depths of 20m).
- Aeration of system is achieved by applying "jet technology".
- Sludge recycling within the system boosts efficiency; it is achieved by a density gradient between downward and upward flow.
- Reduction of smell as reaction takes place within a closed system.
- The sealed system limits potential leakages into the groundwater.
- Foaming is a problem; therefore, irrigation with anti-foaming agents.

BRTs are installed already on site of the factory where the wastewater is generated; doing so enables the operators to feed the pre-treated sewage into the communal sewage system.

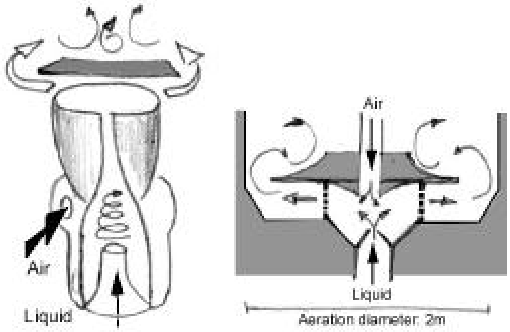
The overall efficiency of the system is directly linked to the aeration capacities. To treat $15 \cdot E^3 m^3$ of wastewater requires $20 \cdot E^3 m^3$ of air per ??? to ventilate the sludge; such quantities can only be provided by using compressed air at a pressure of 2.1 bar (pressure of water column). It is not surprising, though, that about 80% of total energetic requirements of such a plant is used to power the aeration system.

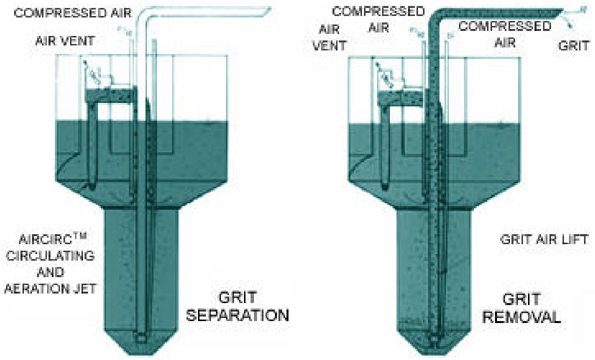


Height of tower versus CO_2 concentration; the optimum is achieved in towers that do not exceed 20m in total height.

To maintain efficient aeration, a particular jet aerator design is required.
Idea and concept behind the “RadioJet”:

- The diameter of the air bubbles and their distribution are crucial for efficient aeration as it determines the overall efficiency of the reaction.
  The RadioJet-design generates a very high concentration of very tiny bubbles.
- Furthermore, bubble coagulation is limited (this would decrease overall aeration efficiency) by gradually increasing the speed of the liquid stream (vortex).
  Rule of thumb: for $100m^2$ use 10 jets.





Variation of the RadioJet-concept: The mixing chamber is built in a way to provide extra mixing caused by a top deflection-plate. This results in intensified turbulent vortex at the edges of the plates.
Advantages: one of this modified jets can roughly cover an aeration area of $20m^2$.
Disadvantage: the plates cause a dead air space right on top of the plate.

Conclusion: So far waste water management (classical and modern) has moved away from an EOP-approach with its "out of sight out of mind" attitude to a process and utility product that could be successfully implemented in agriculture; with the introduction of wildtype and GM microorganisms to the sludge, drip-lawn, etc, it is possible to achieve superb results.
The operating conditions are substrate-dependant, as it is neither constant in terms of quality and quantity, nor homogenous, the use of BT-organisms for the first time guarantee a stable product that does match legal limits as requested by the authorities. Nevertheless, maintenance and operation of any concept require periodic pH and nutrient checks; future organisms, so called "adaptic" bacteria may be implemented in order to minimize human interference.
In any case, to facilitate the biological reaction, the plant still requires a:

- high percentage of easily removable suspended material;
- low percentage of "hard-to-removable" suspended material.

A system that contains contaminants that are very difficult to deal with or are present in ver y low concentrations (a highly dissolved effluents), tend to flush out the bacteria needed for the cleansing process. In such cases it is required that the microorganisms are firmly attached to a carrier substrate that can easily be hold back and kept within the reaction chamber.
Finally, current concepts only use one or a few selected microbial strains to deal with the manifold diversity of suspended pollutants in wastewater.

III. Other Biotech Applications:

III. 1) Biotechnological solutions of air cleaning:

As atmospheric pollution limits keep pace with the onset of new technologies, current trends indicate a further decrease of acceptable pollutants contained in ambient air. Such trends not only increase the pressure to the industry but also ask for low-cost cleansing alternatives; this includes also the increasing number of small polluters that in sum are equivalent to a large emitter.

Source term	**Main components in odor**	**Microorganism**
Pig farm	short-chained fatty acids (FA)	*Streptomycetes sp.*
Water reservoir	mainly fungi (Actinomycetes)	*Bacillus cercus*
Foundry	Formaldehyde	"sludge" fermenting microorgs

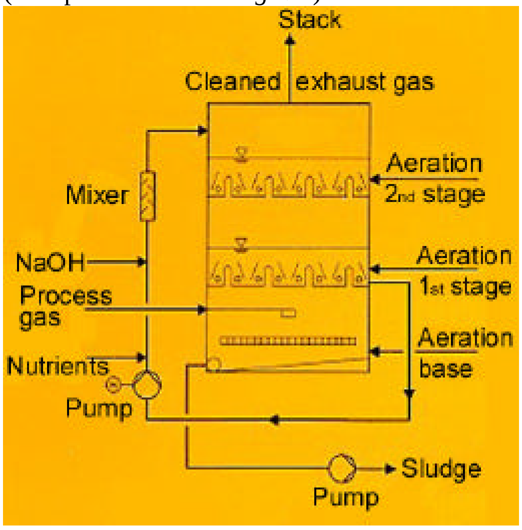
It is essential to control the outflow of exhaust air as people become used to better air-quality standards. Often, people become alerted by the presence of harmless but unpleasant odors; in other cases it is essential to protect the public from harmful but non-detectable fumes.
Microorganisms are capable to literally "soak up" the smelly components of a process gas. Microbial absorption occur in two stages:

- Liquid absorbance stage
- During metabolization

Emitting facility	(input) air-concentration	(output)
Foundry	160 mg/m^3	40 mg/m^3
Animal Waste Processing[1] (cadaver disposal)	20·E^3 GE[2]	100 GE[2]

([1]) Statement of the Industry: "A treatment systems for animal waste management at swine and dairy farms, food processing facilities and other waste streams. In almost all livestock applications, the waste is bio-converted into a nutrient-rich, organic material".
([2]) GE grenzwert-einheiten; unit of limit-value.

Current methods achieve a cleansing result in the order of 20-40%. The technical realization involves a filter that settles on a sheath of air. The filter is kept at a preset humidity level, temperature, and pH. To increase surface area, the microbial filter is best grown onto laminar sheaths that are narrowly placed next to each other. The gap between the microbial mats has to be very tiny. Attached pumps compensate for increased suction resistance. Periodic sprinkling with suspended microorganisms is done in a counter-flow principle. Sprinkling also indemnifies the huge evaporative loss brought about by the turnpver rate of the exhaust gas.
Harvest of the "sludge" is not very practicable, but facilitated by the fact that the filamentous microorganisms can be automatically cut off from underneath. Clogged sheaths have to be removed manually. Even though, the setup below requires periodic maintenance, it is considered to be very robust! (Compare also *Steinberg*-VL)



Generally, any substance with a large surface area can be employed for it; usually, compost, bark, clay, lanae, are used to fill the box.
The pH of the substrate, the toluol content, and humidity are just some of the most crucial parameters, that have to be taken care of.

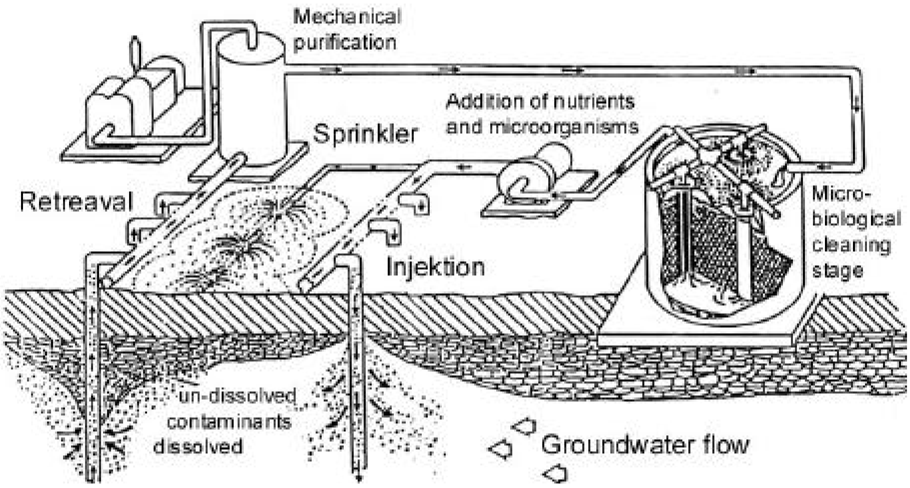
III. 2) Biotechnological solutions in treating solid waste:

- Removal of this underground storage tank eliminated the source of contamination on this site, facilitating further remediation.

Filling stations (petrol stations) are major contributors to soil contamination as the life-span of underground-storage tanks is very limited. It is not surprising though that over time hydrocarbons (HCs) keep leaking into the soil. As a consequence most operators of filling stations rather close it down as it is not feasible to replace leaking tanks.

In-situ treatment of soil with biotechnological means therefore seems to be an attractive option (refer also to Riepe-VL). Nowadays, construction guidelines have to be accompanied with documentation by the vendor that proofs the safe and healthy status of the soil.

a) *In-situ* remediation is cost efficient as it is not necessary to remove the contaminated soil and process it somewhere else; treating it on site, though is a complicated process that requires a totally different approach. Injecting bacteria into the substrate is simply note enough. The substrate has to be aerated, supplied with extra nutrients, and wetted in constant intervals. The addition of worms further facilitates aeration.
The in-situ methods uses the natures own ability to decompose and neutralize oil components during micro biological processes. The soil is put into a respirator so to say. Through a system of borings the soil can be ventilated and supplied with air and nutrients in very carefully adjusted quantities thereby stimulating the development of bacteria capable of decomposing oil-components and other organic substances.
Attention: it is essential to monitor any byproduct that may result out of the metabolic conversion - it could well be that metabolites may be more toxic than the initial components. Furthermore, substrate aeration may result in soil sinking; i.e. leaning of houses, etc.



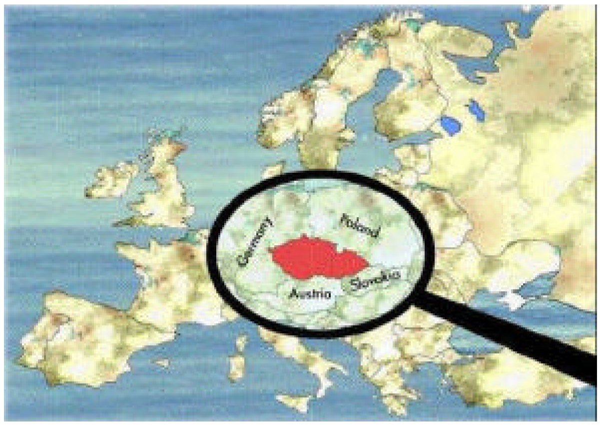
- *In-situ* treatment of a contaminated site. Again, it should be repeated that EOP-repair is always the most expensive approach.

b) *Ex-situ* (off-site) treatment involves the digging out of the soil and processing (or dumping) it somewhere else; the classical “out of sight out of mind” approach. Current applications are involved in the removal, structurization, and treatment of the contaminated substrate, i.e. stratificaiton (see *Riepe*-VL).

II. 3) Biotechnologic solutions for mining activities

The mother of all uranium localities is without doubt Jachymov (St.Joachimstal, Czech Republic) in the Czech Ore mountains (Erzgebirge). Since decades, the silver-mine in Joachimstal created an enormous amount of waste.

Even though the mountains of tailings were deprived of Ag, they contained Uranium, which was used in the 40`s for further exploitation. It is here that the glass industry obtained their uranium for making a greenish yellow color of glass, called Anna-green. Marie Curie obtained a few tonnes of the then near useless uranium ore and found the new elements of polonium and radium in it. Uranium mining seized in Jachymov in 1963.The best known individual mine of Jachymov is the Svornost mine. Uranium minerals from Jachymov are: Albrechtschrauffite, Cejkaite, Jachymovite, Johannite, Liebigite, Meta-Uranopilite, Cuprosklodowskite, Uraninite, Richetite and Weeksite.



As these tailings no longer were considered as waste as it was quite efficient to extract the Uranium; There used to be some troubles with U-mining: problem with workers exposed to radioactive dust, its inhalation, and deposition on the skin; uranium leaching avoids that altogether; the only precondition is a fully functioning infrastructure. Only in late 1963, when it became unfeasible, the tailings became waste piles.

Conventional Mining is usually achieved by mechanical means; it involved the excavation, or the digging out of the ore deposits from the ground. Subsequent steps involve the crushing of the ore, while electrolysis or the blast furnace help to reduce the metal content to its elemental form. All these processing steps require an enormous amount of energy, besides leaving behind a disturbed landscape with huge amount of "waste tailing piles"; i.e. the extraction of aluminum from bauxite is one of the most energy-consuming technologies.

With the help of BT, technologies have been developed that utilize bacteria and fungi to virtually extract a metallic liquids from a leaching tailing. Mineral sulfide-oxidizing bacteria have been utilized in the extraction of copper and uranium from low grade ores for many years in low technology heap and dump leaching operations. It is only relatively recently that bioreactors have been introduced for industrial mineral processing, with several in commercial operation for treatment of gold-bearing mineral sulfides. As Uranium and Gold are the most frequent elements in sea water, it is probably just a question of time and price/ounce when they will be commercially interesting options.

Practical setup to extract metals from a leaching pile:

- A pilot experiment requires a sample of the tailing pile to tune the abiotic conditions in order to maximize conversion rates of the microorganism involved. The “percolator stage”, may take days while only a few mL are used.
- After successfully establishing the proper conditions, the developmental phase enters the “suspension leaching stage” where a larger scale setup is used; this requires a huge amount of acids and a lot of time (months and $1 \cdot E^2$L) while strict safety precautions have to be implemented.
- Finally the “tailings leaching stage” require years and $1 \cdot E^4$L of acid. The environmental problems are enormous; collection of run off is thus a very crucial fact. Furthermore, oxygenation, weather influences, etc. need to be taken into consideration.

III.3a) Mineral leaching is a technique for extracting mineral ore from rock with the help of **microbial** organisms. Mineral leaching is important due to the following two reasons:

- Development of new resources for raw materials, currently unfeasible due to economic reasons. Biotechnology is already used to answer the commercial value of a mineral deposit.
- With the advance of biotechnology existing tailings, and other piles of so-called waste piles will be given the status of raw materials as better and more efficient BT-organisms will become available to boost extraction efficiencies.

In the future, an increasing share of metals will be covered by microbial leaching processes; i.e. the extraction of metals from ore or tailings with the help of bacteria and fungi is a feasible option; these organisms are capable to convert low soluble metals and bring them into solution.
Already many industrial waste piles have turned into lucrative sources of raw material; currently extraction of Cu and U is best done with such microorganisms. The use of the organisms is further enhances when taking into consideration that miners are no longer exposed to toxic inhalants (like radon, radioactive dust, Pb, U, Hg, etc.) especially when dealing with U-mining.

Another interesting field of application is currently analyzed in the protection of the **Environment**. Usually, mining activities left behind a damaged landscape behind; artificial ponds, new streams, leaching mines, etc. became part of the toxic heritage. Conventional clean-up methods (expensive) are becoming almost obsolete when acidophilic microorganism are employed for the rehabilitation of the damaged site.

Which **microorganisms are suitable** for bio-leaching?
Autotrophic and heterotrophic bacteria, are used in mineral leaching. Autotrophic bacteria have the ability to grow solely on inorganic materials, with carbon dioxide as the carbon source, and sunlight (photosynthetics) or the oxidation of inorganic compounds (chemosynthesis), as the energy source. On the other end, heterotrophic bacteria require complex organic compounds as a main carbon source, although some of them may use carbon dioxide to a lesser extent. Most of them require certain vitamins and amino acids because they are unable to synthesize these compounds themselves. The energy source may be chemosynthetic.

What is the role of bacteria in the production of **acidic rock drainage** (ARD) or **acid mine drainage** (AMD)?
Acid rock drainage is the product formed by the atmospheric (i.e. by water, oxygen and carbon dioxide) oxidation of the iron-sulfur minerals pyrite (FeS_2) and pyrrhotite (FeS) in the presence of (catalyzed by) bacteria (*Thiobacillus ferrooxidans*), and any other products generated as a consequence of these oxidation reactions. It is characterized by a low pH and high concentrations of sulfate and dissolved metals, the most distinctive feature of ARD is its bright orange or red colour caused by the precipitation of ferric iron. The bacteria which are usually site-specific strains of Thiobacillus ferrooxidans, utilize the sulfur present as their source of energy. They are autotophic, obtaining their nutritional needs from the atmosphere (nitrogen, oxygen, carbon dioxide and water) and from minerals (sulfur and phosphorus). While these bacteria are not catalysts by true definition, they do act as accelerating agents if their habitat conditions are at or close to optimal and they are a most important factor in the generation of ARD. Scientists have studied wetlands to treat existing ARD problems by using biological reactor systems employing sulfate reduction and biosorption for removal of heavy metals. Present work is being done on the prevention of AMD by testing chemical inhibitors that when applied on mine tailings or waste rocks would inhibit neutrophilic thiobacilli which in turn, decreases the initial oxidation rate of the tailings significantly until a wet or dry cover can be applied.

***Thiobacillus* sp.**: This genus consists of cylindrical ion-oxidizing species; i.e. thermo- and acidophilic prokaryote (among the kingdom Bacteria). It is currently the predominant organism used in mineral leaching.

- most of them grow aerobically by oxidizing elemental sulfur, which is
- followed by a reduction of the sulfur-compound.
  The only source of carbon is the gaseous CO_2 supplied from the air. Ultimately, sulfate is obtained as the end product.

Leaching usually occurs in a very acidic environment; pHs as low as $2 < pH < 3$ are quite common; such a high proton concentration makes sure that the metal-ions do not precipitate but rather remain in solution.

T. ferrooxidans: known since the 1950s, was first discovered in coal mines and was also isolated in waste waters. The aerobic oxidation of iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state is an energy-yielding (exergonic) reaction. Only a small amount of energy is available from this oxidation, and for this reason the iron bacteria must oxidize large amounts of iron in order to grow. Ferric iron forms very insoluble ferric hydroxide [$Fe(OH)_3$]. As *T. ferrooxidans* is also capable to oxidize elemental sulfur it is an obligate acidophil. This in is part because the neutral pH ferrous iron rapidly oxidizes nonbiologically to the ferric state and is thus stable only under anoxic conditions (at acid pH ferrous iron is stable to chemical oxidation).

Characteristics of *Thiobacillus ferrooxidans*

Condition	Characteristic
Optimum growth pH	1.3-4.5
Optimum temperature	30-35°C
Mol% G+C	56-59
Temperature range	10-37°C
Motility	0 to several polar or peritrichous flagella
Gram staining	Gram-negative
Spore formation	none
Shape	rod, (0.5µm x 1-3µm)
Trophy	obligate chemolithoautotroph*
Energy pathway	oxidation of Fe^{2+} and reduced sulfur**
Oxygen requirements	obligate aerobe*
Electron acceptor	oxygen*
Nitrogen source	Ammonium salts, nitrate, fix dinitrogen
Oxygen requirements	obligate aerobe*

T. ferrooxidans cell suspension viewed by an electron microscope magnified 30,000 times

**T. ferrooxidans* is generally assumed to be obligately aerobic, but under anaerobic conditions, *T. ferrooxidans* can be grown on elemental sulfur using ferric iron as an electron acceptor. These results indicate that *T. ferrooxidans* can be considered a facultative anaerobe playing an important role in the iron and sulfur cycles in acidic environments. The ability of *T. ferrooxidans* to grow in oxygen deficient environments may have important implications in bioleaching processes where anaerobic conditions may often exist.

***T. ferrooxidans* may also obtain energy from oxidizing Cu^{+} and Se^{2-} and from the oxidation of tetrathionate, molecular hydrogen, formic acid, antimony compounds, uranium compounds, and molybdenum compounds.

T. thiooxidands known since early 1920s is already a well-studied organism. It is able to survive pH-levels close to 1; such a low pH is able degrade the rock quite quickly. The acidic solution oxidizes only the sulfates but not the sulfides

T. prosperus: A recently discovered organism, isolated in geothermal vents isolated. It can withstand temperatures to a maximum of 85°C.

T. cuprinus: cannot oxidize all bivalent ions; it is able to process copper - this reaction is speeded up if organic substrate like yeast- or meat-extract is added.

Leptosperillum is a microorganisms usually found in waste waters is also able to oxidize bivalent ions (not sulfur) at a pH between 1-2. Although it is not actively involved in the extraction process, it seems that this organism acts as a catalyst for the activity of ***Thiobacillus***; together they are used to leach U, Mo, Ag, etc. *Leptosperillum* is very sensitive to Cu - a concentration exceeding a certain threshold level will ultimately kill it.

In general, almost all **thermophilic microorganisms** are able to oxidize bivalent ions. Elemental S^0, sulfides, Mo^{2+}, and Cu^{2+} can be oxidizes at temperature as high as 70°C. Modified *Thiobaccilus* is even capable to act on Pyrite (within a temperature range of 20 - 60°C, at a pH between 1 - 3); it also oxidizes Zn^{2+}, Pb^{2+}, and other metals common in leaches.

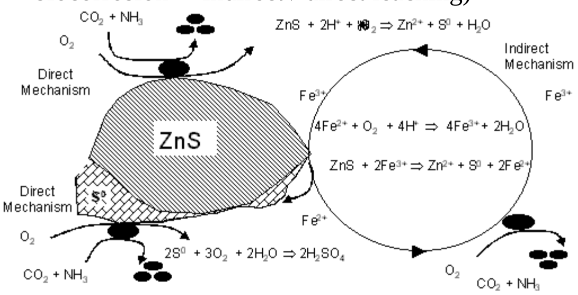
The acid generation process: Sulfides are stable under waterlogged (anaerobic) conditions but when oxygen is introduced into the system, these sulfides are oxidized to sulfuric acid.

Chemical oxidation of pyrite is slow but the reaction is mediated by iron-oxidizing bacteria, in particular *Thiobacillus ferrooxidans*, so that the optimum conditions for sulfide oxidation are the optimum conditions for *Thiobacillus ferrooxidans*, namely oxygen concentration >0.01 Mole fraction (1%); temperature 5-55°C, optimally 30°C; and pH 1.5-5.0, optimally 3.2 (Jaynes et al. 1984). **Progressive oxidation** following drainage of compact sulfidic material. Oxidation appears to be proceeding concentrically from the ped faces. The ped cores remain reduced and dark in color. The pale band is presumed to be a diffusion zone through which iron and acid move outwards and oxidant move inwards.

- Pleistocene marine terrace near Lelydorp, Surinam (Photo: Robert Brinkman)

Reaction Mechanisms - How does bioleaching work and in which circumstances is it used?
In the mineral leaching process, low grade ore is dumped on a large pile (the leach dump) and a diluted sulfuric acid solution (pH ≈ 2) is percolated down through the pile. The metal-compound with low solubility is transferred into an aqueous medium. This is accomplished via two mechanisms. The first mechanism is direct leaching:

1. **Direct leaching**: The *Thiobacillus* attach to the mineral sulfide crystals within the ore matrix, solubilizing the metal by oxidation of the sulfide moiety to sulfuric acid and simultaneously oxidizing associated ferrous iron to ferric. MeS → *T. ferrooxidans* → $MeSO_4$; ($SO4^{=}$ + a the bivalent ion Me^{2+})
   - This requires surface contact of the ore with bacteria (attachment mechanisms of the organism with the ore is not known, but essential).
   - Oxidation (deduction of an electron) via enzymatic transition stages (even though the chemical reaction is known, it is mot definitively answered how the metal-ions are extracted from the ore - "biocorrosion" - indirect / direct leaching)



Biocorrosion of the following compounds are possible: CuS, Cu_2S, ZnS, PbS, MoS_2, Sb_2S_3, CoS, NiS.

← A system for the bio-leaching of sphalerite

2. **Indirect Leaching**: The second mechanism is indirect leaching, wherein the organisms oxidize Fe^{2+} in solution to Fe^{3+}. The ferric ion, in turn, oxidizes the metal ion and is simultaneously reduced to ferrous ion. The bacteria then reoxidize the ferrous ion back to ferric ion, and the cycle is reinitiated.
   $MeS + Fe_2 (SO_4)_3 \rightarrow MeSO_4 + 2FeSO_4 + S°$
   It lacks the direct involvement of microorganisms; they only produce the leaching or regenerate the leaching agent; i.e. the biological reaction reduces trivalent ions (e.g. $Fe^{2+} \rightarrow Fe^{3+}$). Only then the bivalent ions become geo-chemically processed. The acceleration of reaction due to these organisms can be quite impressive: $1E^5$ - $1E^6$ times the normal rate (mostly because the acidic environment favors their proliferation); as the growth rate boosts, the organisms create a mineral layer while they prevent the formation of a sulfur-layer.
   Biotechnology processes for metal extraction and those which contribute to extraction are currently principally associated with gold and copper mining. Other eexamples involve insoluble tetravalent uranium, which is converted to water soluble, hexavalent U:
   $U^{1V}O_2 + Fe_2(SO_4)_3 \rightarrow$ *T. ferrooxidans* $\rightarrow U^{V1}O_2\,SO_4 + 2FeSO_4$
   Indirect leaching is a combination of biochemical and chemical oxidation as these ionic species are cycled through the bi- and trivalent stages.

3. **Mineral precipitation**: The liquid coming out at the bottom of the pile, rich in the mineral, is collected and transported to a precipitation plant where the metal is precipitated and purified:
   $Me^{2+}(aq) + H_2(g) \rightarrow Me°(s) + 2H°(aq)$
   The liquid is then pumped back to the top of the pile and the cycle is repeated. As needed, more acid is added to maintain the low working pH.
   Processes that would address the extraction of zinc, nickel, cobalt and uranium have not been competitive based on actual prices of these metals and the level of technology development. Bio-leaching with stirred tank reactor is used for concentrates of gold ore that are refractory to conventional cyanidation. **Bio-heap leaching** is considered when the ore is low grade and economics cannot sustain the cost of making a concentrate, or the mineralogy is such that the ore cannot be concentrates, or the economics do not support conventional treatment.
   External influences that interfere with bio-leaching: It should be remembered though, that these reaction should take place in a rough, uncovered environment - how can these reactions be influences in a real world? In order to answer this questions, three considerations have to be cleared first:
   1. Activity
   2. Chemical composition of ore
   3. Mineralogical composition of ore

   The optimum leaching conditions for optimal growth have to be maintained at all times. *Thiobacillus* is a tough and moderate organism; it has very low dietary requirements, which are readily provided by the surroundings environment (phosphate, sulfates, and Mg salts are its energetic suppliers
   - i.e. feed on mineral salt while leaching takes places (use of inorganic nutrient)
   - Absorption on the material that they are in leaching.
   - Precipitation on the leaching liquid.

   Nutrients and **abiotic factors**: Several abiotic parameters and nutritional requirements have to be checked before going into the filed:
   - CO_2, additional gassing with essential CO_2 promotes growth, but is difficult and costly in the field.
   - O_2, the supply of O_2 is easily achieved under lab-conditions but hard to maintain in a tailing pile.
   - pH - setting the pH within the working limits is easier said than done. It has to be kept at a level in which metal precipitation is hindered (the leached metal should remain in solution). This can only guaranteed if the pH level is kept within the 2 - 2.5 limits; by adding extra acid, the pH can be stabilized to as low as 1.3
   - Temperature - the typical temperature range is somewhere in-between 28 - 25°C; a temperature lower than 25°C is accomplished by a reduction of the microorganisms productivity.
     Rule of thumb: A temperature reduction by 6°C means a doubling of the generation time of the *T. ferrooxidians*. The lowest tolerable temperature for metabolic activity is +4°C. The introduction of thermotolerant traits of thermophilic bacteria (via GM-technology) may increase its temperature range to as much as +80°C
   - Organic liquids are used to provide essential nutrients to maintain high productivity.
   - Substrate particle size is crucial for their efficiency.
   - Light, particularly, UV-radiation has a negative effect on the bacteria. Therefore, any start-up reaction of a growing culture should take place at night.

   Because leaching efficiency is a direct function of the ore's surface area (the larger the surface area, the higher the metal extraction capabilities), the reduction of particle size facilitates this reaction mechanism. This implies a laborious, energetic, and expensive input of shredding equipment to grind the substrate ore. As *T. ferrooxidians* is only about 3µm long, an optimal leaching result is obtained with a particle size of ≈ 42µm (fine dust); finer fractions do not necessarily increase yield as tailings or waste pile usually do not consist of the ore alone.
   Organically extracted liquids (are usually kept as the company intellectual property) but speculations are mounting that heavy metals are heavily involved. If heavy Metals occur in low concentrations, they are considered as an essential nutrients, higher concentrations are toxic; luckily, *Thiobacillus* is quite tolerant to higher heavy metal concentrations. It tolerates heavy metal concentrations of: U ≤ 10 g/L
   Cu ≤ 55 g/L
   Ni ≤ 50 g/L
   A maturing *Thiobacillus* culture is able to resist even higher concentrations of these elements.

Most applications in bio-leaching do not yetvinvolve genetically modified (GM) microorganisms; currently work is done to optimize the growth of indigenous microorganisms. However, enhanced microbial performance for improved process results can be achieved by searching for better strains or through genetic engineering. Recent work at the Biotechnology Research Institute in Montreal (CDN) has investigated **mineral leaching**, cyanide degradation and sulfate reduction with the help of GM-organisms. Characteristics of microorganisms which could be improved by this approach include:

- increased metabolic rates to produce higher reaction/leaching rates
- increased resistance to toxic and inhibiting cationic and anionic species including metals and chloride
- increased tolerance to cold temperatures for applications in northern climates

Significant developments in this field have been limited due to the requirement for a greater understanding of the basic microbiology of organisms such as *Thiobacillus ferrooxidans*. Although some very significant advances have been made in the area, genetically-engineered microorganisms are not yet available and likely will not be for several years. The use of genetically engineered microorganisms will require careful control to prevent them from competing with natural organisms. This will likely prevent application for less controlled processes such as dump leaching, heap leaching and environmental processes which are performed in the “open”. Similarly, control might be difficult if a process requires microbial consortia. If genetically-engineered strains are used, they would only apply to controlled plant processes.

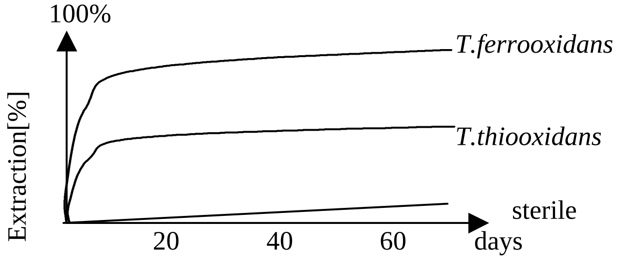
III.3.b) Real world application - lab versus field trials

What types of bioreactors are being used to perform research in biotechnology?
Different type of bioreactors are commonly used by scientists for effluent treatment. The most commonly used are the **rotating biological contactor** (RBC), the trickling filter, the packed bed reactor and stirred tanks reactors.
The RBC provides an effective means of alternately submerging a film of microorganisms in a substrate medium and then exposing the film to air for oxidation. A very large surface area is provided in a small volume of substrate, thereby permitting excellent transfer of nutrients and oxygen necessary for bacterial metabolism. It offers several significant advantages such as low energy requirements; low space requirements, simplicity of operation; low maintenance requirements, high treatment efficiency and resistance to shock loads. This system was used in biotech-labs for the demonstration of selenite reduction to selenium by *Escherichia coli* at a mine site and was patented . It was also demonstrated and patented for the degradation of oxalate (it is presently applied in Jamaica).
A **trickling filter** consists of a bed of stones or sand that support the biomass, and the waste-water is allowed to trickle down over the medium. The contaminant contained in the liquid flowing over the bed is oxidized by the organisms. The trickling filter has the advantage that the throughput is not restricted by wash-out., making it highly suitable for wastewater treatment. However, it has a number of disadvantages: the biomass cannot be recovered from the bed; not all microorganisms adhere to surfaces. The thickness of growth on the support particles must be controlled, otherwise the accumulation will block the bed and; aseptic operation is difficult if not impossible. This type of system has been used for a pilot-scale demonstration on a mine site for a nitrification process.
A **packed bed reactor** resembles a trickling filter reactor but the wastewater is pumped through the bed of stone or other bacterial support from the bottom of the reactor. It is used for bacterial processes that require a certain level of anaerobic condition, it is presently used in our lab the case for the treatment of nitrate containing effluents.
A **continuos stirred tank reactor** (CSTR) is essentially a cylindrical vessel in which the contents are well mixed. Basically, it is a batch reactor with provision for continuous feed and removal of product. The main advantage of the CSTR, apart from simplicity of construction, is the ease with which the temperature and pH can be controlled. The open construction of the CSTR makes it easy to clean the internal surfaces, which is an important factor in maintaining aseptic conditions in large-scale operations. However, since microorganisms are in suspension they can be easily washed-out at high flow rates. CSTR fermenters are limited in throughput as a result of this phenomenon. CSTR are used in the mineral industry for bacterial leaching of concentrates.

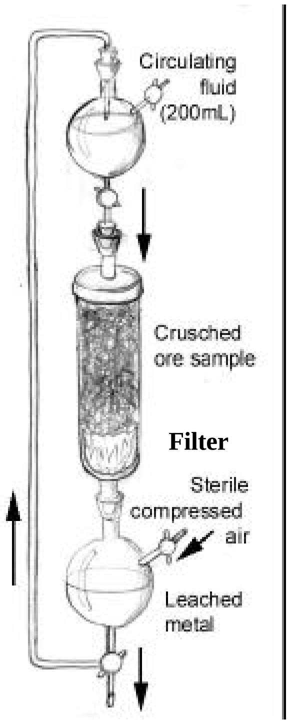
i) Laboratory style bio-leaching works like a coffee-making facility; **percolation** is the flow or trickling of a liquid downward through a contact or filtering medium. The liquid may or may not fill the pores of the medium. Also called filtration. *In-situ*, it is the movement of process solution in streamline flow through small interconnected and saturated interstices of rock or earth, principally of capillary size. In any case percolation still requires:

- monitoring of pH and temperature;
- analysis of solution; to tune T, and pH to optimal ranges;

Lack of control of oxygenation and surface area.
Time frame: 3 months till 1 year per percolator.
Replacing the percolator with a shaker (submersed leaching) boosts reaction using finely grounded ore reduces reaction times, increased surface area, increased O_2 to have higher yields.



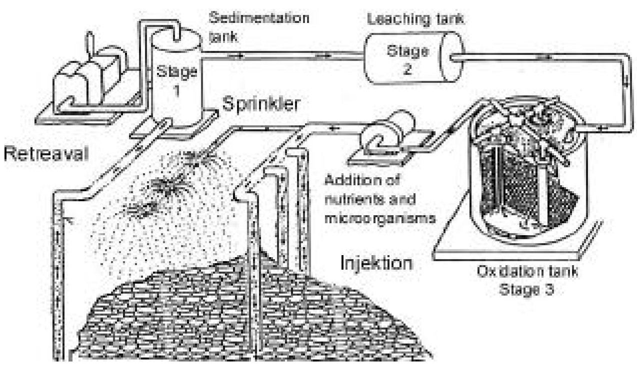
U-leaching with optimum abiotic conditions and the proper organisms.



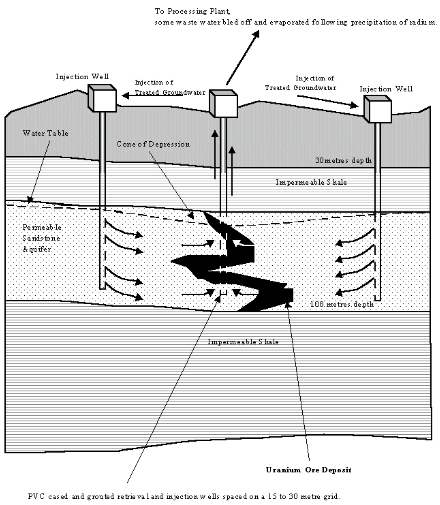
Percolator setup

Bioreactor: mining industry design bioreactors, where O_2, CO_2, and pH can be accurately set;
"**Air Lift Reactor**": Supply of microorganisms with needed air occurs simultaneously and the suspension of finely grounded substrates; i.e. mud waste. Up to 2 tons per load can be processed. Current models are capable of handling up to 200 tons per leaching event.

i) *In-Situ* style bio-leaching: The situation of interest here is so-called "in situ" leaching, where the idea is to remove the mineral directly, without first having to dig up the rock (and bring it to the surface for crushing) or relocating the tailing pile. The way this is done is to introduce a corrosive fluid (such as acid) into the rock, and dissolve the mineral of interest. This is then pumped to the surface as a solution. The major advantage of on site processing is the enormous cost saving effect on transportation costs.



- *in-situ* bio-leaching



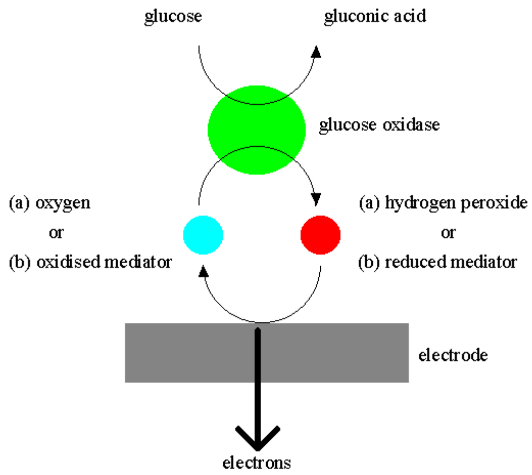
Generalized cross section of *in-situ* leaching process applied to a sandstone hosted roll front type uranium deposit (South Australia) ®

Pile of waste material is exposed to acids, which leaches through the pile, explain produce...... a sprinkler system which provides nutrients and additional acids may loose its liquid fraction due to evaporation; furthermore, pumping corrosive substances through pipes is quite risky and expensive (higher energy cost for pumping liquids).
Consequently alternative methods have been tired: In the Finger dump approach tailing piles just half a meter thick but 100m's long achieve the same results with lower energetic requirements. This approach improves ventilation conditions while lowering internal reaction temperatures suitable for the organisms); ore of the basis take $\approx 50E^3$ tons; i.e. double chambered reaction tanks that have to be leakage proof, and provide optimal results when operated in a counter-flow within chamber (increases loading).

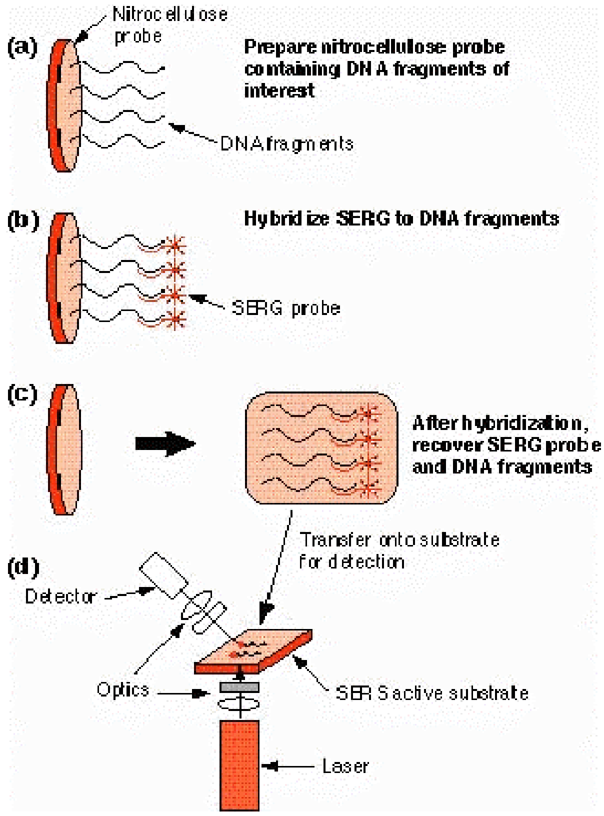
Future trends: The accumulation metals (**Biosorption**) is another trend that is not only interesting on an environmental aspect but seems to become commercially valuable (sulfate reduction plant to remediate contaminated groundwater in the Netherlands).Certain species of microorganisms have been observed to accumulate large quantities of metals of interest. These metals of interest include metals that are toxic to humans (e.g. cadmium) and metals of economic value (e.g. copper, silver). The ability to accumulate a particular metal varies among the different microbial species; some metals are accumulated to a greater degree than others. Metal accumulation has been observed to occur in association with extracellular products (e.g. polymers) at or within the cell membrane, and intracellularly. Biosorption is a physico-chemical reaction between the dissolved metal and the cellular components, in many respects similar to ion exchange. It was also found that certain species of fungi are able to absorb uranium on their cell walls. These fungi also has the capacity to remove copper and nickel from solution.
Automation of bio-leaching is the trend in the industry. Computer controlled processing of tailing piles, atomated reworking of tailing pile, monitoring of pH, T, O_2, particle size, etc.
Recent developments will soon make it possible to extract Ni, Co, Mo, Zn. Engineering of new strains (mixed cultures) enables the extraction several metal components from the ore. Currently, research in this field favors monopolization as potentials GM-microorganisms are patented by just a few multinationals. Even though the chemical reaction mechanisms involved are quite simple, it may proof difficult to maintain and operate the complex technical setup if the plant is operated in developing countries.

III.5) Biosensors: are analytical devices which are capable of providing either qualitative or quantitative results. Biosensors combine the exquisite selectivity of biology with the processing power of modern microelectronics and optoelectronics to offer powerful new analytical tools with major applications in medicine, environmental diagnostics and the food and processing industries. Biosensors consist of bio-recognition systems, typically enzymes or binding proteins, such as antibodies, immobilised onto the surface of physico-chemical transducers. The term immunosensor is often used to describe biosensors which use antibodies as their biorecognition system. In addition to enzymes and antibodies, the biorecognition systems can also include nucleic acids, bacteria and single cell organisms and even whole tissues of higher organisms. Specific interactions between the target analyte and the complementary biorecognition layer produces a physico-chemical change which is detected and may be measured by the transducer. The transducer can take many forms depending upon the parameters being measured - electrochemical, optical, mass and thermal changes are the most common. Biosensors are at the forefront of multi-disciplinary science involving the marriage of the biological world and the physical electronic world. A biosensor is analytical device incorporating a deliberate and intimate combination of a specific biological element (that creates a recognition event) and a physical element (that transduces the recognition event).

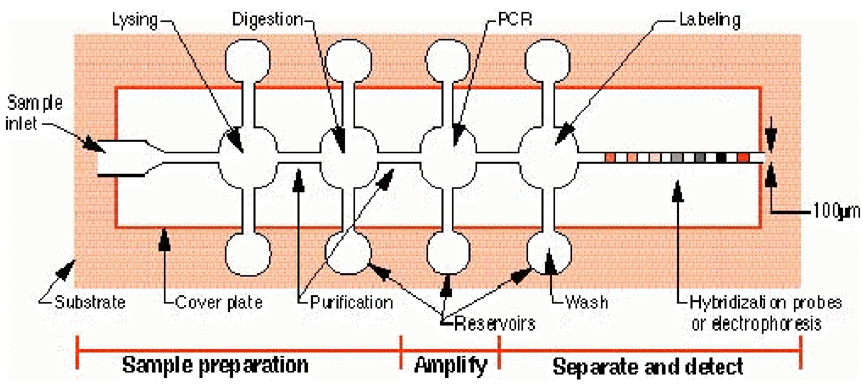
The bioelement is usually an enzyme or an antibody, both of which merit further presentation. Enzymes are large protein molecules that catalyse chemical reactions. They are synthesised by cells and are used as tools to do the cell's. chemistry. In a simplified scheme, the enzyme participates actively in the transformation of chemical A (the substrate) to chemical B (the product) but remains unchanged at the end of the reaction



↑ Glucose Oxidase Sensor (GOD). glucose reacts with the oxidised form of the enzyme to form gluconic acid, but leaves behind two electrons and two protons, thus reducing GOD. Next, oxygen dissolved in the surrounding fluid reacts with GOD, accepting the aforementioned electrons and protons to form H2O2 (hydrogen peroxide) and regenerating oxidised GOD- which is ready to react once more with glucose.



- ORNL's surface-enhanced Raman gene (SERG) probes can locate free DNA molecules that have hybridized to other DNAs fixed on a surface. The technique has use in medi cine, forensics, agriculture, and environmental bioremediation.



¬ Conceptional design of DNA processing and analysis chip.

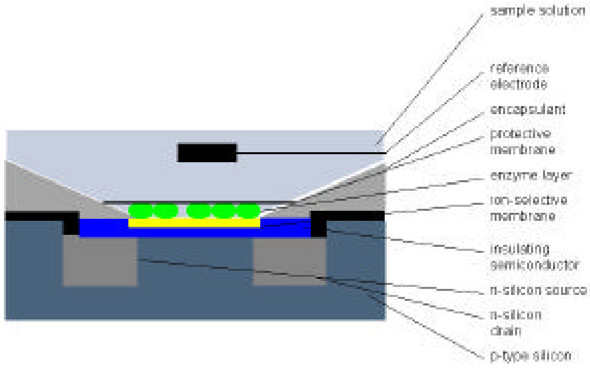
Basically two concepts:

a) **Binding-type sensor**: Bio-componentttt should be a coloring agent, receptor a protein
   Lective Vs analytical part glycoprotein
   Antibody Vs. Prosthetic group

b) **Enzymatic catalytic sensor**:
   Organelle and Co-factor (analytics)
   Enzyme and substrate analytic counterparts
   Tissue slice and Enzyme activity (resulting)

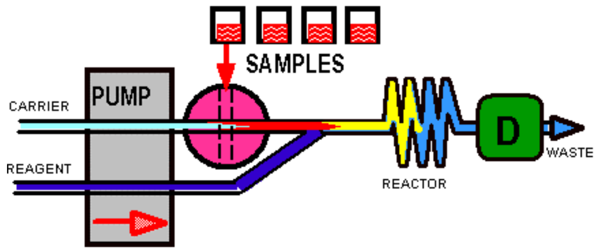
Irrespective which type is used, all approaches require the conversion of the biological signal into an electrical signal; this is usually done with an:

- Optoelectronic transducer (conversion of a photonic into an electronic signal)
- Field-effect transistor (FET - conversion of an minute voltage gradient into a processible signal)
- Semiconductor electrode (very selective bio-chemical sensors)
- Thermistors (temperature sensitive semiconductors).



Schematic diagram of an ENFET

Flow injection analysis (FIA) is a rapidly growing analytical technique based on the injection of a liquid sample into a nonsegmented carrier stream. The injection sample forms a zone, which is transported into a detector or sensor. The continuous change in absorbance, electrical potential or other physical parameter yields a response curve which is the basis of an assay or information on a system under study. A very specific macromolecule which is directly attached to a sensor membrane triggers an electrical response by the transducer (key and lock principle).

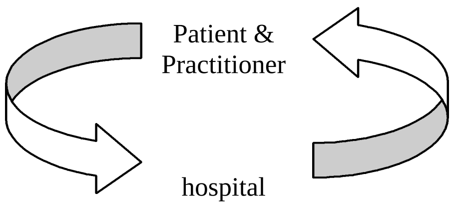


The FIA concept

Still, reaction kinetics of such a setup in-between compounds mix varies substantially:
Immune reactions require 10 min;
Enzyme reactions just about 60 sec.

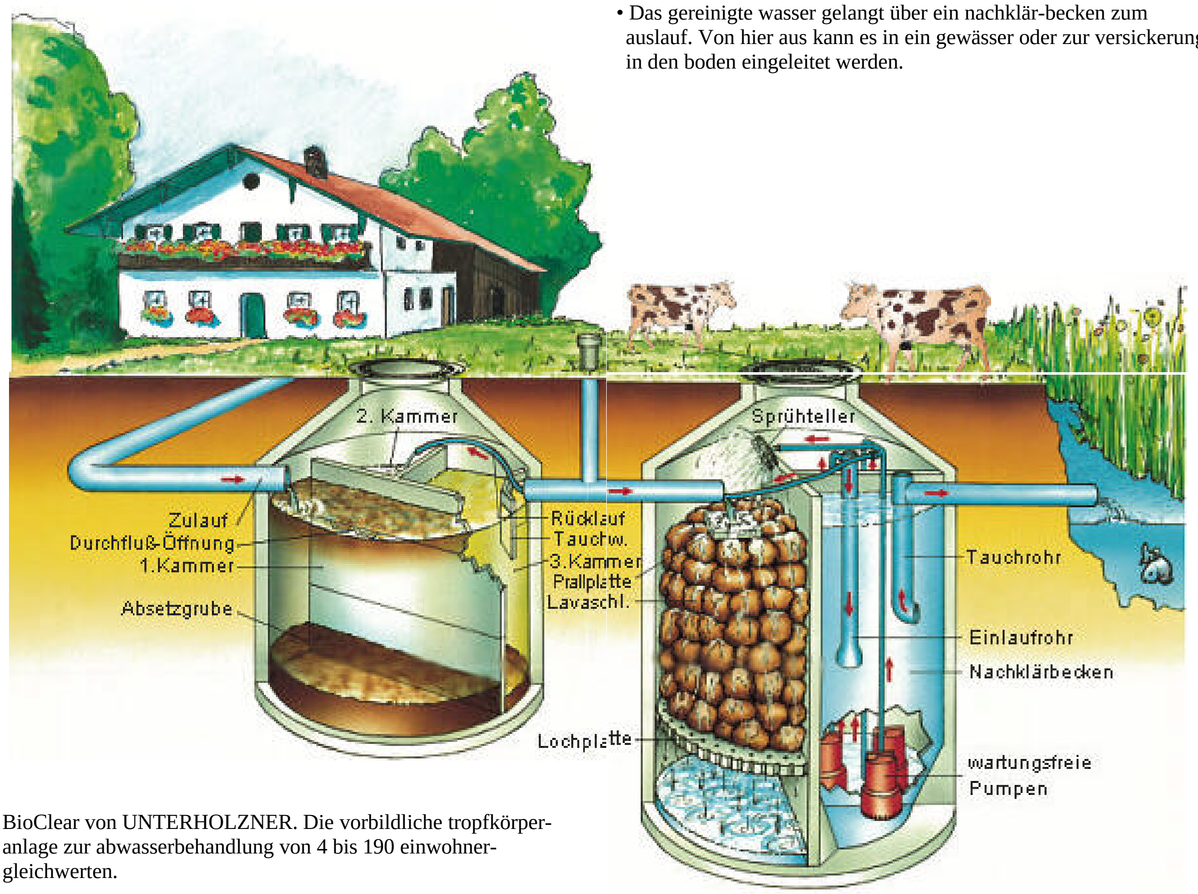
Simple to use **Immune ASSY assessments** are currently widespread in any ambulatory and amongst practitioners. More sophostocated sensors still have some disadvantages like being sensitive to light, leakage, etc.; therefore, they are not yet that robust to be suitable for everyday applications in ambulatories; they will become more widespread once the test is simple to use.
Currently, biosensors for the following low molecular substances are available: viruses, enzymes, lectines, polymerase-carbohydrates, and many other biochemical reaction mediators and metabolites within the body.

Future trends: Automated self-controlled health monitoring applied by the patient itself: e.g. home-useable glucose detector that is connected to the online service of one's practitioner or the local district health service. Patient supervision of this kind not makes preventive medicine possible enables while limiting hospital-access to life threatening situations only (the so-called "Medical closed looped approach").



APPENDIX - Biologische abwasser-entsorgung durch klein-kläranlagen von UNTERHOLZNER.

Die Arbeitsweise der tropfkörper-anlage BioClear prüfzeichen PA-I 3953
- Die absetzgrube nimmt sämtliche dickstoffe auf und hält diese zurück. Von hier gelangt das abwasser in die eigentliche tropfkörper-anlage.
- Die tropfkörper-anlage teilt sich in drei bereiche: tropfkörper-bereich mit lavaschlacke, pumpenraum, n-achklärbecken.
- Nach der vorreinigung in der absetzgrube gelangt das wasser in freiem gefälle auf den tropfkörper. Hier siedeln sich nach kurzer zeit mikroorganismen an, die sich von den inhaltsstoffen des vorgereinigten wassers ernähren. Unter dem tropfkörper sammelt sich das abwasser, welches mittels rücklauf-pumpe zwecks gründlicher reinigung mehrfach zurückgepumpt und teilweise auf den tropfkörper versprüht bzw. zwecks schlammablagerung in die 2. kammer der absetzgrube zurückgeleitet wird.
- Das gereinigte wasser gelangt über ein nachklär-becken zum auslauf. Von hier aus kann es in ein gewässer oder zur versickerung in den boden eingeleitet werden.



BioClear von UNTERHOLZNER. Die vorbildliche tropfkörper-anlage zur abwasserbehandlung von 4 bis 190 einwohner-gleichwerten.

- BioClear ist eine vollautomatisch gesteuerte, normalerweise ins erdreich eingebaute klein-kläranlage.
- BioClear arbeitet geräusch- und geruchfrei.
- BioClear trägt das prüfzeichen PA-I 3953.
- Damit ist sichergestellt, dass das genehmigungsverfahren bei der wasserbehörde zügig und kostengünstig erfolgen kann.

Diese vorteile bietet ihnen nur BioClear von UNTERHOLZNER.
- geringer stromverbrauch.
- selbstreinigung der kläranlage durch spüleinrichtungen.
- ungestörter weiterbetrieb selbst bei ausfall einer pumpe.
- anzeige eventueller unregelmässigkeiten im pumpenbetrieb.
- prüfzeichen PA-I 3953.
- ausgezeichnete prüfergebnisse, z.B. 13 mg/l BSB5.

Zuverlässiger service und kundendienst.
- Die anlage wird von UNTERHOLZNER oder vom bauunternehmer auf die baustelle geliefert.
- Ein bauunternehmer - auf wunsch von UNTERHOLZNER vermittelt - sorgt für fachmännische ausschachtung und einbau.
- Montage von pumpen und rohrleitungen sowie herstellung der elektrischen verbindungen durch unseren kundendienst.
- Inbetriebnahme und einweisung ebenfalls durch unseren kundendienst.
- Notdienst bei störungen.

UNTERHOLZNER übergibt Ihnen damit eine voll funktionsfähige anlage und steht Ihnen jederzeit für eventuelle fragen zur verfügung.

Wohin mit dem abwasser ?
Vor allem in dünnbesiedelten gebieten ist die abwasser-entsorgung individuell zu lösen. Freigefälle-kanalsysteme wie in dichter bewohnten oder gewerblich genutzten gegenden sind aus wirtschaftlichen gründen nicht realistisch. Was bleibt, ist die eigene hausklär- oder in manchen fällen eine gemeinschafts-kläranlage für mehrere wohnhäuser ebenso wie für gebäude mit gewerblicher nutzung.

Von der natur abgeschaut - abbau organischer stoffe.
Alles, was die natur organisch abbaut, wird wieder zu fruchtbarer erde. Für genau diese natürlichen vorgänge finden die daran beteiligten mikroorganismen in UNTERHOLZNER kläranlagen ein optimales miliö. Durch langjährige erfahrung und intensive forschung ist es uns gelungen, diesen natürlichen prozess wirkungsvoll auf den arbeitsablauf von klein-kläranlagen zu übertragen. Hierdurch erreichen UNTERHOLZNER klein-kläranlagen ausgezeichnete ablaufwerte. Das UNTERHOLZNER - lieferprogramm bietet Ihnen für alle gegebenheiten das massgeschneiderte system.
- Die mehrkammer-grube nach DIN 4261 teil 1 und 3 als einfachste und bekannteste ausführung.
- Die tropfkörper-anlage BioClear als technisch hochentwickelte lösung.

Alle diese anlagen sind tausendfach bewährt. Nutzen sie daher den UNTERHOLZNER beratungsservice.

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Mineral leaching

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