Added value of microbial life in flocs

W. Verstraete; P. De Schryver; T. Defoirdt; R. Crab

Laboratory for Microbial Ecology and Technology
Faculty of Bioscience Engineering
Ghent University - Belgium

LabMET

UNIVERSITEIT GENT

Faculteit Bio-ingenieurswetenschappen
Faculty of Bioscience Engineering

Greetings from the UGent

W. Verstraete

P. De Schryver

T. Defoirdt

R. Crab

Breakthroughs in the field of Microbial Resource Management (MRM)

- The *Beijerinck axioma*:

  "all micro-organisms are omnipresent"

→ only valid for "open" contiguous enivronments

→ in closed environments: inoculation may be necessary

- The *Darwin "niche theory"* is out; the *Hubbell "neutral theory"* is in:

→ the biodiversity is determined by the influx, the arrival of new species

→ the microbial community makes its own niche

→ ecosystem engineering occurs by the inhabitants themselves

Breakthroughs in the field of Microbial Resource Management (MRM)

- The *Pareto law* is valid for microbial communities:

→ The energy/food distribution as it occurs between micro-organisms corresponds to an 80/20-ratio

→ 20% of the species have 80% of the energy/food-flux

→ measured by DGGE

- The *Power law* is valid for microbial ecosystems:

→ the species diversity relates to the physical size of the system

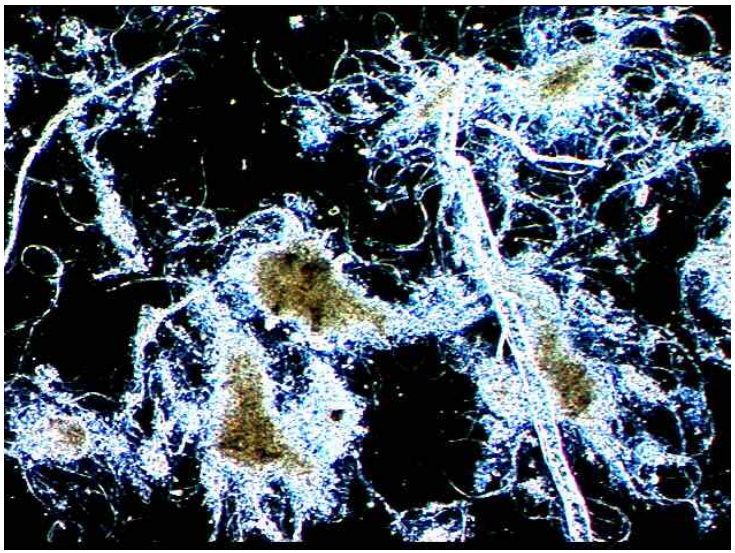
→ $S = c.V^Z$ with S = number of species

V = volume (m^3) of the system

c & Z = coefficients

Breakthroughs in activated sludge operation and control

- Control of filamentous/zoogloeal species within the flocs



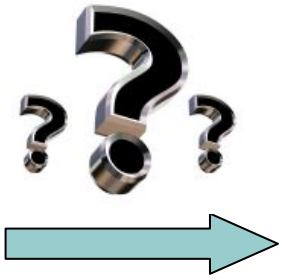
- Co-existance of
  - heterotrophs
  - denitrifiers
  - nitrifiers
  - poly-P/PHB/glycogen accumulating species

Research question

How can production of microbial flocs in activated sludge systems be <u>upgraded</u> to

<u>Bio-Flocs Technology (BFT)</u>

in aquaculture

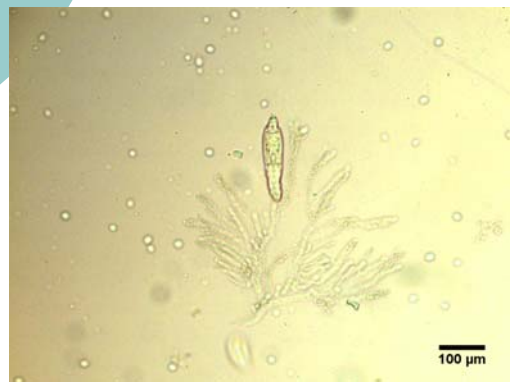


Themes

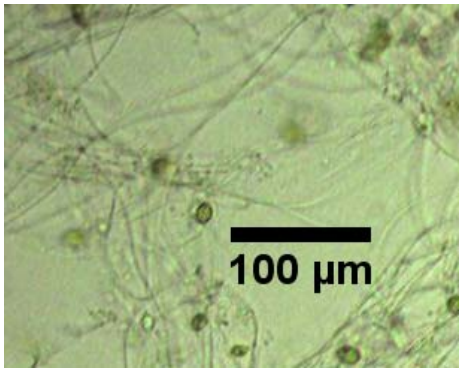
I. What are bioflocs?
II. Motives of micro-organisms for living in microbial structures
III. Mechanisms of binding cells into flocs
IV. Methods to characterise flocs
V. Special nutritious compositions of flocs for aquaculture
VI. Overall conclusions

I. What are bio-flocs?
Biological constituents in bio-flocs

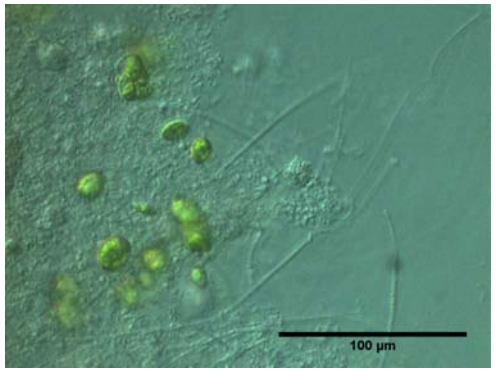
- Sizes can range from a few to several thousands µm
- Main biological constituents :



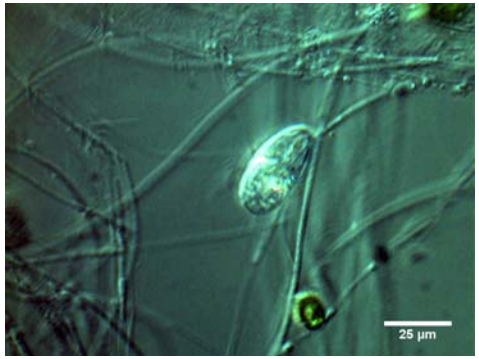
Floc forming bacteria
e.g. *Zoogloea*



Filamentous bacteria



Algae

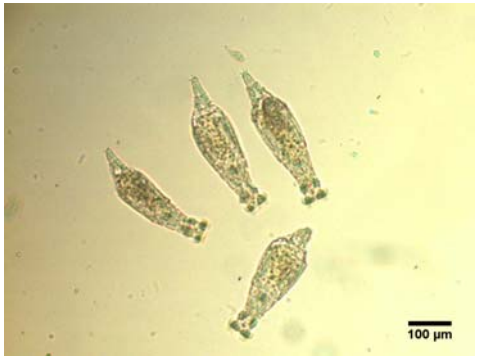


Predating
micro-
organisms like:

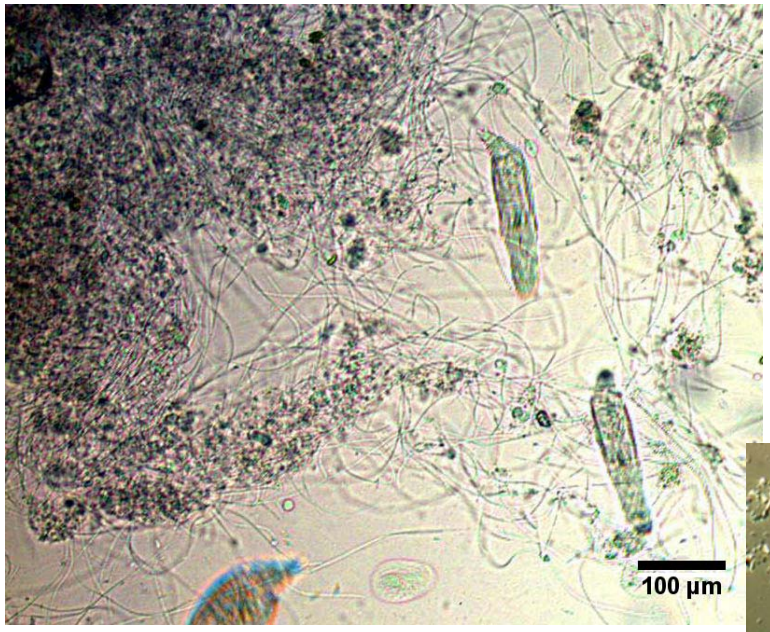
← Protozoa

Rotifers →

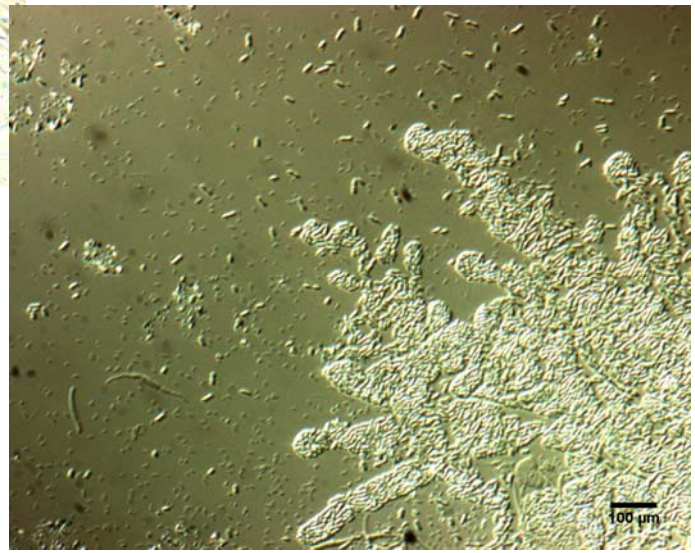
…



I. What are bio-flocs?
Bio-flocs are heterogenous mixtures

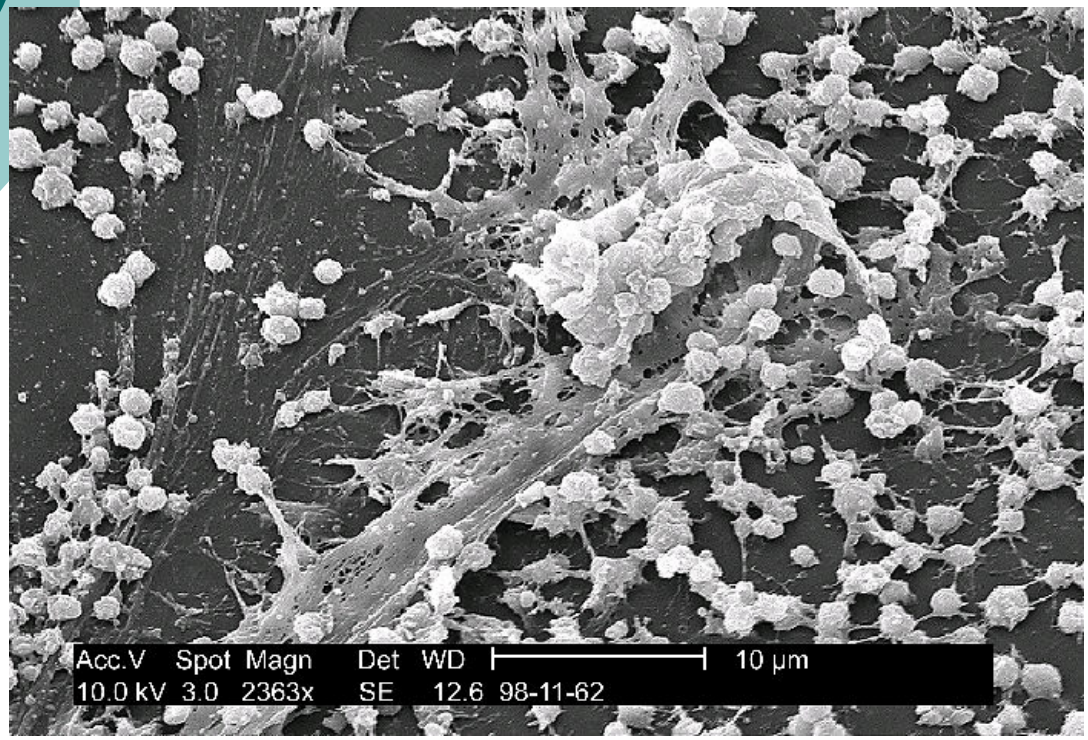


Various compositions depending on environmental factors: C/N-ratio, predation, light, shear rate, temperature,…



- **10 - 90 % percent is living**
- **C/N-ratio ≈ 10**
- **Concentration: from a few to 40 g/dm³**

I. What are bio-flocs?
Special components in bio-flocs



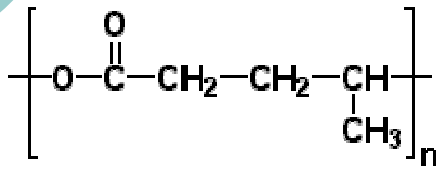
- Extracellular polymeric substances (EPS)
- Storage polymers (PHB, glycogen and polyphosphate)

EPS production*:*

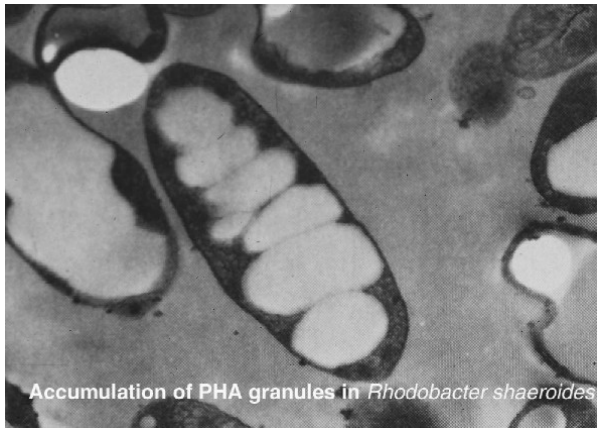
CDC – PHIL – IOWA (USA)
http://phil.cdc.gov/phil/home.asp

I. What are bio-flocs?
Storage polymers in bio-flocs

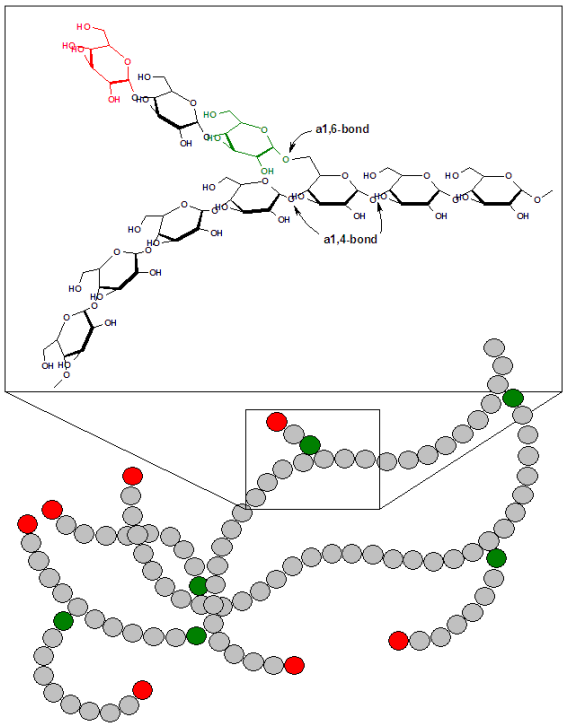
- PHB/glycogen/polyphosphate: accumulate as carbon/energy or reducing-power storage material in microbial cells



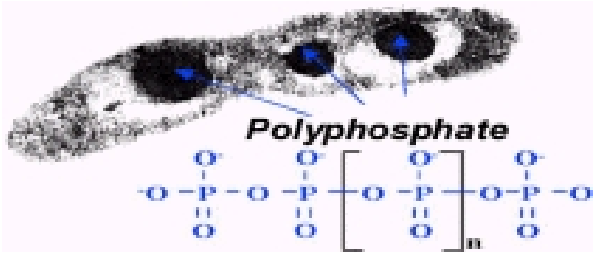
poly(3-hydroxybutyrate)



PHB in bacteria



Glycogen structure



I. What are bio-flocs?
Storage polymers in bio-flocs

- Glycogen accumulating organisms (GAO) en polyphosphate accumulating organisms (PAO):

  Use stored energy to accumulate PHB in the cells (Salehizadeh & Van Loosdrecht, 2004. Biotechnology advances 22, 261-279)

→ GAO: use energy from glycolysis to accumulate substrate (e.g. glucose) fermentation products (e.g. acetate) in the form of PHB

→ PAO: use energy stored as poly-P to store exogenous substrate in the form of PHB

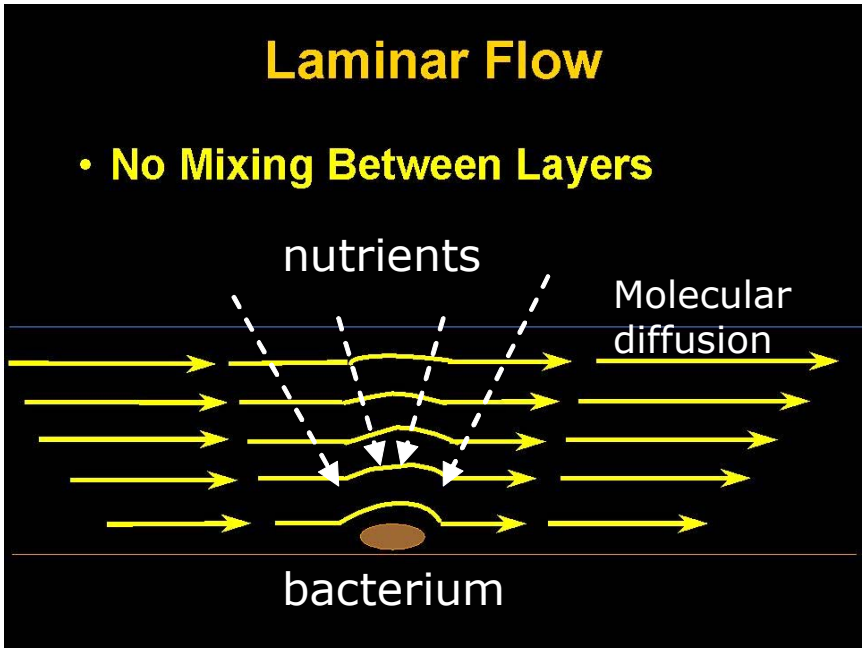
II. Motives of micro-organisms for living in floc structures

1) Avoidance of wash out

2) Food supply:

Individual cells ≈ 1 µm

→ hampered nutrient transfer through laminar layers



SOLUTION!!!

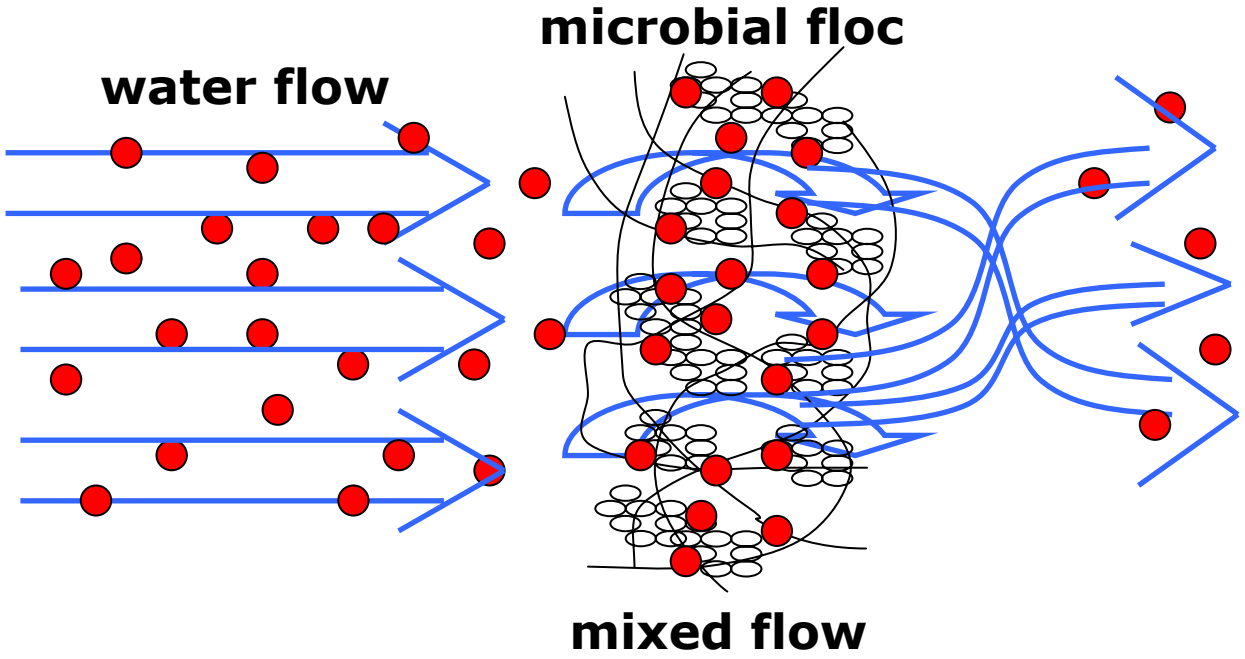
Grouping of the micro-organisms into larger structures = microbial flocs

II. Motives of micro-organisms for living in floc structures

Motive for living in flocs

= advective flow

≈ Harvesting nutrients from water



II. Motives of micro-organisms for living in floc structures

Hypothesis:
Bioflocculation with high porosity (up to 99%) allows advective flow through flocs →

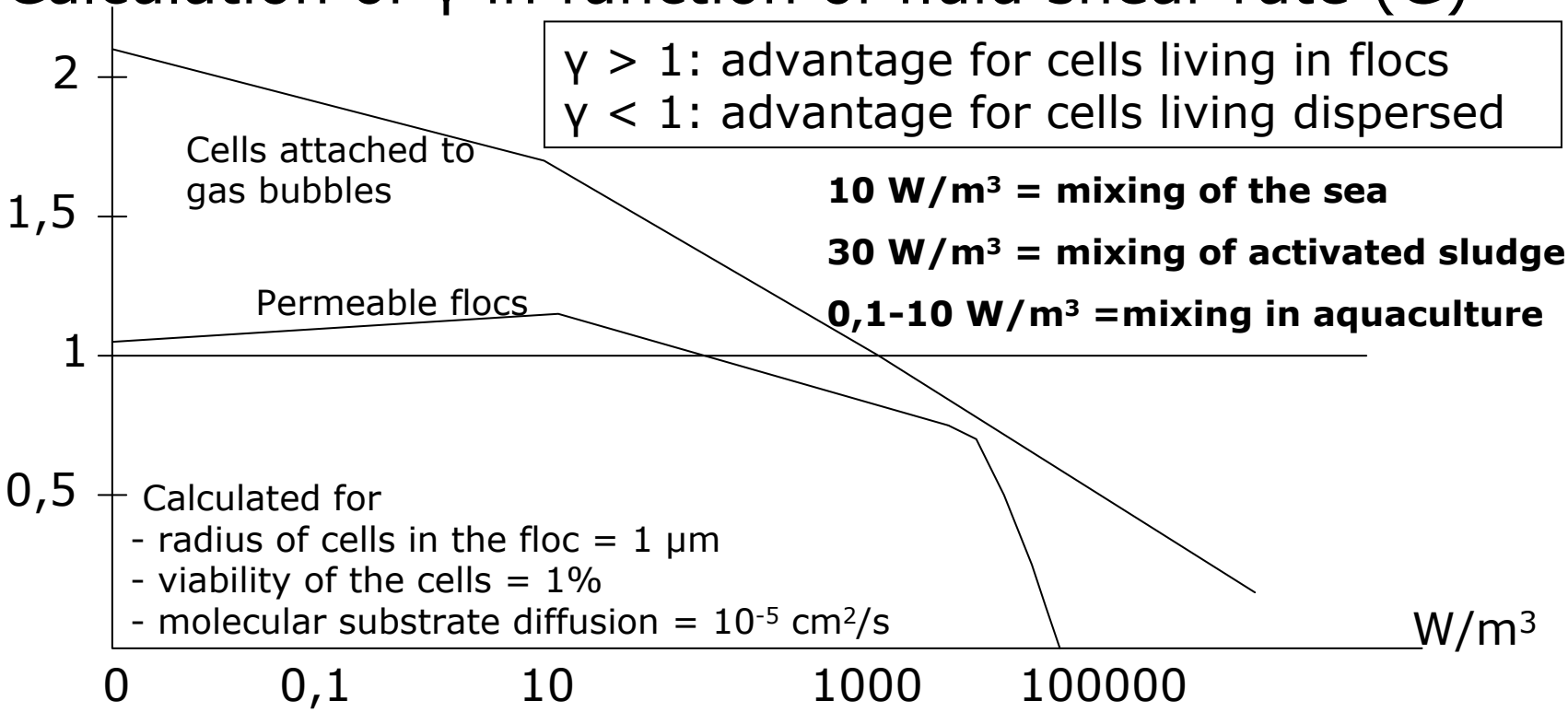
Relative uptake (γ) =

$$\frac{\text{Observed uptake rate by cells in flocs}}{\text{Uptake rate if cells are dispersed}}$$

→ Function of
- Fluid shear rate G (s^{-1}; W/m^3)
- Size of the microbial cells
- Viability of the cells

II. Motives of micro-organisms for living in floc structures

Calculation of γ in function of fluid shear rate (G)



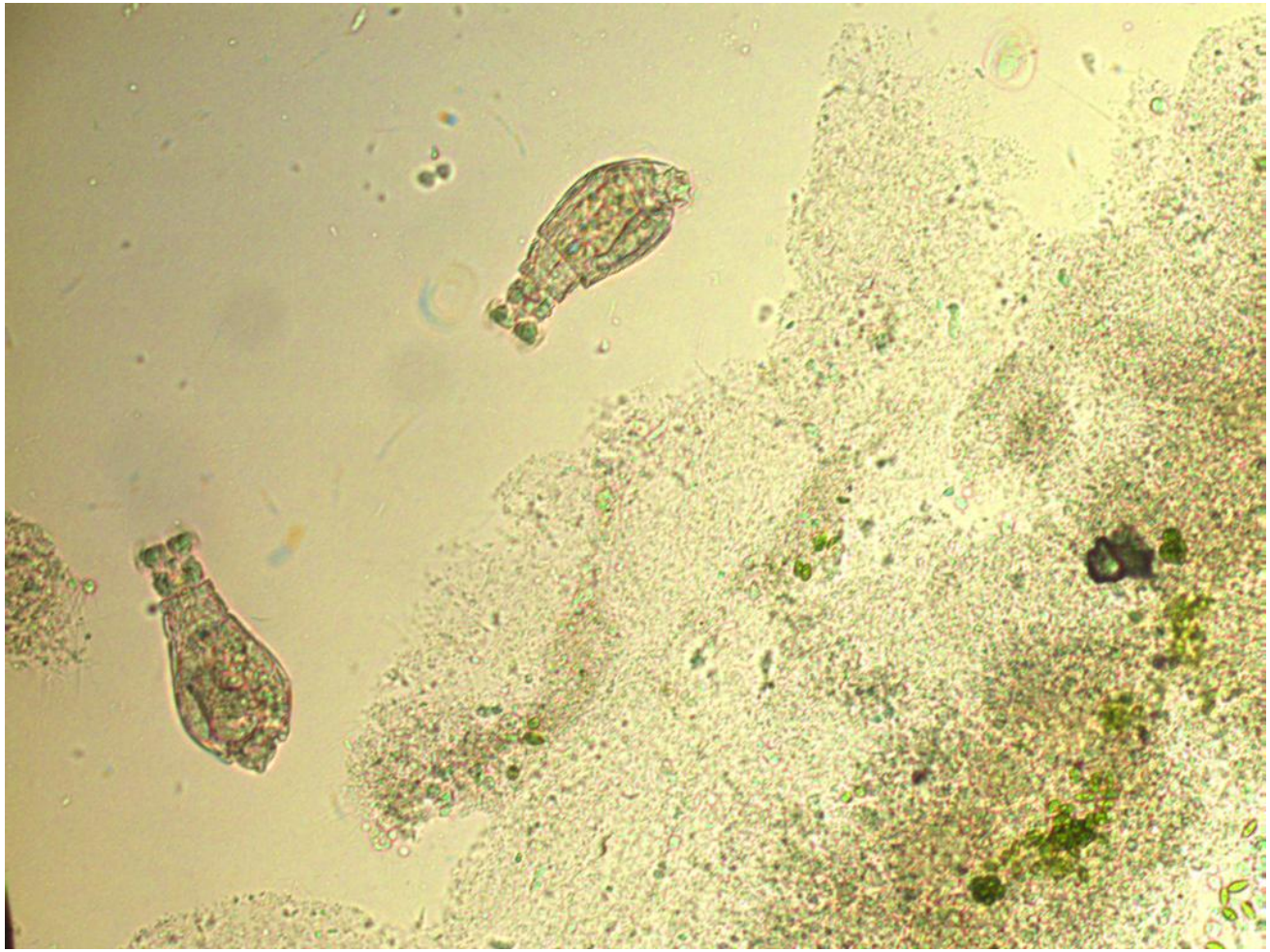
MESSAGE: Importance of choosing the appropriate aeration device and power input/m³ pond !!!

(Logan & Hunt, 1988. Biotechnology and Bioengineering 31, 91-101)

II. Motives of micro-organisms for living in floc structures

3) Lower predation by natural ennemies

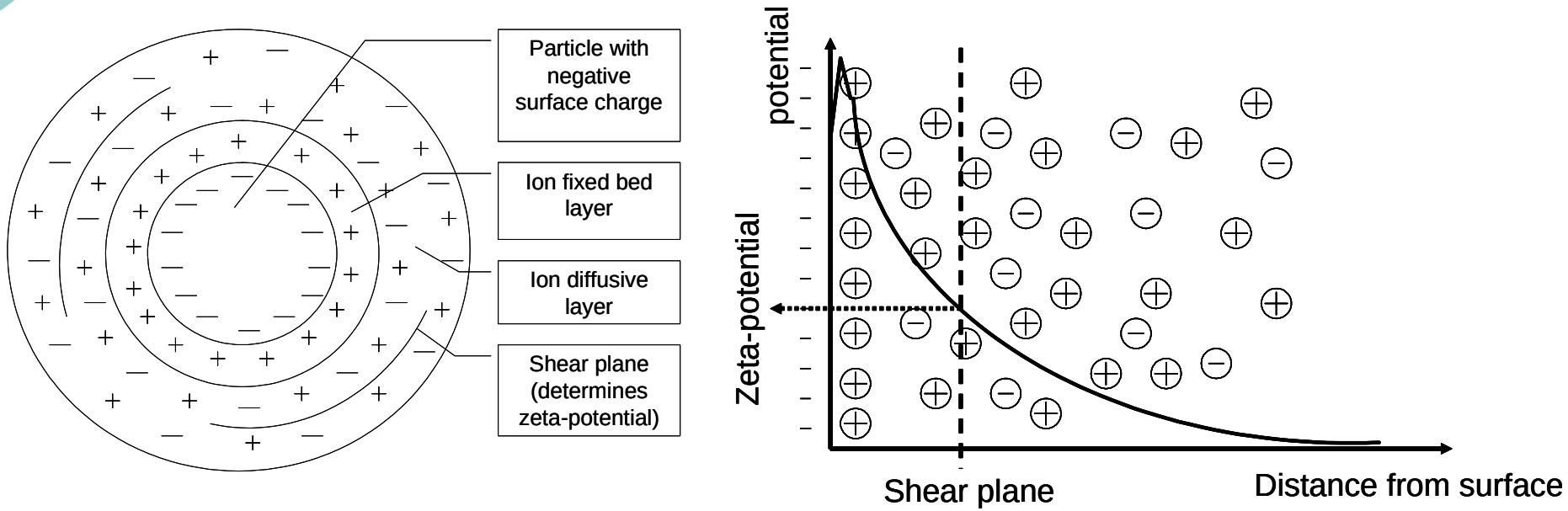
Rotifers and protozoa are predating the egde of a floc



Size exclusion: Only the edge of the floc can be grazed

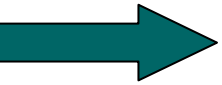
III. Mechanisms of binding cells into flocs

1) Interplay between repulsing and attracting powers: DLVO-theory

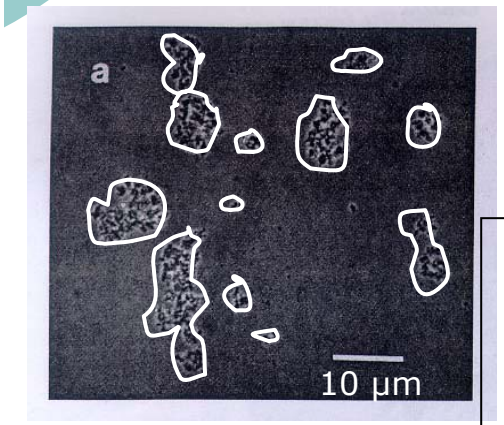


III. Mechanisms of binding cells into flocs

Electrostatic repulsion between equally charged particles (Coulombic powers)

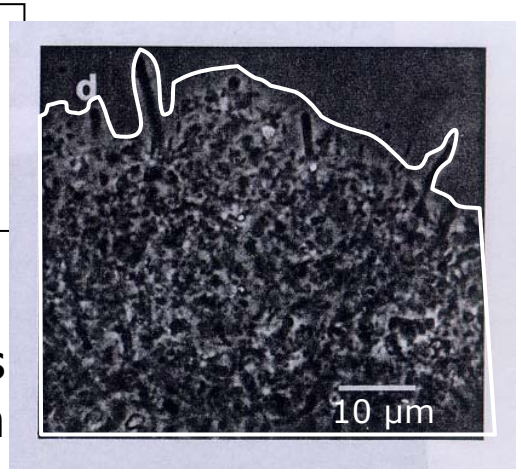


Van der Waals attraction originating from the induction of molecular polarization into dipoles



High ζ-potential: repelling forces > attractive forces:
→ dispersion

Jenkins et al. 1993, Manual on the Causes and Control of Activated Sludge Bulking and Foaming, p.191, CRC press LLC.



Low ζ-potential: repelling forces < attractive forces
→ aggregation

III. Mechanisms of binding cells into flocs

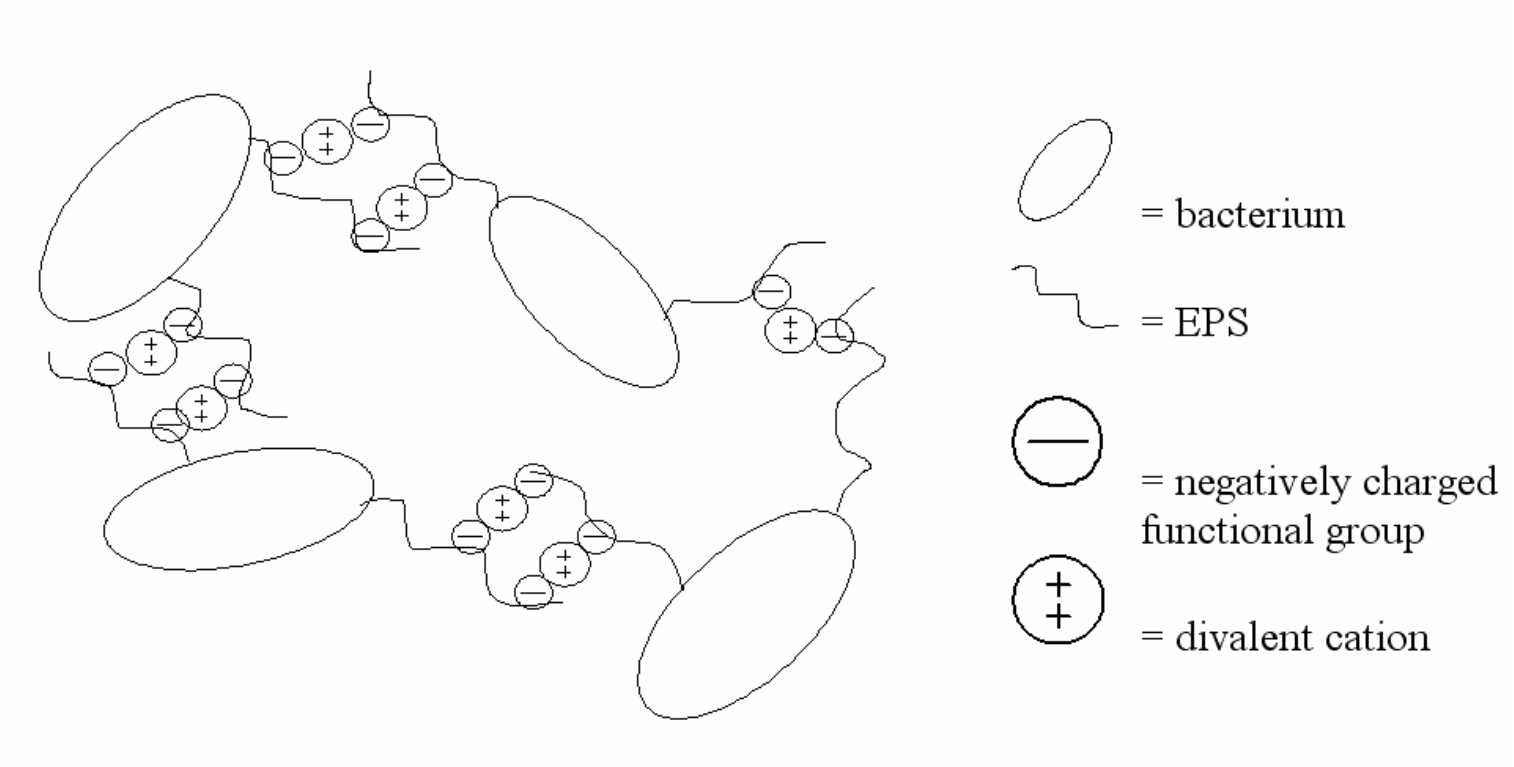
DLVO-theory:
influence of the so-called "surface protonation concept" ???

(Tay et al. 2000. Journal of Environmental Engineering 126, 403-418)

➔ Do cells actively pump out protons (and thus invest energy) to become less ionic and clump better?

III. Mechanisms of binding cells into flocs

2) Cations can help floc formation:



Divalent cation bridging (DCB) theory

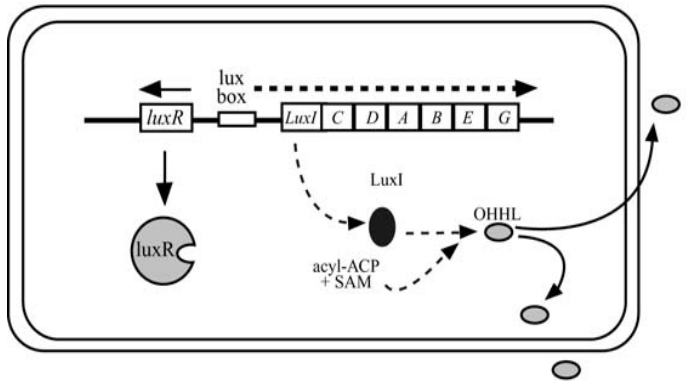
III. Mechanisms of binding cells into flocs

3) Quorum sensing:

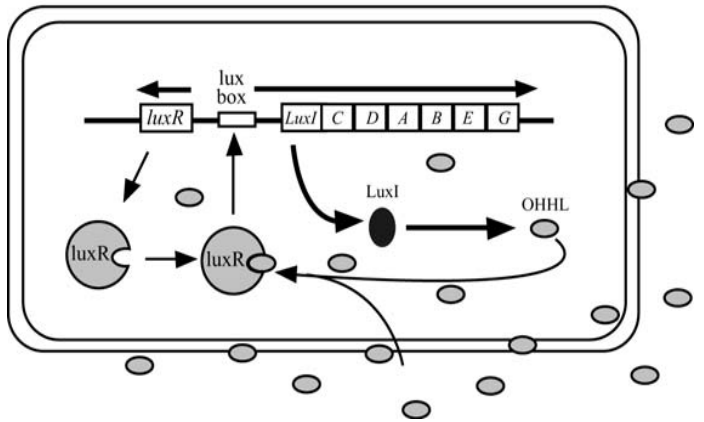
Signaling molecule-dependent induction of microbial activity

→ Production of auto-inducers

A. Low signaling molecule concentration



B. High signaling molecule concentration

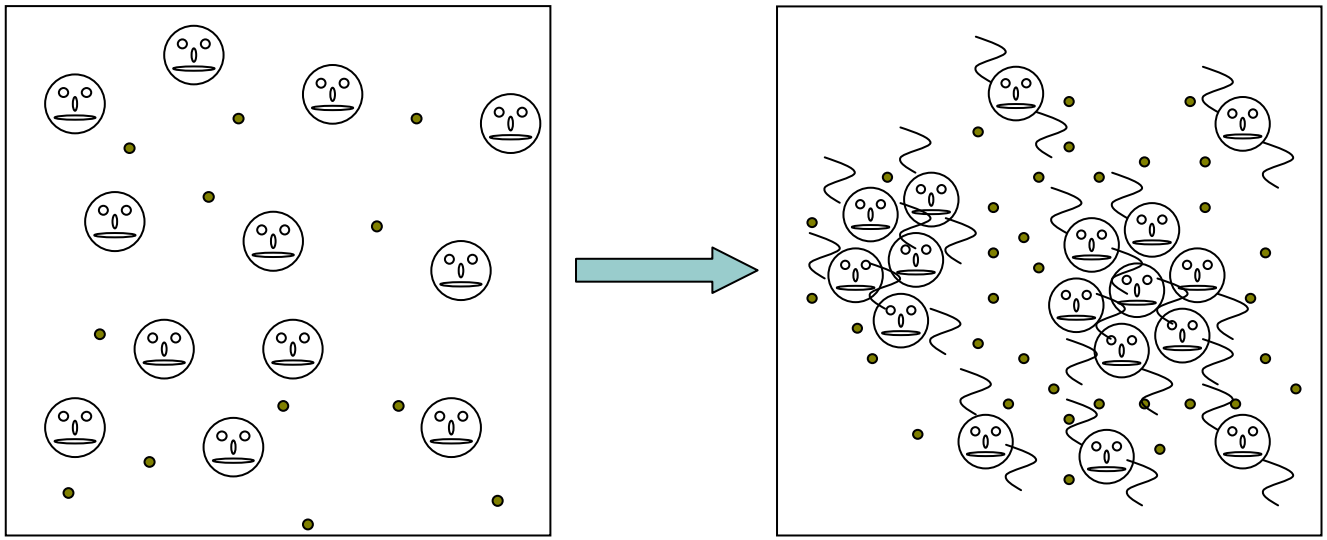


III. Mechanisms of binding cells into flocs

HYPOTHESIS

HYPOTHESIS

Communication affects floc structure: quorum sensing



Signaling molecule concentration low → no effect induced

Signaling molecule concentration high → induces dispersed MO for increased EPS-production

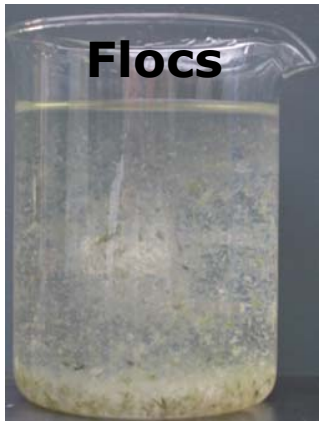
MESSAGE: Influence quorum sensing in biofilm formation is known, thus probably also active in free flocs!!!

(Morgan-Sagastume et al. 2005. Canadian Journal of Microbiology 51, 924-933)

IV. Methods to characterise flocs:

Process parameters influencing floc formation:

influence on floc formation is well known,
combined influence on aquaculture organisms as well
still needs to be established.



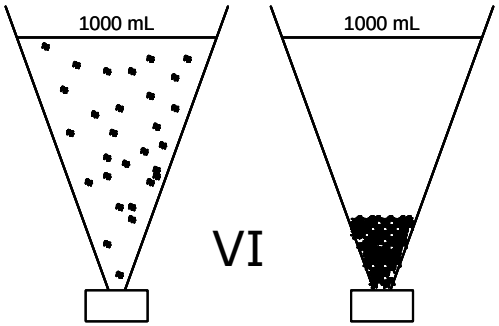
VERSUS

MESSAGE:	Research needed concerning optimal balance between floc formation and culture organism growth.

IV. Methods to characterise flocs:

Physical parameters

Suspended solids (SS)	**g SS.L^{-1}**
Volatile suspended solids (VSS)	**g VSS.L^{-1}**
Volume index (VI)	**mL. (g dry weight)$^{-1}$**
Porosity (ε)	**---**
Floc size distribution	**size (µm) & frequency (%)**



	Bio-flocs technology	Activated sludge systems
VI	???	40-60 mL. g DW^{-1}
VSS	???	≈3 g VSS.L^{-1}

MastersizerS

IV. Methods to characterise flocs:

Chemical parameters

Chemical oxygen demand (COD)	**g $O_2.L^{-1}$**
Biological oxygen demand (BOD)	**g $O_2.L^{-1}$**
Extracellular polymeric substances (EPS)	**mg.g VSS^{-1}**
Oxygen uptake rate (OUR)	**mg O_2.g VSS^{-1} $.h^{-1}$**
Protein-, PHB-, glycogen-, ash-content	**mg.g VSS^{-1}**

BOD-measurement: Analysis based on biological oxidation in Oxitop bottle

COD-measurement: Chemical analyses based on oxidation with $K_2Cr_2O_7$ in acid environment

IV. Methods to characterise flocs:

Biological analysis

Parameter	Units
Cell yield (Y): the amount of biomass produced per unit of substrate utilized	**g VSS-C.(g feed-C utilized)$^{-1}$**
Apparant cell yield (Y_{app}): the amount of biomass produced per unit of substrate added	**g VSS-C.(g feed-C added)$^{-1}$**
Specific substrate removal rate (q): rate of substrate removed per unit of biomass	**g feed-C.(g VSS-C)$^{-1}$. day^{-1}**
Volumetric substrate removal rate (q_v): rate of substrate removed per unit of pond volume	**g feed-C.(m^3 pond)$^{-1}$.day^{-1}**

In general: 1 g VSS ≈ 0,5 g C ≈ 1,33 g COD

IV. Methods to characterise flocs:

Parameter	Units
Removal efficiency (E): amount removed per unit of a compound added by feed: carbon	**%**
nitrogen	**%**
phosphorous	**%**

<u>MESSAGE</u>: THE USE OF UNIFORM UNITS CONCERNING BIO-FLOCS TECHNOLOGY IS NEEDED!!!

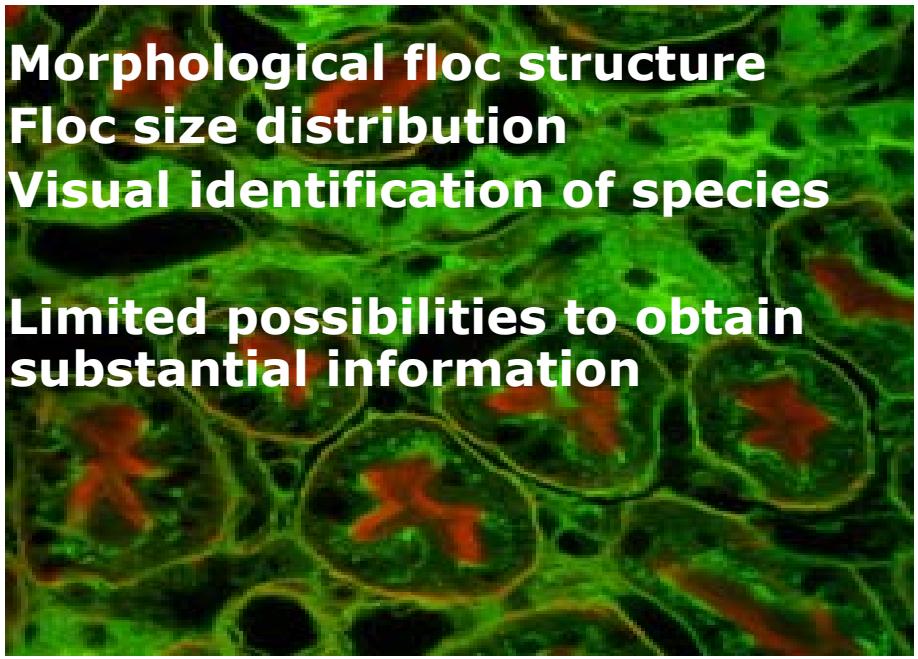
IV. Methods to characterise flocs:

Biological analysis

- **Microscopy**

→ **Morphological floc structure**
→ **Floc size distribution**
→ **Visual identification of species**

→ **Limited possibilities to obtain substantial information**

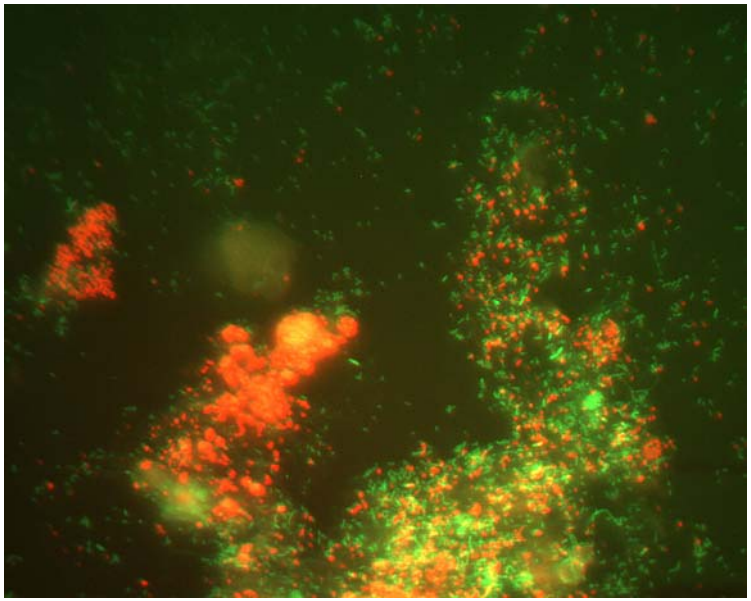


- **Molecular analysis**

- FISH: Fluorescent In Situ Hybridisation
- Real-time PCR: Quantitative Polymerase Chain Reaction
- DGGE: Denaturing Gradient Gel Electrophoresis
- Biosensor strains

IV. Methods to characterise flocs: - LabMET labtests

- **FISH:** Flourescent in situ hybridisation, the fluorescent visualisation of a certain group of micro-organisms in a sample



FISH pictures of Anammox:

Red/yellow: Anammox

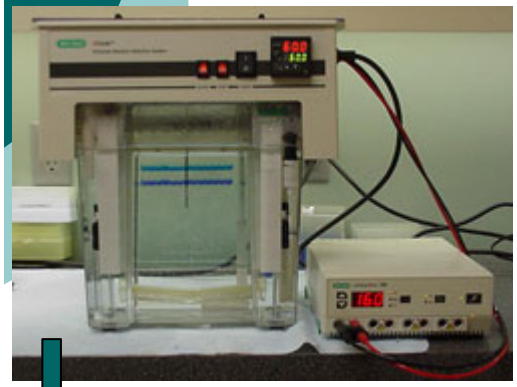
Green: all bacteria

(Vlaeminck et al. 2007. Applied Microbiology and Biotechnology. In press.)

- **Real-time PCR:** determination of the amount of phylogenetic/functional DNA of a group of micro-organisms in a sample

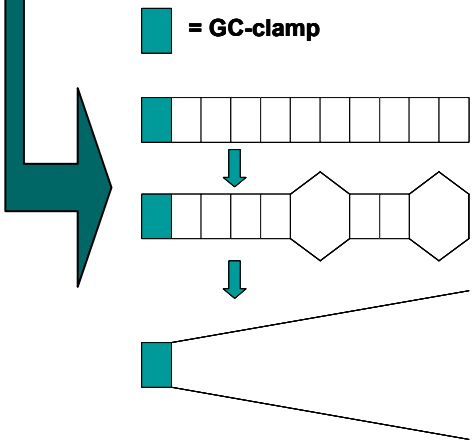
IV. Methods to characterise flocs: – LabMET labtests

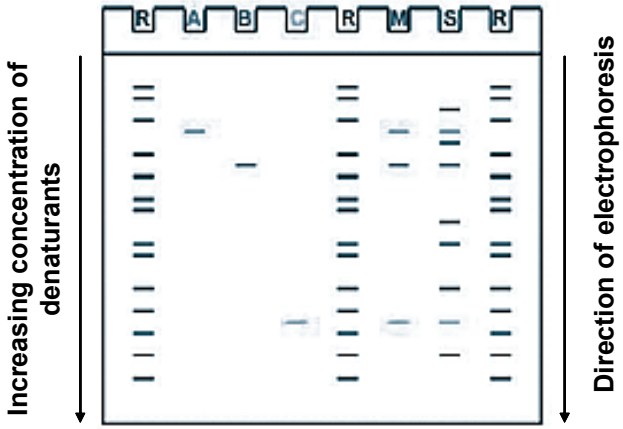
DGGE: Denaturing Gradient Gel Electrophoresis



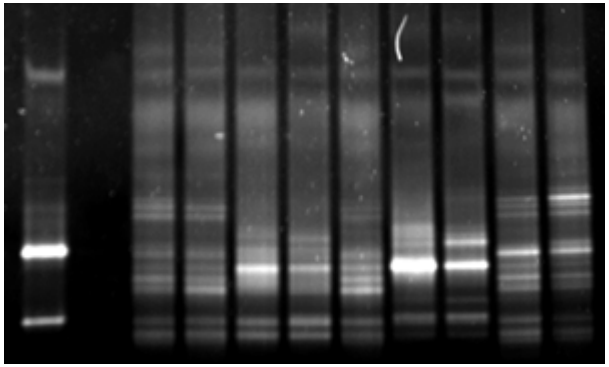
In aquaculture hatcheries: ± 600 culturable bacterial species!!!!!!!!!! (Schulze et al. 2006, Aquaculture 256, 50-73)
(only about 1% of all bacteria can be cultured)

→ Hard work to make and interpret DGGE-patterns





R = reference pattern, A = Organism 1, B = organism 2, C = organism 3, M = Mix of organisms 1, 2 and 3, S = unknowm sample



IV. Methods to characterise flocs: – LabMET labtests

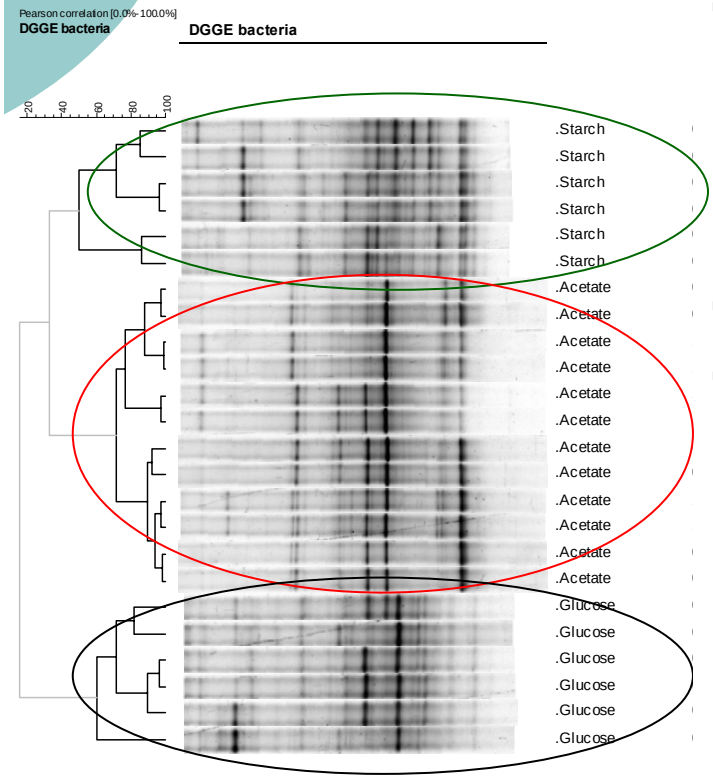
- **DGGE → general application: qualitative changes in community in function of time**

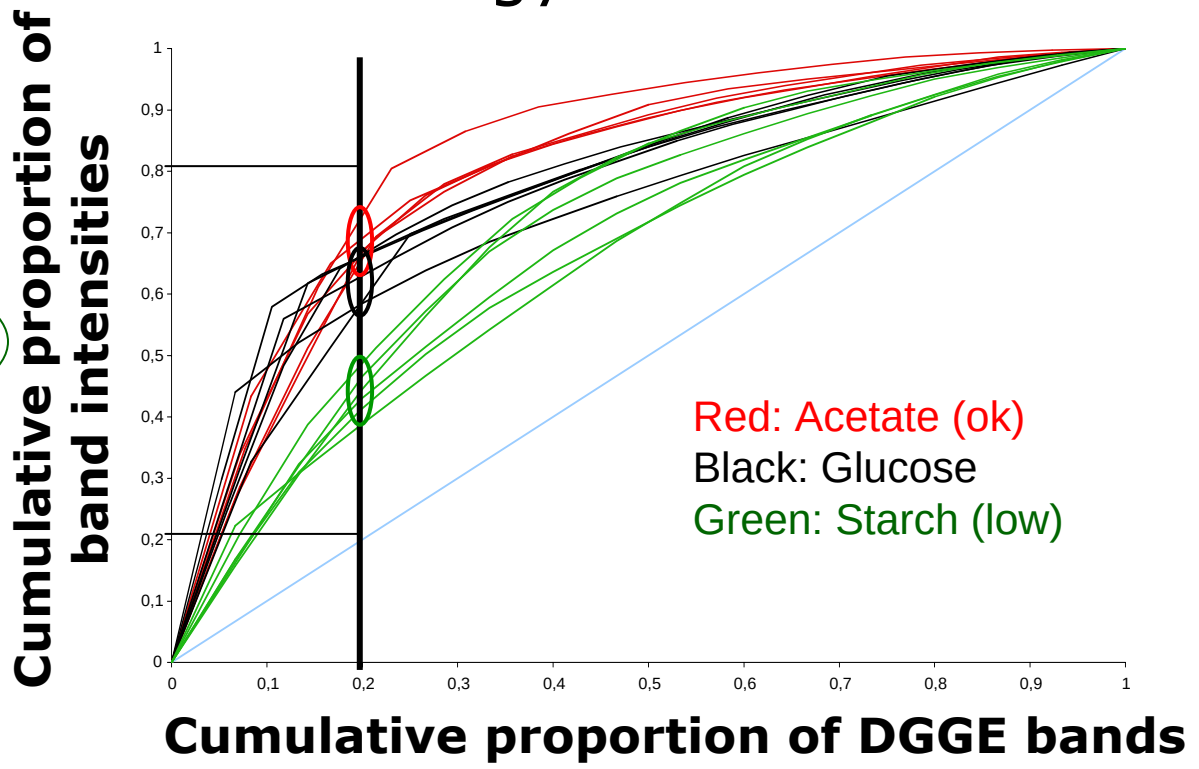
NOW: NEW MICROBIAL APPROACHES

- **Pareto-principle**
- **Moving window analysis**
- **Q-array**

IV. Methods to characterise flocs: – LabMET labtests **(Crab et al. 2006, unpublished work)**

- Pareto-principle: 20% of the species → 80% of energy-flux



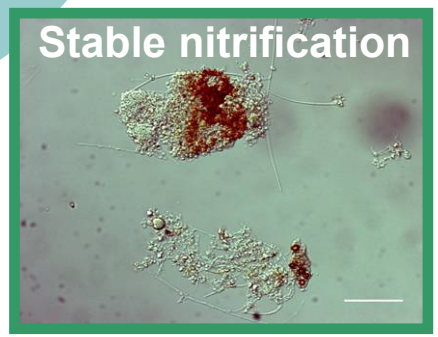


	Acetate	Glucose	Starch
Pareto	75%	60%	40%

IV. Methods to characterise flocs:
– LabMET labtests (Wittebolle et al. 2006, unpublished work)

- Moving Window Analysis:

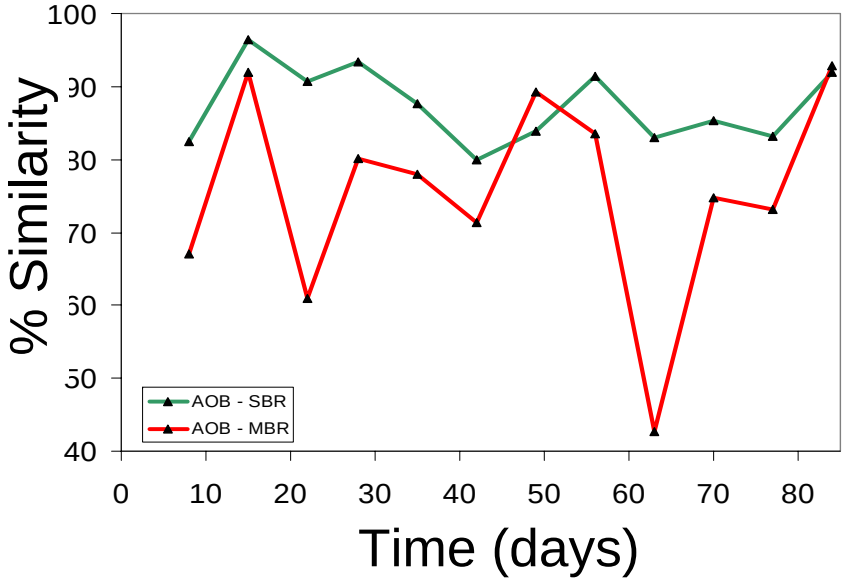
Quantitative comparison of the change in band patterns between succesive periods → indication for the stability of a microbial ecosystem

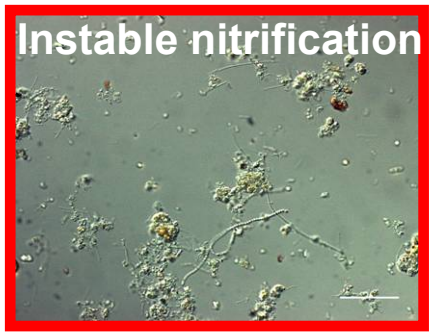


$\Delta_{t(week)}$

12.6 ± 5.2

24.6 ± 14.3





$\Delta_{t(week)}$

To quantify the CCC

Moving Window Analysis

To visualise the Cooperative Community Continuum

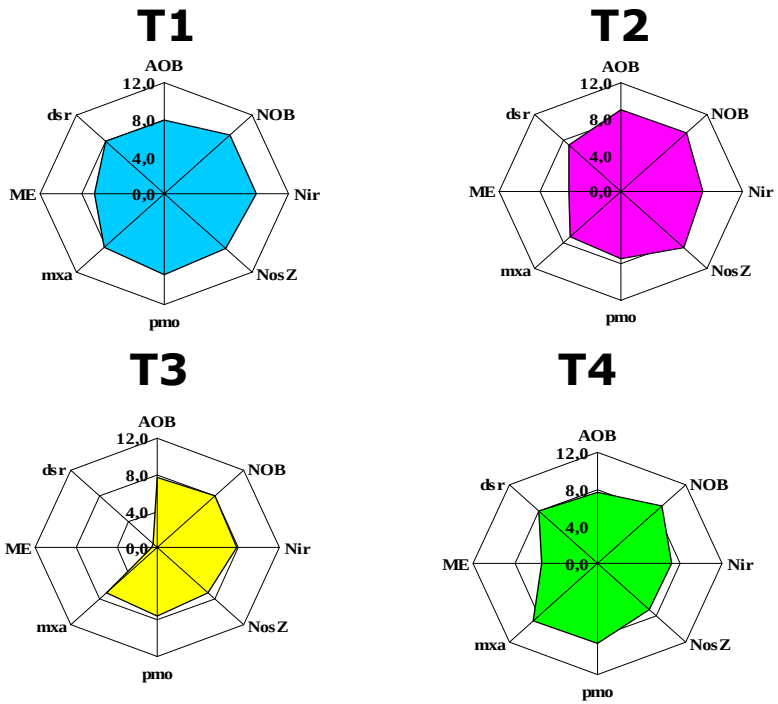
IV. Methods to characterise flocs: – LabMET labtests (Geets et al. 2006, Applied Microbiology and Biotechnology, in press)

Q-array:

Assay that allows the simultaneous quantification of phylogenetic and functional genes → provides rapid and detailed insight in community structure

E.g.

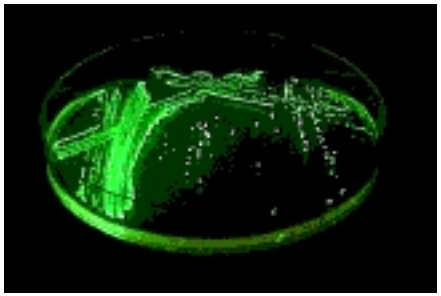
follow up on nitrification and denitrification processes in wastewater treatment plant



In number of DNA-copies (log_{10}/mL)

IV. Methods to characterise flocs: - LabMET labtests

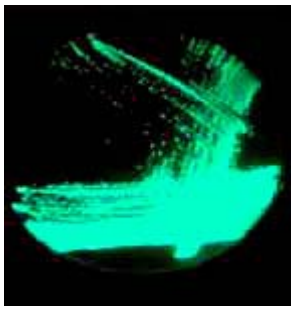
- **Detection of quorum sensing:** biosensor strains



Escherichia coli JB523: green fluorescent protein induced by AHL signal molecules

Chromobacterium violaceum CV026: purple pigment induced by AHLs, TLC with biosensor overlay for identification

(Morgan-Sagastume et al. 2005. Canadian Journal of Microbiology 51, 924-933)



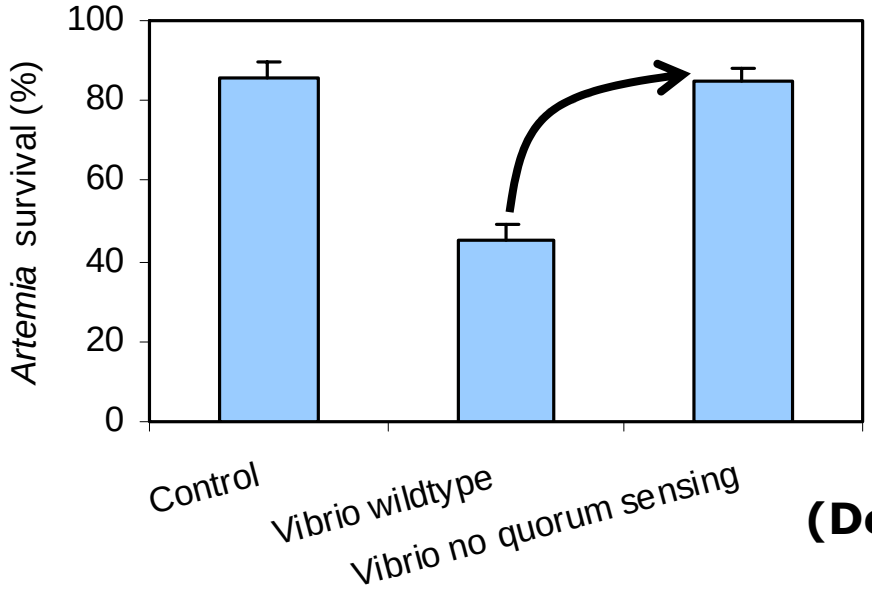
Vibrio harveyi JMH597: luminescence induced by the autoinducer-2 signal

(Defoirdt et al. 2006. Applied and Environmental Microbioly 72, 6419-6423)

IV. Methods to characterise flocs: - LabMET labtests

- Disruption of quorum sensing by bacteria & algae in flocs ➔ biocontrol effect
  - Production of antagonistic molecules
  - Inactivation of signal molecules (degradation)

(Defoirdt et al. 2004. Aquaculture 240, 69-88)



Example: disruption of quorum sensing in luminescent vibrios ➔ increased survival of *Artemia*

(Defoirdt et al. 2005. Environmental Microbiology 7, 1239-1247)

V. Special nutritious compositions of flocs for aquaculture – LabMET labtests

- Nutritional composition of bio-flocs:
  0,3-0,4 g VSS/L – HRT = 1 day

	Bio-flocs grown on carbon source			Fish feed
	Glucose	**Starch**	**Acetate**	
Protein (% of dry weight)	32	21	19	20-50
Lipid (% of dry weight)	39	17	21	10-25
Ash (% of dry weight)	2	3	7	< 8,5
Carbohydrate (% of dry weight)	27	59	53	15-60

V. Special nutritious compositions of flocs for aquaculture – LabMET labtests

- Nutritional composition of bio-flocs:
  - 0,3-0,4 g VSS/L – HRT = 1 day

	Bio-flocs grown on carbon source			**Fish feed**
Fatty acids content (mg.g DW^{-1})	**Glucose**	**Starch**	**Acetate**	
18:2(n-6)	0.5 ± 0.3	0.7 ± 0.2	0.4 ± 0.2	13.2
18:3(n-3)	0.05 ± 0.006	0.04 ± 0.03	0.06 ± 0.03	0.09
20:5(n-3)	0.5 ± 0.1	0.15 ± 0.02	0.08 ± 0.03	0.8
22:6(n-3)	0.04 ± 0.01	/	/	1.17
sum n-6	1.0 ± 0.3	1.0 ± 0.1	0.6 ± 0.1	13.4
sum n-3	0.8 ± 0.03	0.3 ± 0.07	0.19 ± 0.08	2.38

V. Special nutritious compositions of flocs for aquaculture – LabMET labtests

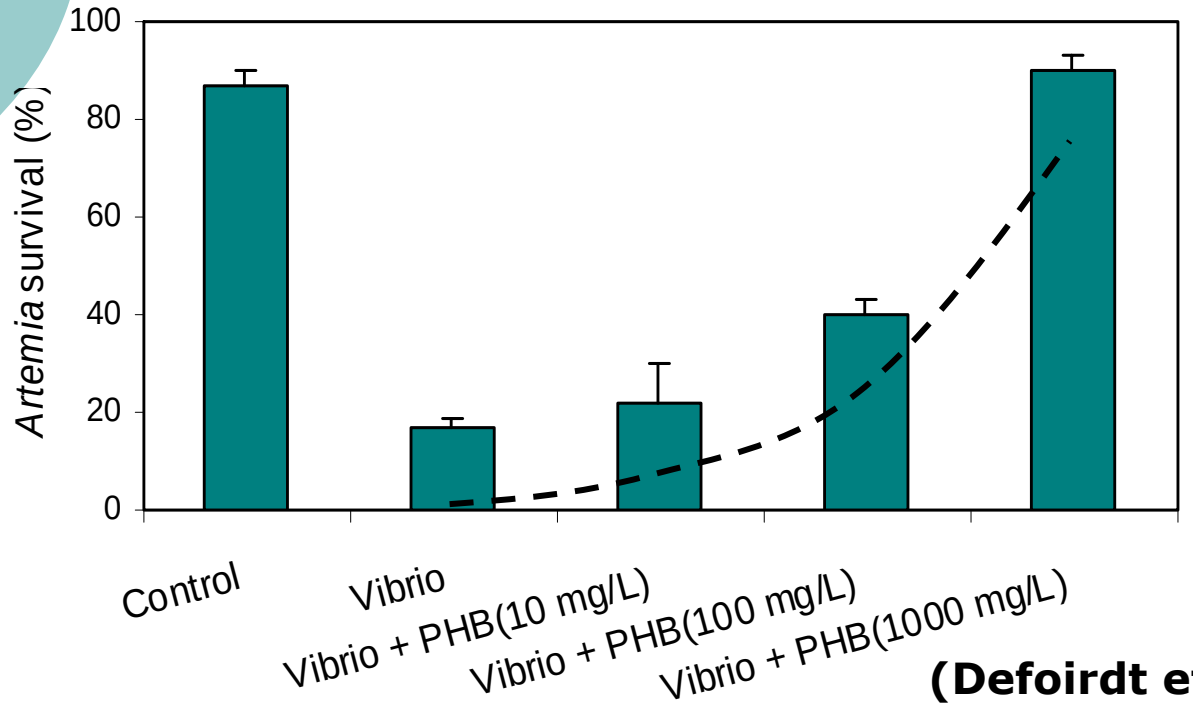
- Nutritional composition of bio-flocs:

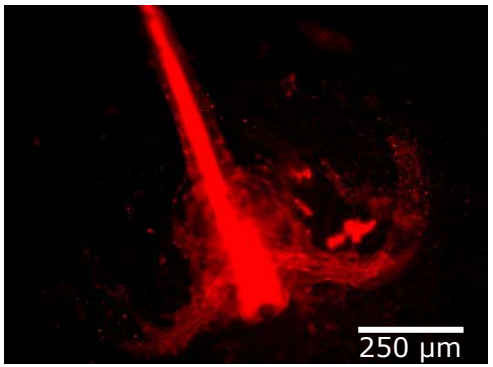
- PHB-content

	Acetate as carbon source		
C/N-ratio	10	20	30
Feeding frequency (times/24h)	**1x**	**1x**	**1x**
PHB -content (% of dry weight)	Up to 18%	Up to 64%	Up to 48%

V. Special nutritious compositions of flocs for aquaculture – LabMET labtests

- *In vivo* tests with gnotobiotic brine shrimp *Artemia franciscana*: protection against luminescent vibriosis





Fluorescence microscopy image of stained *Artemia* nauplius fed PHB

(Defoirdt et al. 2007. Environmental Microbiology 9, 445-452)

MESSAGE: PHB STRONGLY PROTECTS ARTEMIA

VI. Overall conclusions

The road to go for Bio-Flocs Technology based aquaculture

1. Microbial Resource Management (MRM) is important for adequate BFT:
   - *The Beijerinck axioma*
   - *The Hubbell "neutral theory"*
   - *The Pareto-law*
   - *...*

2. Nutritional aspect of bio-flocs warrants further R&D:
   - Influence of carbon source
   - Quorum sensing
   - PHB content
   - ...

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Our affiliation:

Laboratory for Microbial Ecology and Technology (LabMET)

Faculty of Bioscience Engineering

Ghent University – Belgium

http://labmet.ugent.be