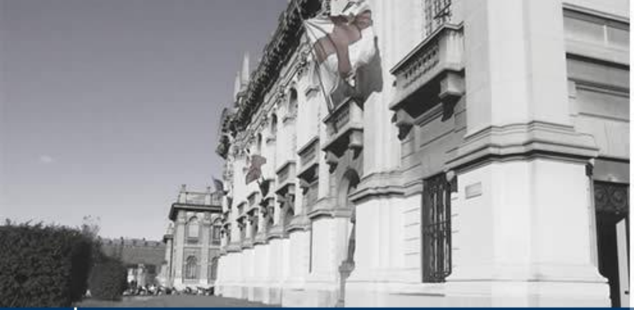


POLITECNICO DI MILANO

Coadvise + Treasure programmes

Specialist Course

Tlemcen, 7th – 11th February 2010

Biomass activity measurements

Part 1 – Microbiology and wastewater characterisation

Roberto Canziani

General Index (1)

1) **Fundamentals of Microbiology** ***(short hints)***

2) **Main microbial substrates in wastewater: organic substances and nitrogen compounds**

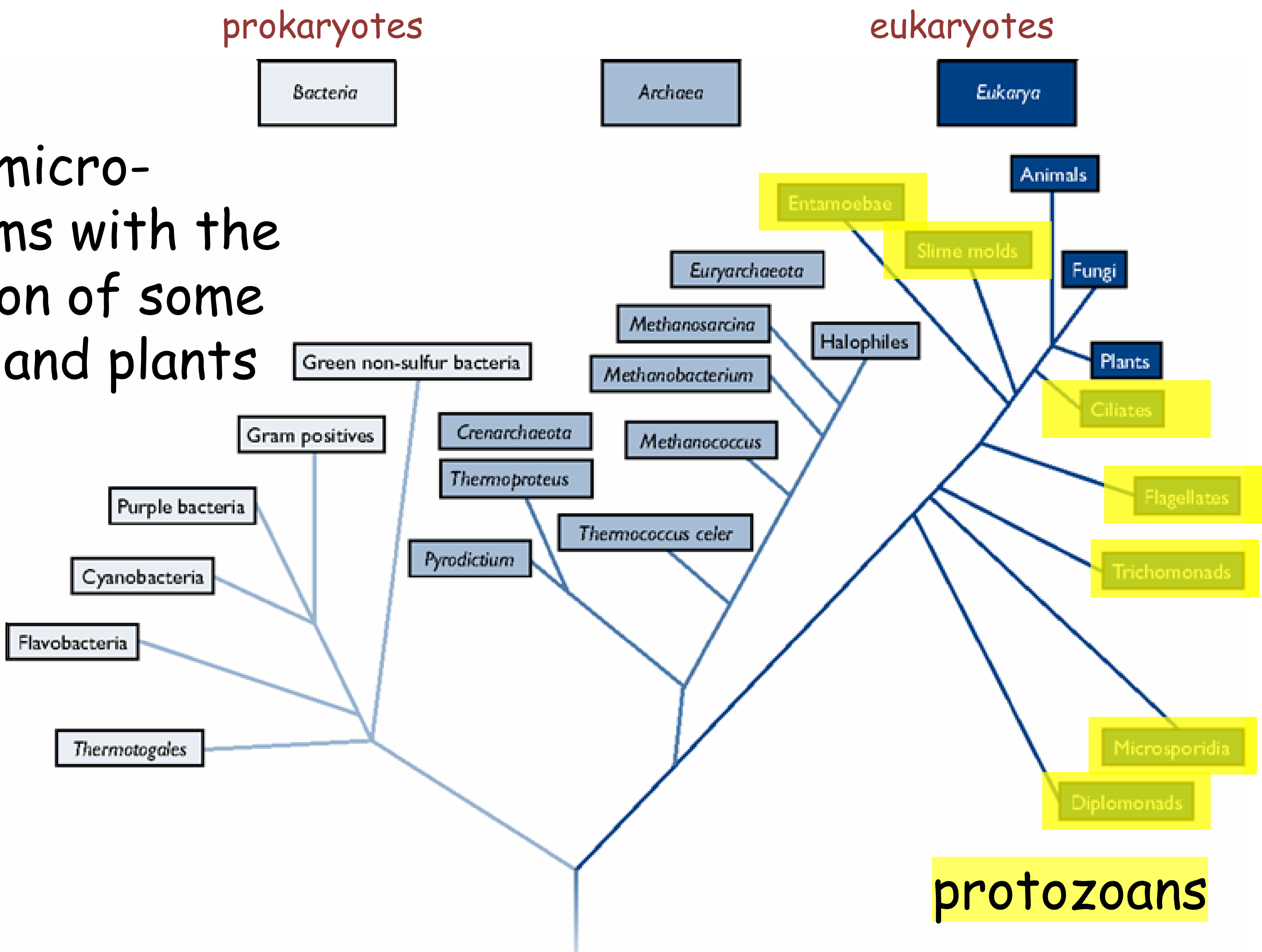
3) **Bacterial activity assessment techniques**

- Respirometry
- Titrimetry
- Manometry
- Calorimetry

Fundamentals of microbiology

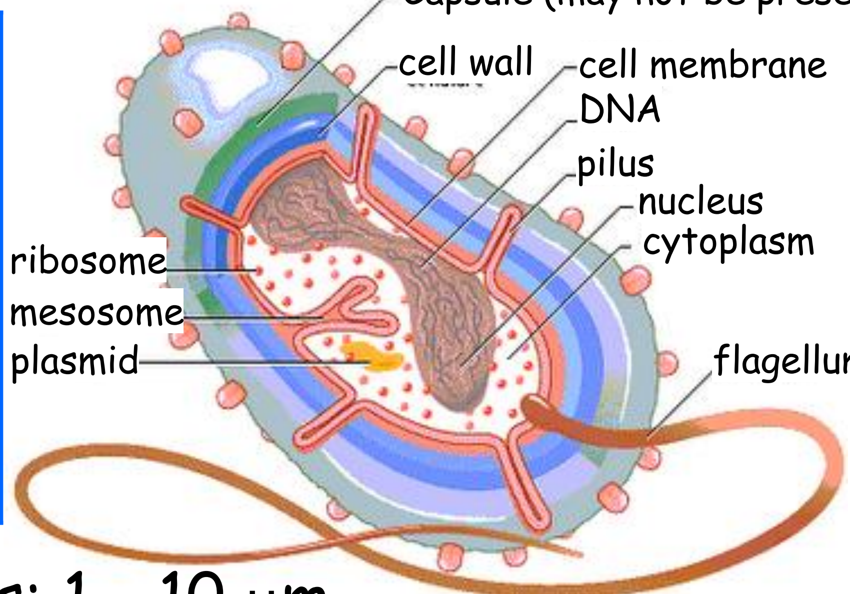
Three domains of life

All are micro-organisms with the exception of some animals and plants



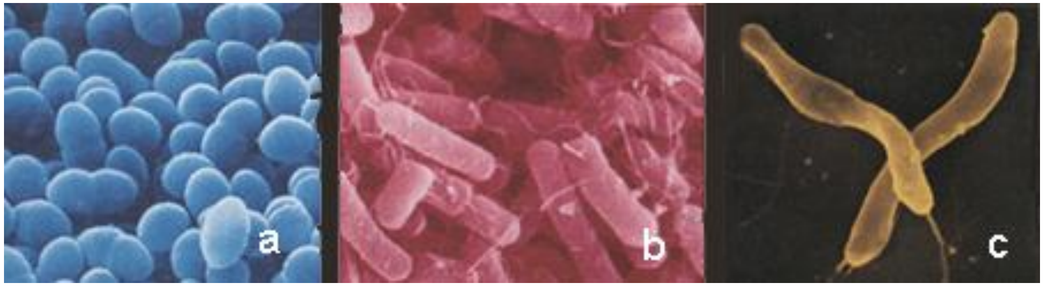
From: depts.noctrl.edu/biology/courses/340/**powerpoint**/27.pps, Dr J. Visick

Algae & Blue Algae	**Microorganisms:** **Prokaryotes and some Eukaryotes**
Protozoans	
Fungi (Yeasts and molds)	Single-cell or multicellular organisms without tissue differentiation
Prokaryotes (Bacteria & Archaea)	
(Viruses)	



Linear dimensions: 1 – 10 μm

Some bacteria can protect themselves in a **spore**, very resistant to environmental agents. Others have a protective **capsule**.



a – cocci (spheric shaped)

b – bacilli (cylindric shaped)

c – spirilla (spiral shaped)

BACTERIA

~ 80% is water. Solid mass can be represented by empirical formulas such as: $C_5H_7O_2N$ or $C_{60}H_{87}N_{12}O_{23}P$.

Only **soluble compounds** can cross their semi-permeable membrane by a **passive** transfer (osmotic, as for water) or **active** (mediated by enzymes).

CRITERIUM	CLASSIFICATION		
Origin	faecal	environmental	
Hygiene - based	Pathogens *(pathogenic bacteria)*	non-pathogenic	
Temperature	psycrophilic (2-20°C)	mesophilic (20-45°C)	thermophilic (45-75°C)

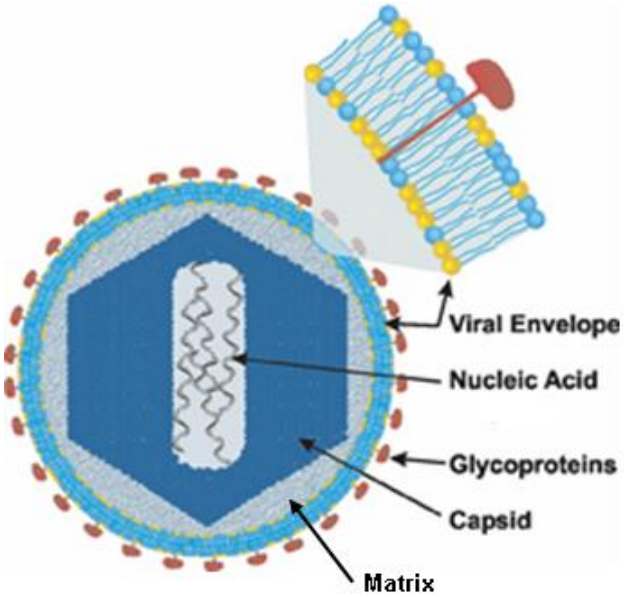
Energetic	photosynthetic (energy from solar radiation)	chemiosinthetic (energy from chemical reactions)	
Trophic	phototrophs	lithotrophs	organotrophs
Carbon source	autotrophs (inorganic C, i.e.: CO_2)	heterotrophs (organic C)	

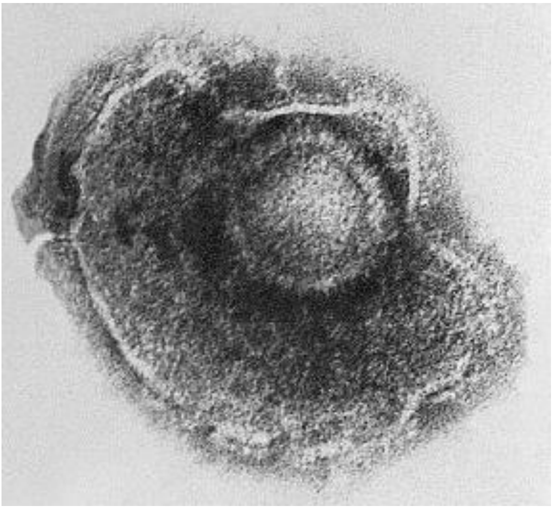
Redox reaction	aerobes (O_2 as electron acceptor)	anoxic/anaerobes (a different substrate acts as electron acceptor)

VIRUSES

Single-cell organisms (0,01 - 0,1 μm), often pathogens and parasites as they live at the expense of a host-organism.

They are made of a viral envelope, a protein capsule (capsid) containing long-chain DNA and/or RNA. In spite of being simpler, more primitive and smaller than bacteria, they are often resistant to disinfection (Enterovirus spp. as Coxsackie and **Poliovirus**, **Haepatitis A/B**, **HIV**…).





Varicella (Chickenpox) virus

MICROALGAE

photosynthetic single-cell organisms (~ 10 μm), empirical formula $C_5H_8O_2N$ or $C_{106}H_{180}O_{45}N_{16}P$: useful to determine their nutrient requirement: C:N:P = 106:45:16 (see later: **eutrophication**)

PROTOZOANS

Single-celled organisms, often cause severe **diseases** (es.: Criptosporidium, Plasmodium, Giardia → Amoeba, Leishmania).

See a complete list on:

http://www.microbiologyprocedure.com/eukaryotes-microbes/list-of-protozoan-diseases-in-human.htm

HELMINTHS (PARASITIC WORMS)

Three Phyla: Plathelminths (classes of Cestodes and Trematodes) and Nematodes, also cause severe **diseases** (e.g.: schistosomiasis)

See more on WHO: Controlling disease due to helminth infections (2003)

http://www.who.int/wormcontrol/documents/en/Controlling%20Helminths.pdf

Atlas of Medical Parasitology: **http://www.cdfound.to.it/_atlas.htm**

Metabolism: the chemical changes in living cells by which energy is provided for vital processes and activities and new material is assimilated:

CATABOLISM

Biochemical reactions that produce energy and stable waste residues.

ANABOLISM

Biochemical reactions that lead to new cell synthesis.

$$CO_2 + H_2O + energy \xrightarrow{clorophyll} CH_2O + O_2$$

Energy source:

- solar: phtosynthesis,
- chemical: respiration.

$$CH_2O + O_2 \rightarrow CO_2 + H_2O + energy$$

- aerobic: oxidation with O_2 as an electron donor
- anaerobic: no free O_2; if oxides are used (SO_4^{2-}, NO_3^-) the process is called anoxic respiration.

  When no external electron acceptors are available, organic substance (o.s.) is degraded by heterotrophic fermentation, an oxidation-reduction process where part of o.s. is oxidised to CO_2 and part is reduced. The last step of fermentation leads to acetic acid. Further degradation is carried out by **heterotrophic methanogens**: $CH_3COOH \rightarrow CH_4 + CO_2$

AEROBIC AUTOTROPHIC BACTERIA

$$NH_4^+ + 2O_2 \longrightarrow NO_3^- + H_2O + 2H^+$$

oxidation of inorganic compounds with O_2 . *(e.g.: nitrifiers)*

ANOXIC AUTOTROPHIC BACTERIA

$$5S + 2H_2O + 6NO_3^- \longrightarrow 5SO_4^{2-} + 3N_2 + 4H^+$$

oxidation of inorganic compounds with combined oxygen (NO_2^-, NO_3^-, SO_4^{2-})
(e.g.: anoxic S oxidation; methane oxidizers utilize CH_4 as a substrate in conjunction with the reduction of sulfate and nitrate; ***Anammox*** *bacteria reduce nitrite and oxidize ammonia to N_2 and water:* $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$*)*

ANAEROBIC AUTOTROPHIC BACTERIA

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$$

oxidation of inorganic compounds without O_2. *(e.g.: autotrophic methanogens)*

AEROBIC HETEROTROPHIC BACTERIA

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

Organic compounds oxidation with O_2 *(e.g.: aerobic heterotrophs).*

ANOXIC HETEROTROPHIC BACTERIA

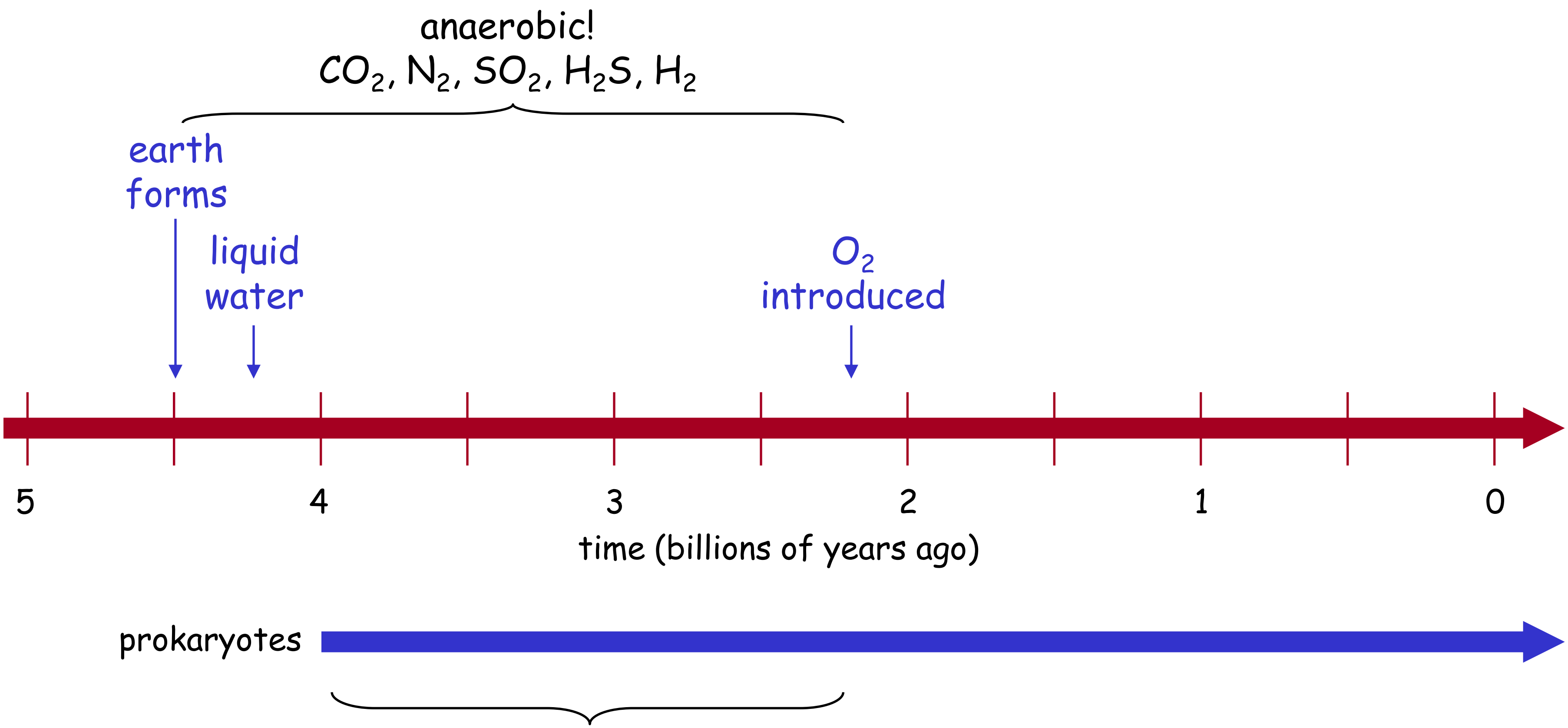
$$C_6H_{12}O_6 + 4NO_3^- \longrightarrow 6CO_2 + 2N_2 + 6H_2O$$

Organic compounds oxidation with combined oxygen (NO_3^-. SO_4^{2-})
(e.g.: heterotrophic denitrifiers).

ANAEROBIC HETEROTROPHIC BACTERIA

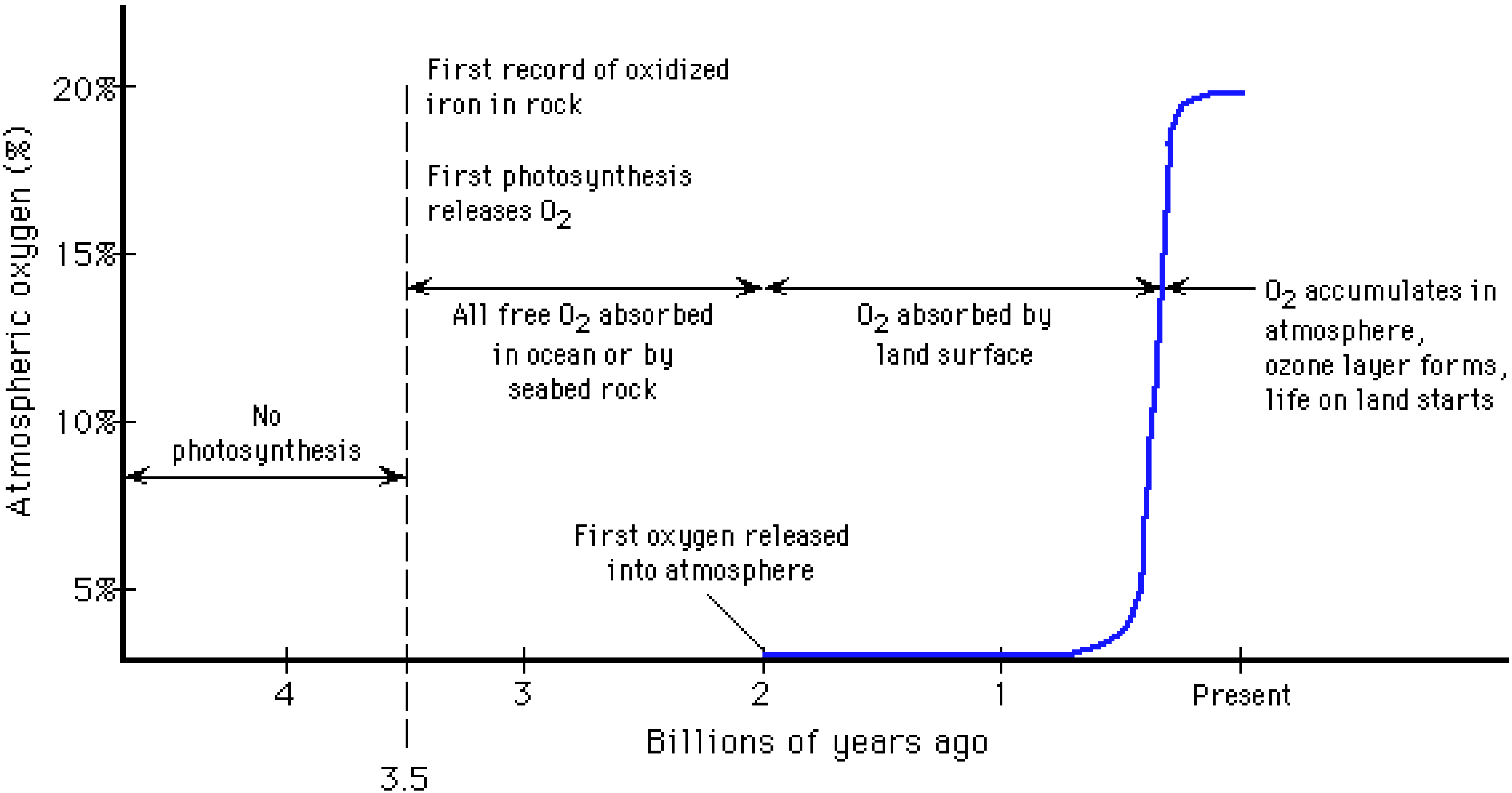
$$C_6H_{12}O_6 \longrightarrow 3CH_4 + 3CO_2$$

Oxidation-reduction of organic compounds *(e.g.: heterotrophic methanogens)*



- anaerobic chemoautotrophs (methanogens?)
- heterotrophs (fermentation and anaerobic respiration)
- anoxigenic photosynthetic bacteria

*From: depts.noctrl.edu/biology/courses/340/**powerpoint**/13.pps*

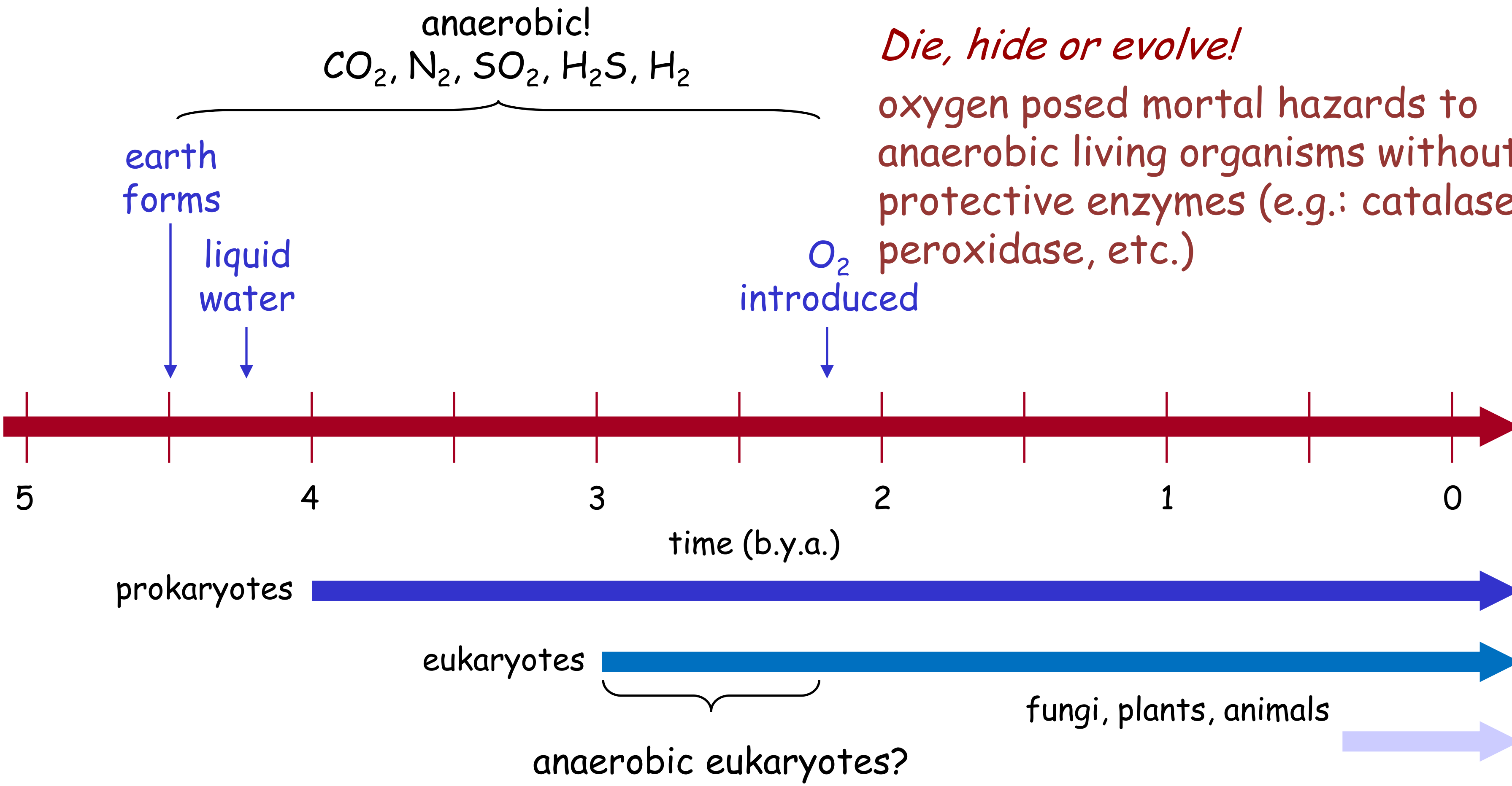


*From: depts.noctrl.edu/biology/courses/340/**powerpoint**/13.pps*

Microbiology applied to Environmental Engineering – The Oxygen Revolution (3)

Die, hide or evolve!

oxygen posed mortal hazards to anaerobic living organisms without protective enzymes (e.g.: catalase, peroxidase, etc.)



Enzymes and antioxidants to protect from toxic oxygen

Then, potential to develop aerobic respiration. **Why?**

*From: depts.noctrl.edu/biology/courses/340/**powerpoint**/13.pps*

- One of the main sources of pathogens are human and animal faeces.
- In faeces only 1 out of 1 million bacteria is pathogenic…
- … but they are ALWAYS present.
- An ill individual or one who bears pathogens in higher proportion can diffuse the illness

	QUANTITY
Organic matter in soil (humus)	HIGH
Lake and river sediments	HIGH
Sewer sediments	VERY HIGH
Animal manure	VERY HIGH
Human faeces	VERY HIGH
Sewage sludge	VERY HIGH
Clean natural surface water	Low
Polluted natural surface water	Medium
Open air (rural areas)	~ 0
Air emissions from air conditioning	Low
Air emissions from cooling towers	Medium
Air emissions from activated sludge plants	Medium
Air recycled from confined environment	Low

What's the advantage of aerobic respiration?

Higher energy yield! (more biomass production…)

	electron acceptor		energy yield	
Chemoautotrophy				
Methanogens	CO_2	-0.38 V	H_2	57 kcal/mole
Sulfur oxidizers	S	-0.28 V	S	119 kcal/mole
Anaerobic respiration				
Sulfate reducers	SO_4^{2-}	-0.22 V	glucose	22 kcal/mole
Nitrate reducers	NO_3^-	+0.42 V	glucose	160 kcal/mole
Aerobic respiration				
Aerobes	O_2	**+0.82 V**	**glucose**	**248 kcal/mole**

From: North Central College Illinois (USA); Course in "Microbiology" Dr J. Visicks
*http://depts.noctrl.edu/biology/courses/340/**powerpoint**/13.pps*

Main microbial substrates in wastewater:

organic substances

and

nitrogen compounds

Analytical need: estimate **ALL organic matter** (which is a mixture of many heterogeneous compounds) which is present in a water or wastewater sample.

Their complete degradation to **H_2O e CO_2** requires **OXYGEN**

Methods which measure the oxygen required for organic matter oxidation are: T.O.D. **B.O.D.** **C.O.D.**

T.O.C. is a method which measures the organic carbon which is converted into **CO_2**

BOD – definition and measurement - first order degradation kinetics (1)

The **Biochemical Oxygen Demand** (BOD), estimates the oxygen required for the oxidation of organic matter by the aerobic metabolism of the microbial flora.

BOD(t): BOD as a function of *time*

First-order kinetics: $\frac{dS}{dt} = -k \cdot S \longrightarrow S_t = S_0 \cdot e^{-k \cdot t} = S_0 \cdot 10^{-k' \cdot t}$

S, S_t = substrate (org. matter) concentration [mg/L] at time t

k = biodegradation constant $[t^{-1}]$

k' = 0,43 k

Measurement is performed at 5 (BOD_5) and 20 (BOD_{20}) days (in Norway, BOD_7 is common)

BOD(T): BOD as a function of *Temperature*

Biodegradation gets quicker at increasing temperature; this is shown by the dependence of k with T: k = k(T) : $k_T = k_{20} \cdot \theta^{(T-20)}$ (θ = 1,04 – 1,06)

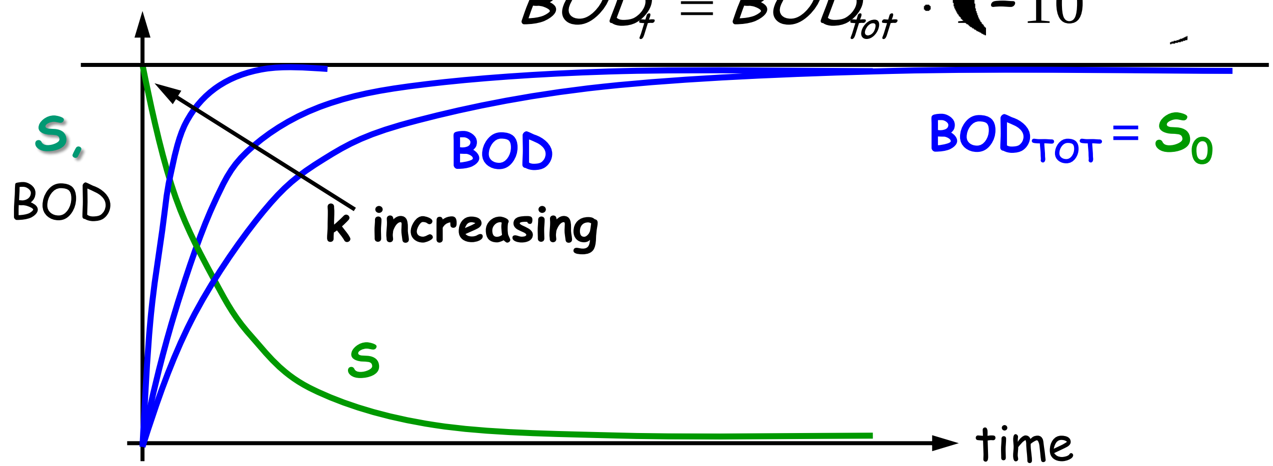
Measurement is performed at 20°C

BOD – definition and measurement – first order degradation kinetics (2)

$S_t \quad = \quad S_0 \quad - \quad (S_t - S_0)$

- S_t → residual substrate at day t
- S_0 → biodegradable substrate at start t=0 (total substrate) → BOD_{tot}: oxygen necessary to biodegrade all biodegradable substrate
- $(S_t - S_0)$ → substrate biodegraded in "t" days → BOD_t: oxygen necessary to biodegrade biodegradable substrate in "t" days

$$BOD_t = BOD_{tot} \cdot \left(1 - 10^{-k \cdot t}\right)$$



BOD – dilution method (1)

METHOD

1. A sample is preserved in the field at 4°C and the analysis started within 4 hours.

2. The sample is incubated until it gets T = 20°C.

3. The sample is aerated to bring the dissolved oxygen content to saturation

4. Comparison of the dissolved oxygen content at the beginning and the end of the incubation period is the measure of the BOD (Biochemical Oxygen Demand).

more details at: http://www.ungiwg.org/openwater/?q=node/98

BOD – dilution method

Direct method:

If BOD < 7 mg/L, it is determined directly by measuring the dissolved content of the water sample before and after a five days incubation period at 20°C.

Unseeded dilution method:

If BOD > 7 mg/L, appropriate sample aliquots are diluted using dilution water, saturated with oxygen, and the oxygen content is determined before and after the incubation period.

A minimum of three dilutions per sample, with a final content between 40% and 70% of the original oxygen concentration, will give best results.

more details at: http://www.ungiwg.org/openwater/?q=node/98

Seeded dilution method:

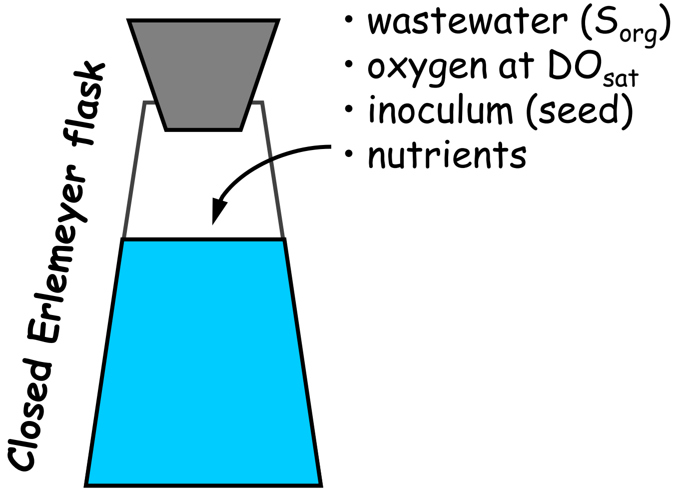
conditions must be appropriate for the living organisms to function unhindered during the incubation period.

Toxic substances should be absent

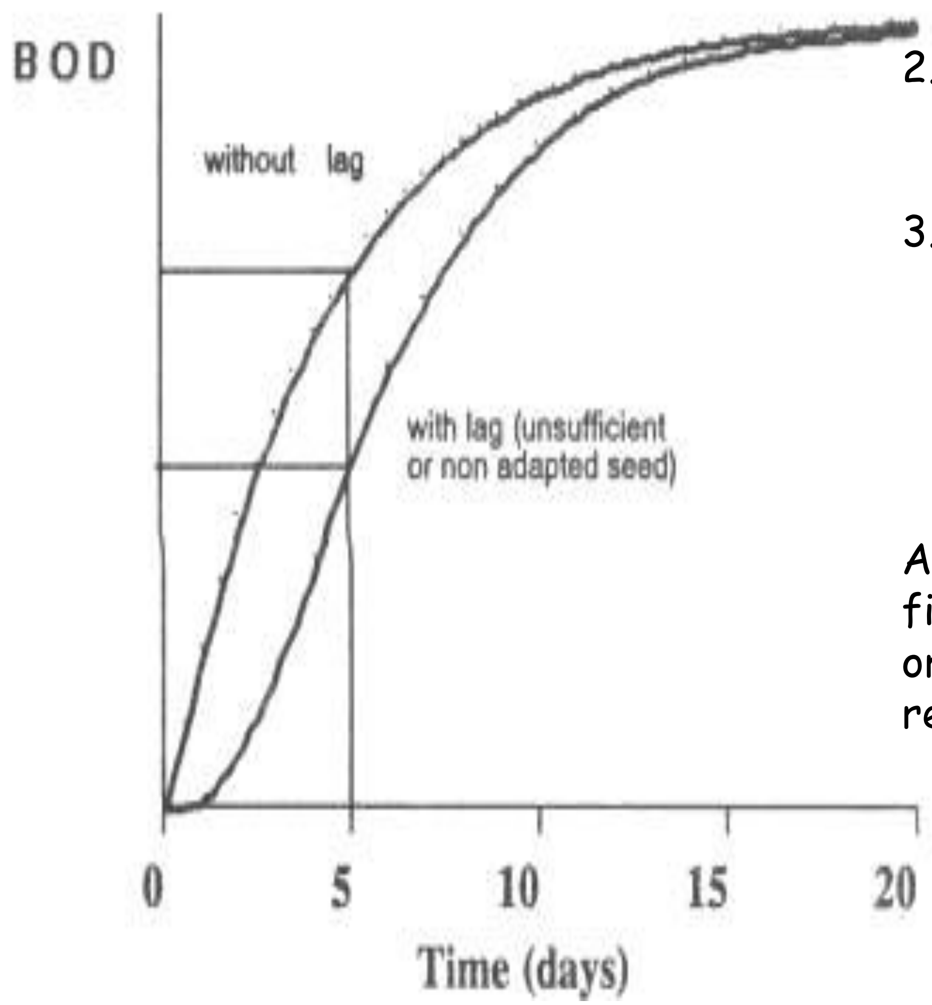
Necessary nutrients, such as N and P, should be present.

It is important that a mixed group of organisms (called "**seed**" or "**inoculum**") be present during the test.

The dilution water is usually seeded with a little quantity (2 – 3 drops in a liter) of activated sludge and saturated with oxygen (overnight) before the BOD test.



BOD – seeded dilution method (2)

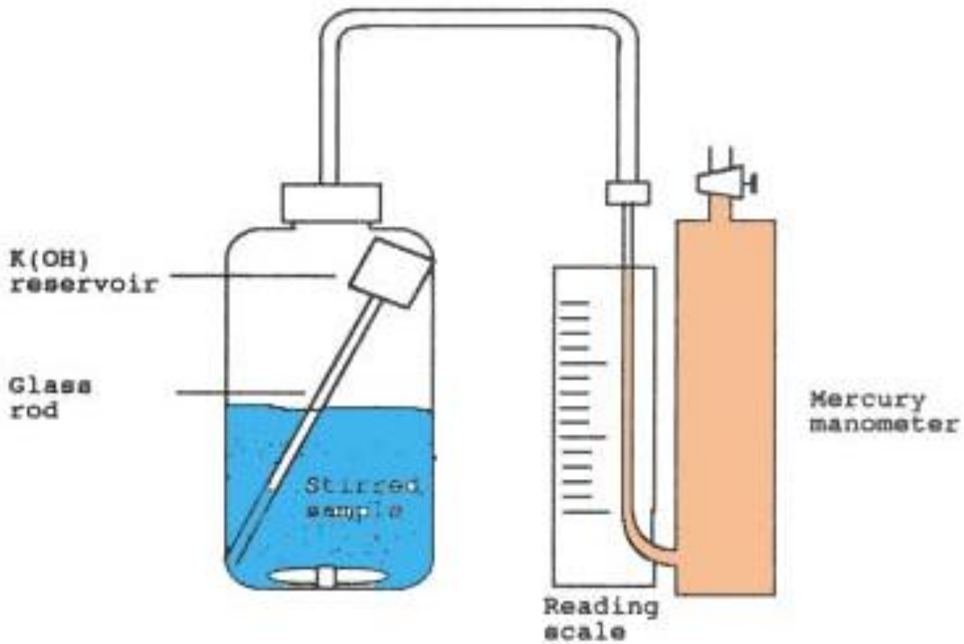


Siphon the diluted sample to fill **three** BOD bottles:

1. one for incubation and BOD_5 measurement (BOD after five days),
2. one for the determination of the dissolved oxygen content (measured and recorded as "initial DO"),
3. one for the determination of the immediate dissolved oxygen demand (IDOD), after a 15 minutes incubation period (to eliminate the oxygen demand from sulfide, sulfite and/or ferrous ions).

A minimum of three dilutions per sample, with a final content between 40% and 70% of the original oxygen concentration, will give best results

BOD analysis – manometric method



The sample is kept in a sealed container fitted with a pressure sensor. A substance that absorbs carbon dioxide (typically KOH or LiOH) is kept above the sample level.

Oxygen is consumed and carbon dioxide is released, but is absorbed: a pressure drop can be observed, as the total amount of gas decreases.

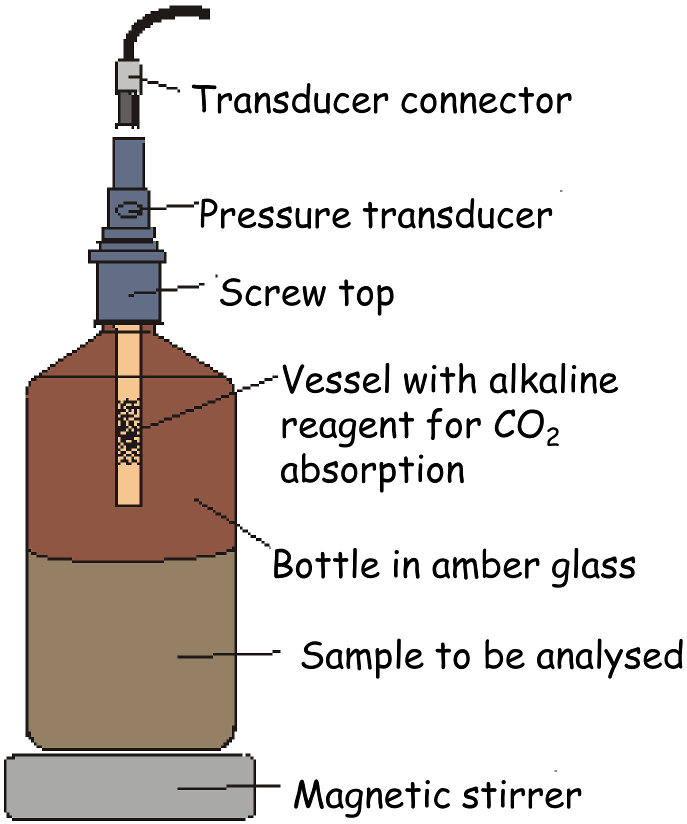
From the drop of pressure the consumed quantity of oxygen can be derived.

Advantages

simplicity: no dilution of sample required, no seeding, no blank sample

direct reading and continuous display of BOD value (a graph of its evolution can be plotted).

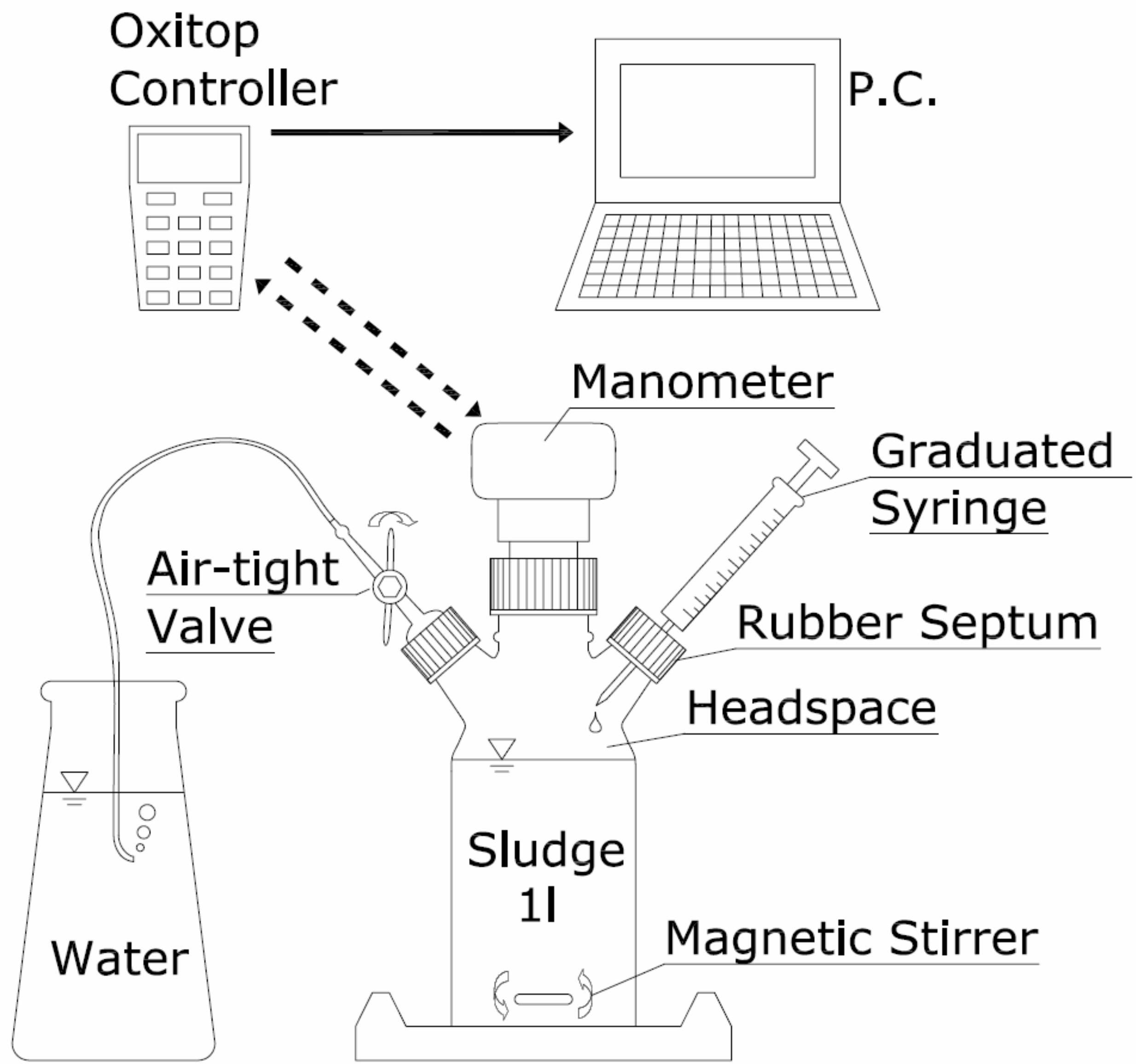
BOD analysis – Warburg respirometer



Bottle equipped with pressure-measuring head

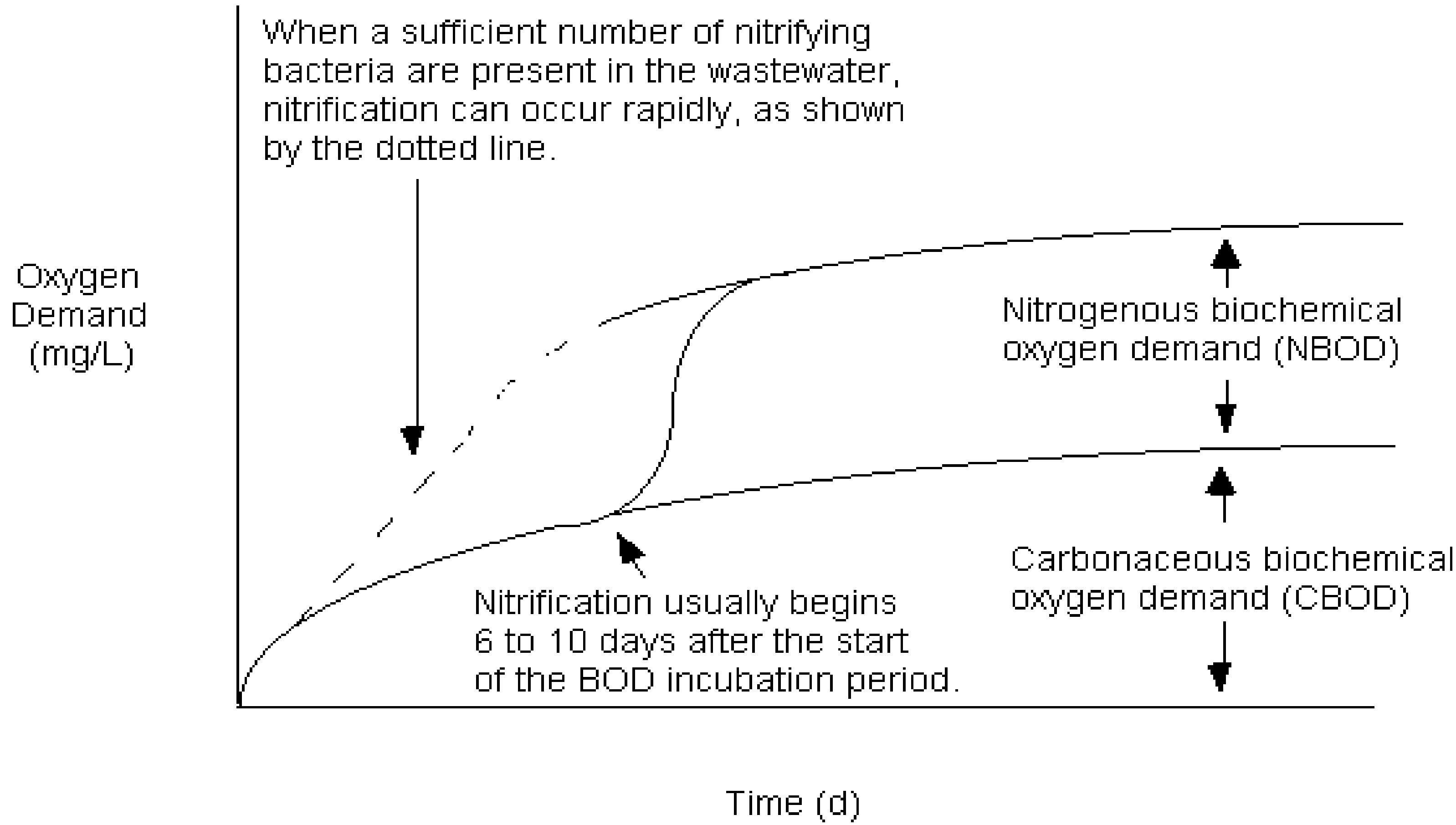
Bottle equipped with pressure-measuring head and data logger and transfer device (Oxitop)

BOD analysis - Oxitop



BOD analysis - Oxitop

BOD analysis – suppression of nitrogenous demand



Nitrogenous oxygen demand can be suppressed by adding Allyl-thiourea (ATU)

Chemical oxygen demand (COD) – definition and measurement (1)

Chemical Oxygen Demand (COD) is the amount of oxygen in water consumed for chemical oxidation of pollutants.

$$C_nH_aO_bN_c + \left(n + \frac{a}{4} - \frac{b}{2} - \frac{3}{4}c\right) O_2 \rightarrow nCO_2 + \left(\frac{a}{2} - \frac{3}{2}c\right) H_2O + cNH_3$$

COD can be estimated if org. matter composition is known

For example, if a sample has 500 ppm of phenol:

$C_6H_5OH + 7O_2 \rightarrow 6CO_2 + 3H_2O$ COD = (500/94)(7)(32) = 1191 ppm

Chemical oxygen demand (COD) – definition and measurement (2)

In the analytical method potassium dichromate ($K_2Cr_2O_7$) is used as oxidant and H_2SO_4 is added, boiling for 2h):

$$C_nH_aO_bN_c + dCr_2O_7^{2-} + (8d + c)H^+ \rightarrow nCO_2 + \frac{a+8d-3c}{2}H_2O + cNH_4^+ + 2dCr^{3+}$$

excess $K_2Cr_2O_7$ is titrated with
ferrous ammonium sulfate FAS: $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$

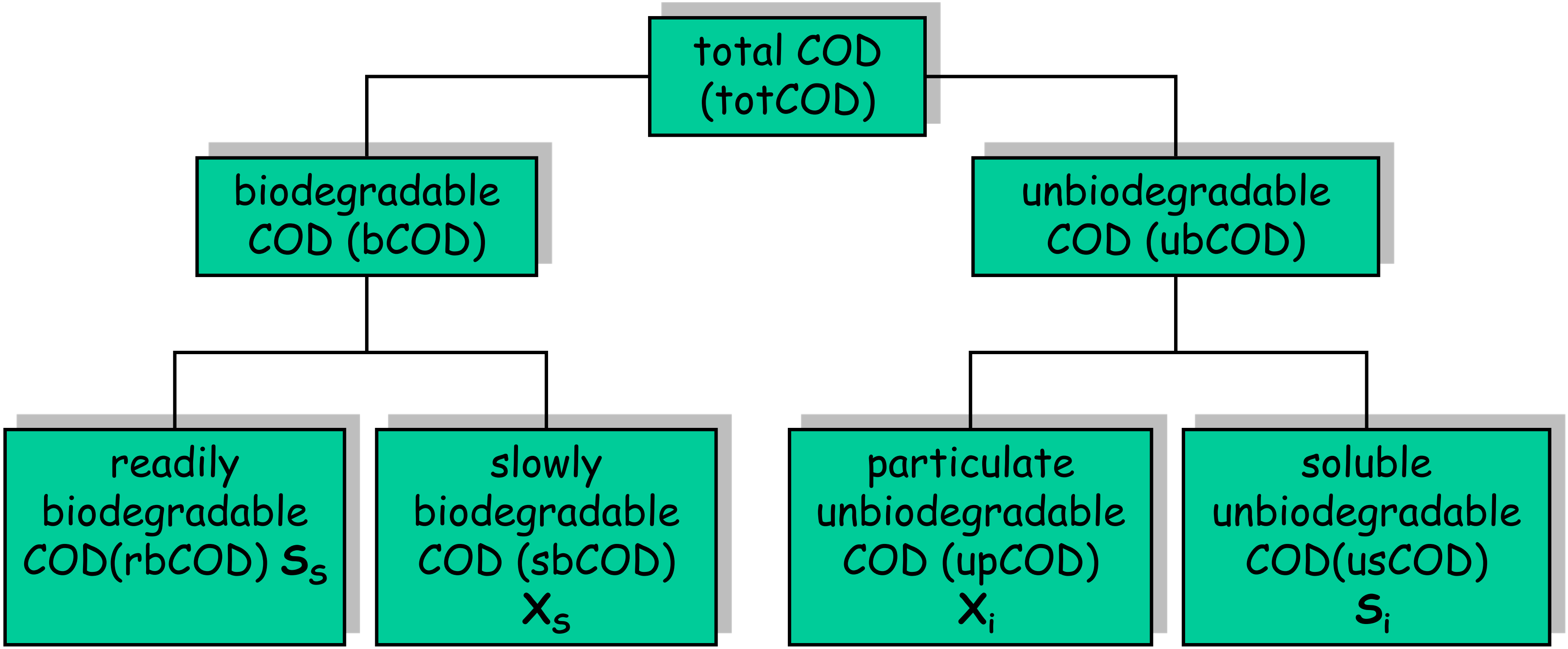
until all of the excess oxidizing agent has been reduced to Cr^{3+}.

Ferroin (*) is used as indicator as it changes from blue-green to reddish-brown when all dichromate has been reduced to Cr^{3+} (1 eq O = 1 eq FAS).

(*) Ferroin = $C_{36}H_{24}FeN_6O_4S$, MW: 692,24

Chemical oxygen demand (COD) – definition and measurement (3)

For modelling purposes, total COD in wastewater is divided into the following fractions:



rbCOD *(Readily Biodegradable COD):*

It is part of the soluble fraction

- **acetate** and VFAs,
- **glucose** and simple sugars,
- **ethanol** and simple alcohols
- other small molecules

that bacteria assume directly through the cell membrane.

sbCOD *(Slowly Biodegradable COD)*

any organic compound that can be biodegraded through previous **hydrolysis**, which is carried out by exocellular enzymes.

upCOD *(Unbiodegradable Particulate COD)*

Any substance that is not biodegraded under the operational conditions of the biological system. Removal may occur through bio-flocculation.

usCOD *(Unbiodegradable Soluble COD)*

It is usually defined as the soluble residual COD after extensive biological treatment.

NOTE: usCOD in the effluent includes usCOD of the influent + unbiodegradable microbial soluble products derived from cell decay.

bCOD = rbCOD + sbCOD $\cong BOD_{20}$

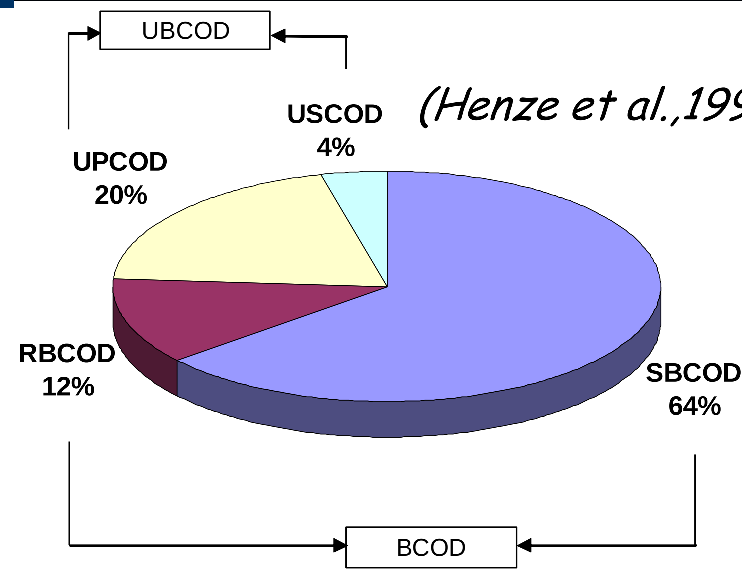
rbCOD can be estimated as

1) the soluble COD after flocculation with $ZnSO_4 \cdot 7H2O$ at pH>11 and filtration on a 0,45-μm membrane(*)
2) Respirometric tests

As a first approximation, usCOD can be estimated as the soluble effluent COD from a conventional AS process with sludge age > 5 days.

(*)Mamais D., Jenkins D., Pitt P. (1993) A rapid physical-chemical method for the determination of readily biodegradable soluble COD in municipal wastewater, Water Research, 27 (1), 195-197

Chemical oxygen demand (COD) – definition and measurement (7)



(Henze et al.,1997)

Influent total COD (tCOD)
350 – 700 mg/L = 100%

biodegradable COD (bCOD)
60% – 85%

unbiodegradable (inert) COD (uCOD)
15% – 40%

Soluble readily biodegradable COD (rbCOD)
5% - 30%

Particulate slowly biodegradable COD (sbCOD)
30% - 80%

Soluble unbiodegradable COD (usCOD)
2% - 10%

Particulate unbiodegradable COD (upCOD)
18% - 30%

Organic nitrogen (N_{org}): is degraded to **ammonium N (NH_4^+-N)**

proteins ⇒ polypeptides ⇒ peptones ⇒ aminoacids ⇒ urea ⇒ NH^+_4

In wastewater, usual ranges are

20 - 60 % N_{org}

80 - 40 % NH_4^+-N

Total Kjeldahl Nitrogen = TKN = N_{org} + N-NH^+_4

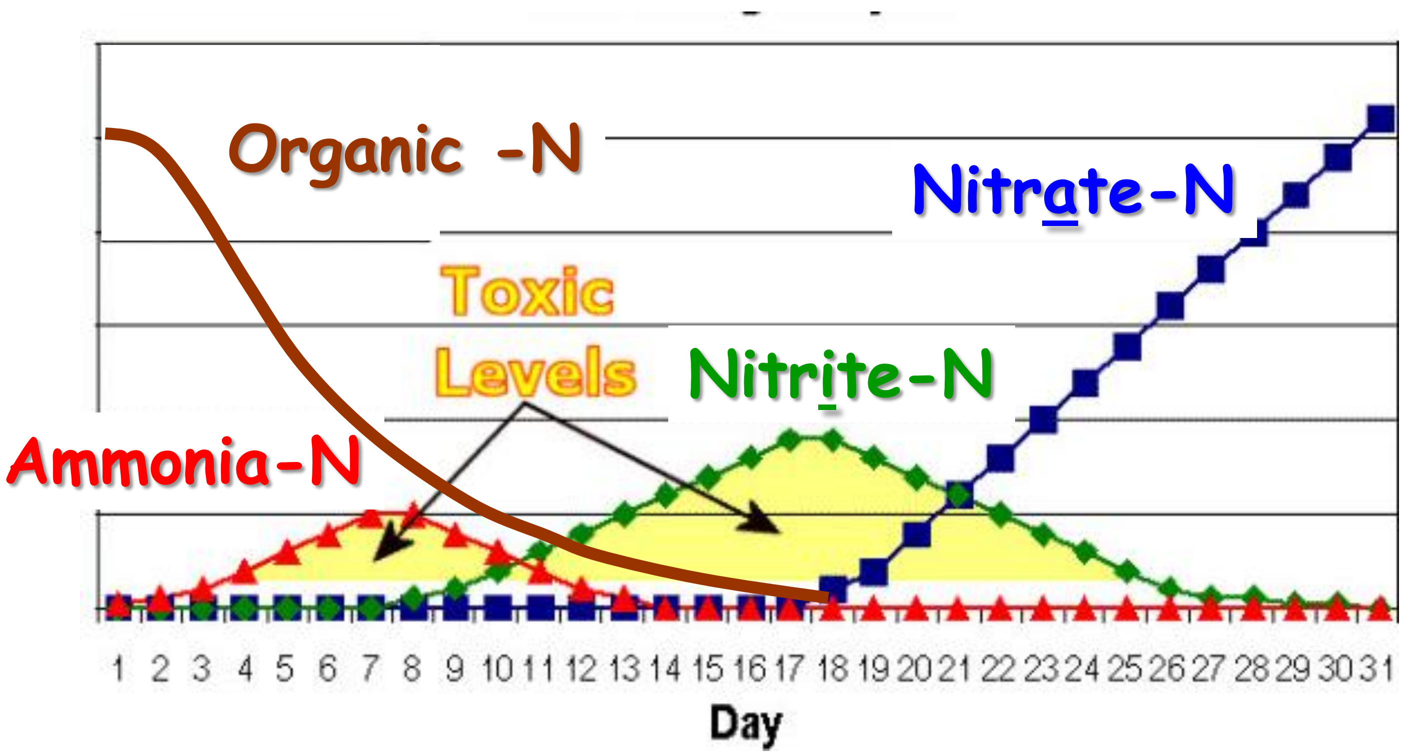
Nitrogen compounds (2)

a) urine → aminoacids + urea (N_{org}) → NH^+_4 -N

b) autotrophic nitrifiers **convert NH^+_4 to NO^-_2 and these to NO^-_3:**

Nitrosomonas, Nitrosospira, Nitrosococcus: $NH^+_4 + 3/2\ O_2 \Rightarrow NO^-_2 + H_2O + 2H^+$

Nitrobacter, Nitrospira, Nitrococcus: $NO^-_2 + 1/2\ O_2 \Rightarrow NO^-_3$

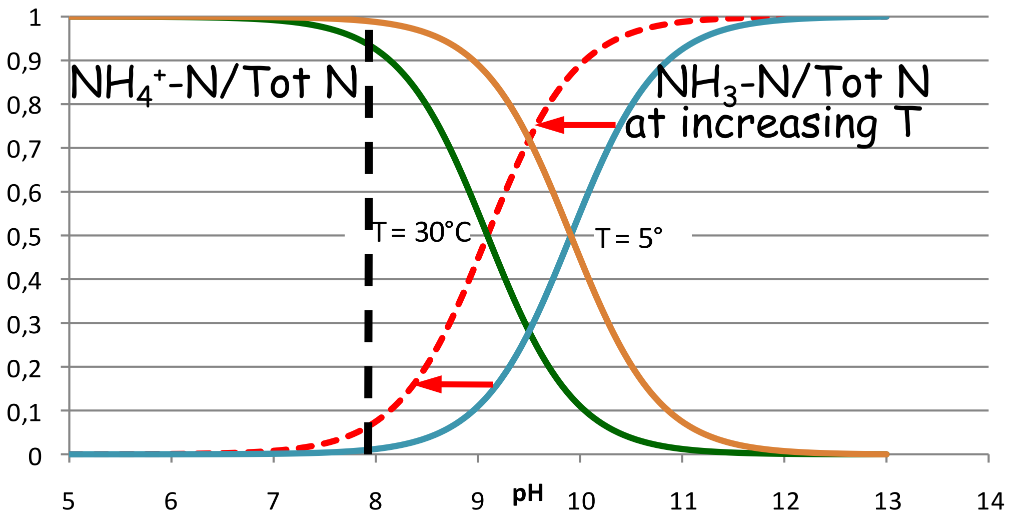


NH^+_4 can take days to be converted into NO_3^-.

The following equilibrium holds

$$H^+ + NH_3 \rightleftharpoons NH_4^+$$

NH_3: toxic at 0,1 mg N/L; NH_4^+: non toxic to fish even at 10 mg/L



$$[NH_3 - N] = \frac{[NH_4^+ - N] \times 10^{pH}}{e^{\frac{6344}{273+T}}}$$

If an alkaline discharge occurs, causing pH = 8 in water, toxic effects may occur at T > 20°C

Nitrogen compounds (4)

Measuring units:

Es: 30 mg/L TKN

10 mg/L Ammonia (NH_3, MW 17)

25 mg/L NO_3^- (MW 62)

2 mg/L NO_2^- (MW 46)

how much is Total Nitrogen?

Total-N = 30 + 25 x 14/62 + 2 x 14/46 = 36.25 mg Tot-N/L

as Ammonium-N is included in TKN

Ammonium-N = 10 x 14/17 = 8.24 mg N-NH_4^+/L

Org-N = 30 – 8.24 = 21.76 mgN/L

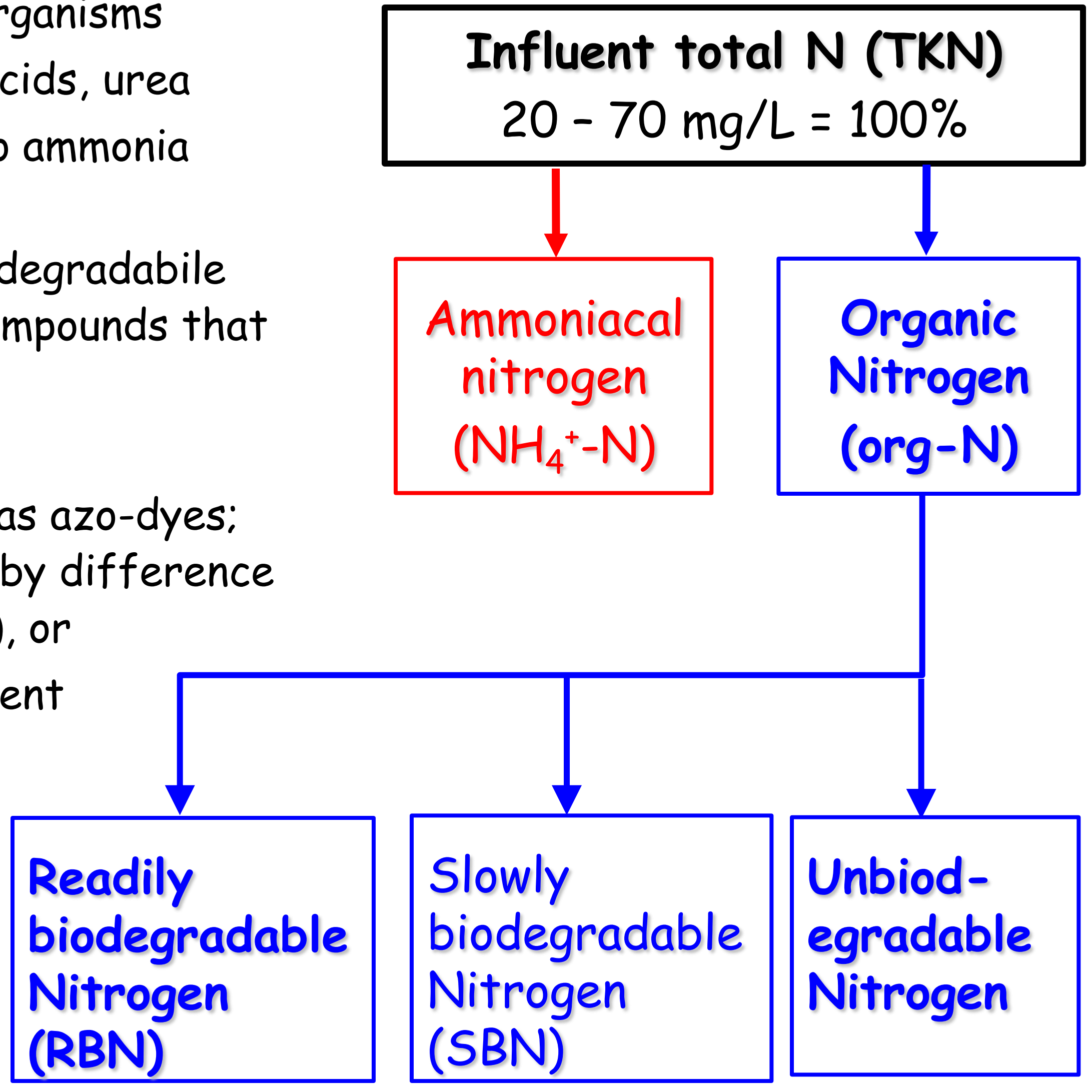
Nitrogen compounds (5)

- **Ammonia**: substrate of nitrifying organisms
- **Org-N**: proteins, peptone, amminoacids, urea
- **rbN**: can be rapidly hydrolyzed into ammonia (S_{ND})
- **sbN**: azoto organico lentamente biodegradabile (X_{ND}); nitorgn in complex organic compounds that need hydrolysis

  $X_{ND}/S_{ND} \cong$ sbCOD/rbCOD
- **nbN**: non-biodegradable TKN, such as azo-dyes; usually soluble. It can be measured by difference nbN = (TKN in – NH_4-N - X_{ND} – S_{ND}), or

  as residual soluble TKN in the effluent

  of a full nitrification process.



Bacterial activity assessment (1)

The diffusion of models for the description of bioprocesses has lead to the following needs

1. ***Wastewater characterization***, as traditional parameters (BOD and COD) were not enough to describe the process adequately.
2. Simpler and cheaper methods for the ***calibration of stoichiometric and kinetic parameters*** of the biomass

↓

In the last decades of the last century new techniques have been developed to fulfil these needs

Bacterial activity assessement (2)

Bacterial activity can be evaluated in batch tests by tracking:

- The concentration of a substrate/product by:
  - manual sampling and analysis
    - ✓☺ simple and conventional
    - ✓☹ time consuming
  - using an on-line probe: titrimetry / respirometry
    - ✓☺ simple and convenient
    - ✓☹ dependent on probe availability/stability/reliability
  - measuring reaction by-products: manometry (gas production) and calorimetry (heat exchanged)
    - ✓☺ simple and convenient
    - ✓☺ simple and convenient (on-line data)
    - ✓☹ dependent on instrument reliability/sensitivity