

L'extrapolation des bioréacteurs

Un problème de génie des procédés ou de physiologie microbienne?

Frank Delvigne

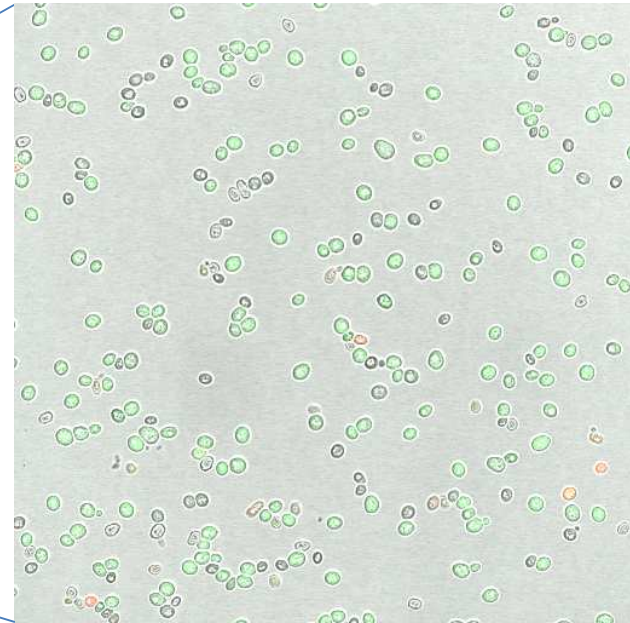
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Ecole Thématique CNRS | INGÉNIERIE DES
BIOSYSTÈMES : DE LA CELLULE AU BIOREACTEUR

1. Introduction



Diversité des bioréacteurs

Epuration des eaux



Biogaz



- Matériaux de construction (acier inox 316L, matières plastiques, béton)
- Agitation mécanique, recirculation, pneumatique
- Mode de fonctionnement (batch, continu, *fed-batch*)

Bioéthanol



Production d'enzymes



Cas particulier des photo-bioréacteurs



Relation « prix de revient » - « volume réactionnel »

- Epuration des eaux
- Biogaz
- Biocarburant
- Acides organiques
- Alcools
- Acides aminés
- Enzymes
- Polymères
- Arômes
- Antibiotiques
- Protéines recombinantes

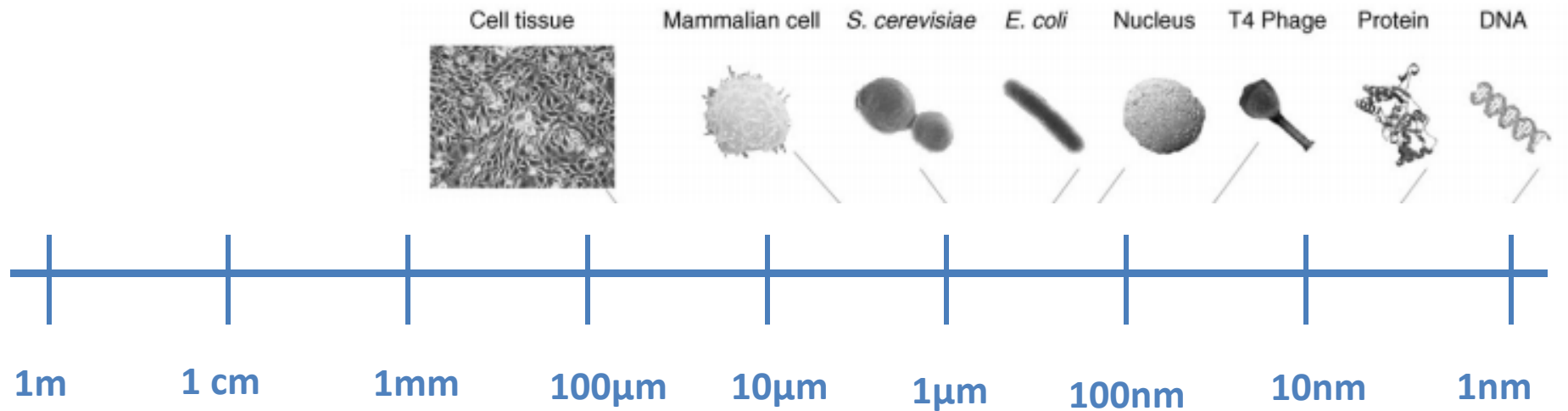


Prix de
revient

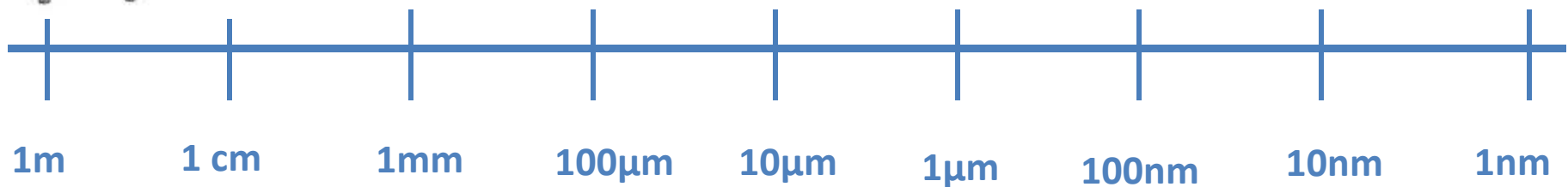


Volume du
réacteur




Maîtriser la biologie...

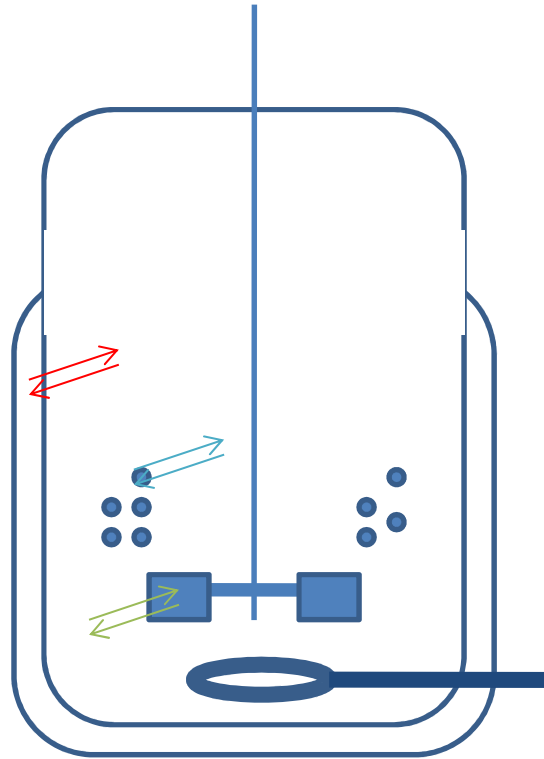


... dans des systèmes de culture industriels



Intensification des bioprocédés

- Transfert de masse 
- Transfert de chaleur 
- Transfert de quantité de mouvement 

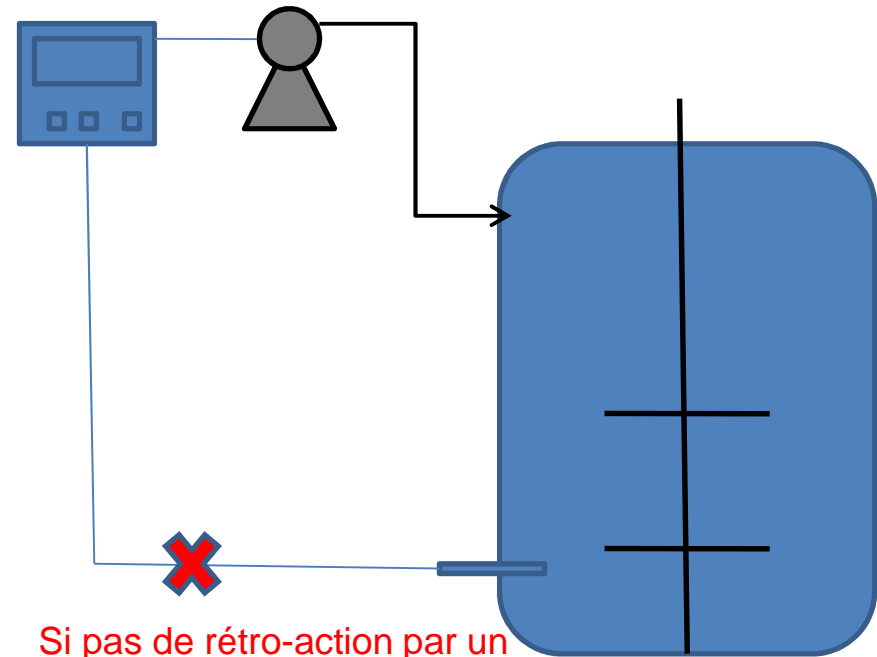


Intensification des bioprocédés : paramètres à contrôler

- Agitation
- Température
- pH

- Oxygène dissous
- Concentration en substrat
- Potentiel rédox
- Apport de lumière

Besoin d'un système senseur-actuateur



Le développement des bioréacteurs va de pair avec le développement de capteurs adéquats

Physiologie microbienne ou génie des procédés ?

Deux exemples préliminaires :

1. Formation de mousse lors d'un procédé de production de lipases par *Yarrowia lipolytica*
2. Choc thermique lors de la culture industrielle de *Bifidobacterium bifidum*

Exemple 1

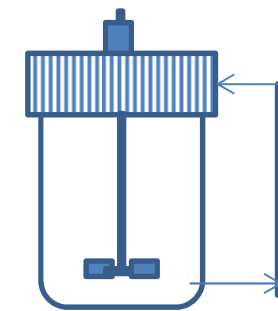
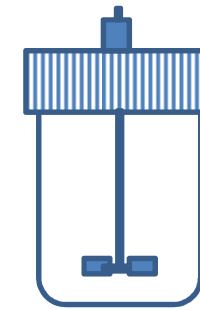
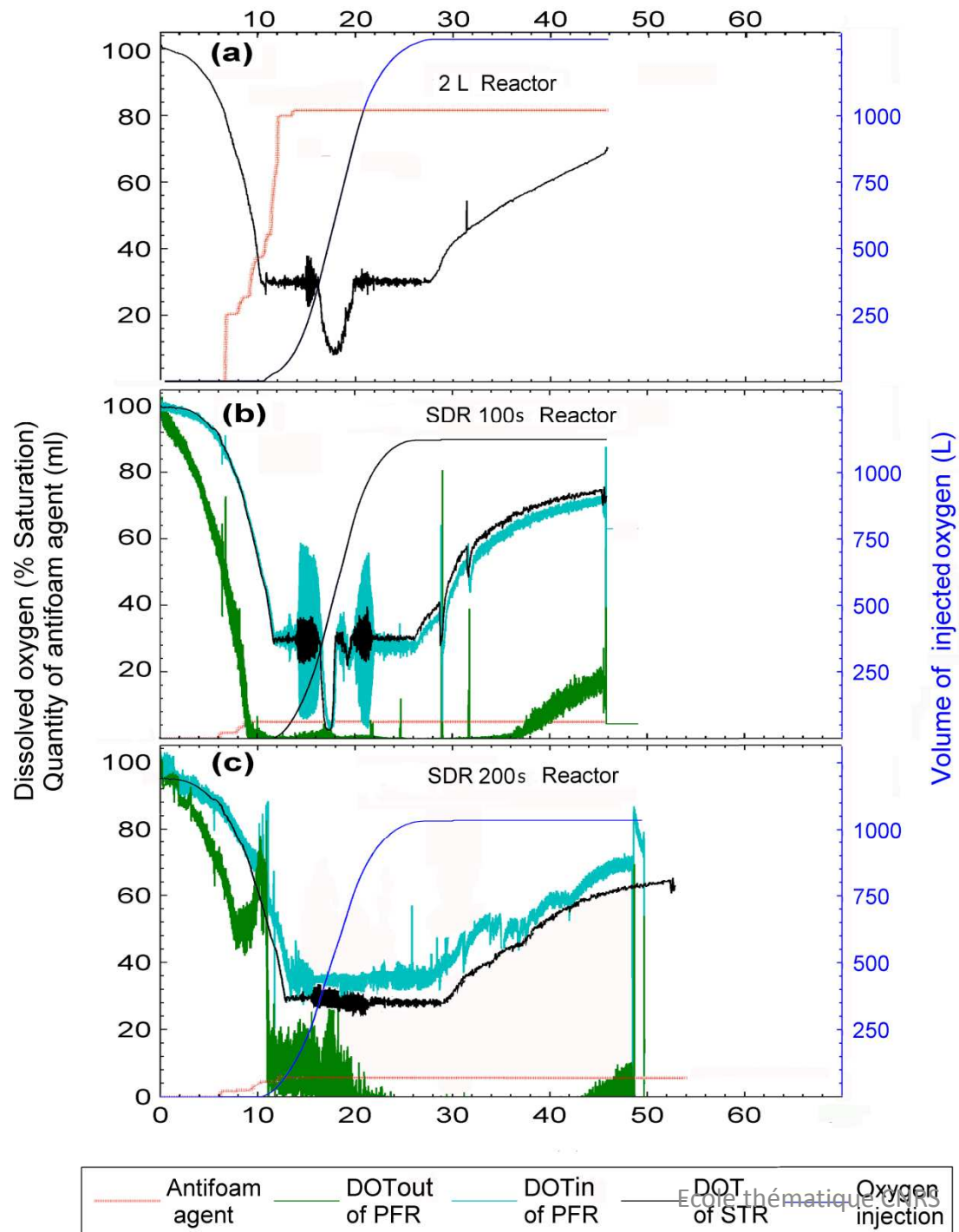
Formation de mousse lors d'un procédé de production de lipases par *Yarrowia lipolytica*

→ Intensité de formation de mousse = $f(G/S)$

→ Extrapolation des procédés aérobies sur base de G/V

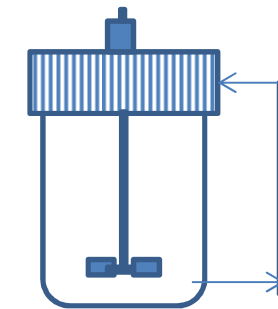
Les règles d'extrapolation classique prédisent donc une intensification de la formation de mousse lors de la montée en échelle

Kar, T., Destain, J., Thonart, P., Delvigne, F., (2012) Physical and physiological impacts of different foam control strategies during a process involving hydrophobic substrate for the lipase production by *Yarrowia lipolytica*. *Bioprocess and biosystems engineering* 35, 483-492



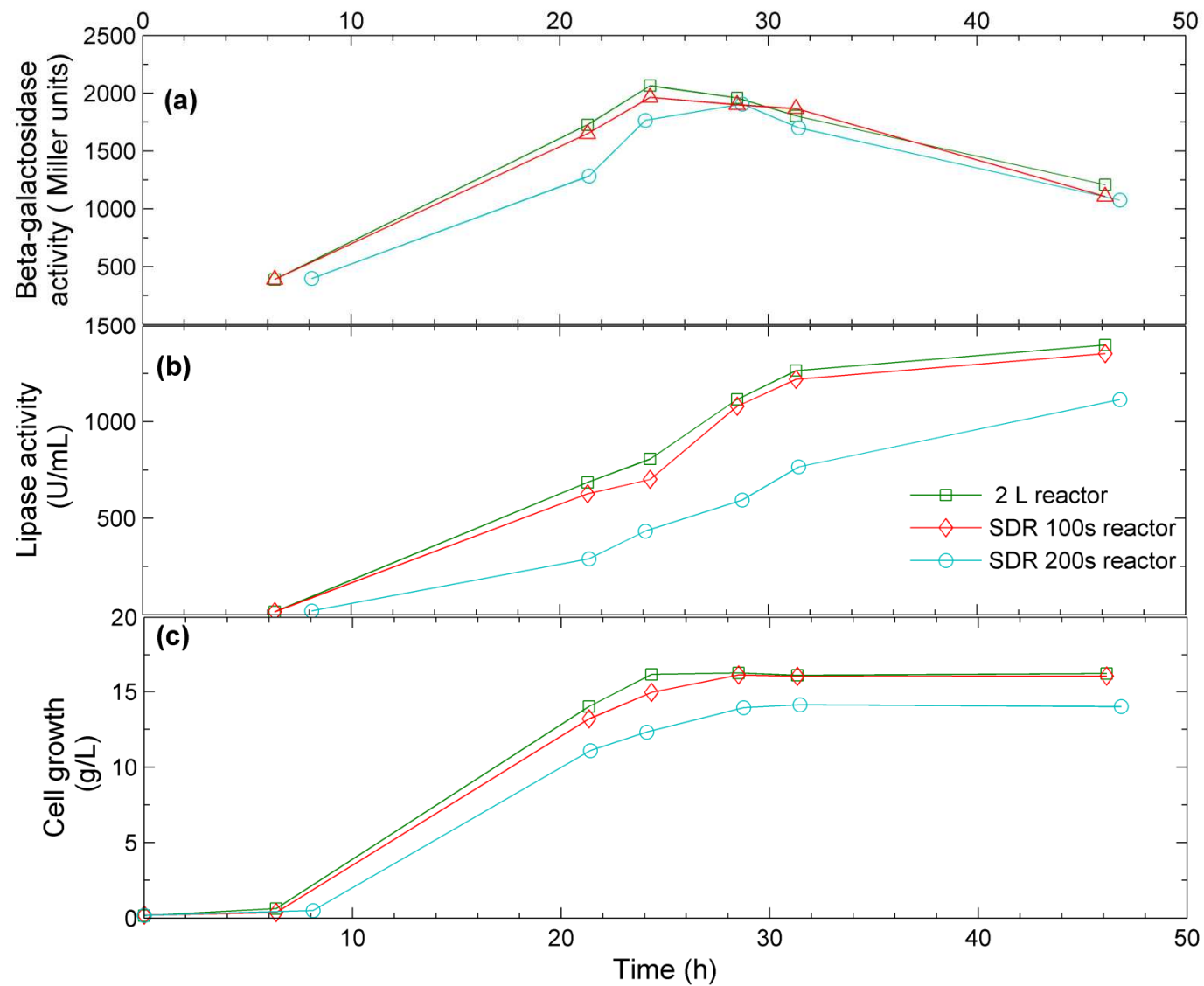
← C_L OUT

← C_L IN



← C_L OUT

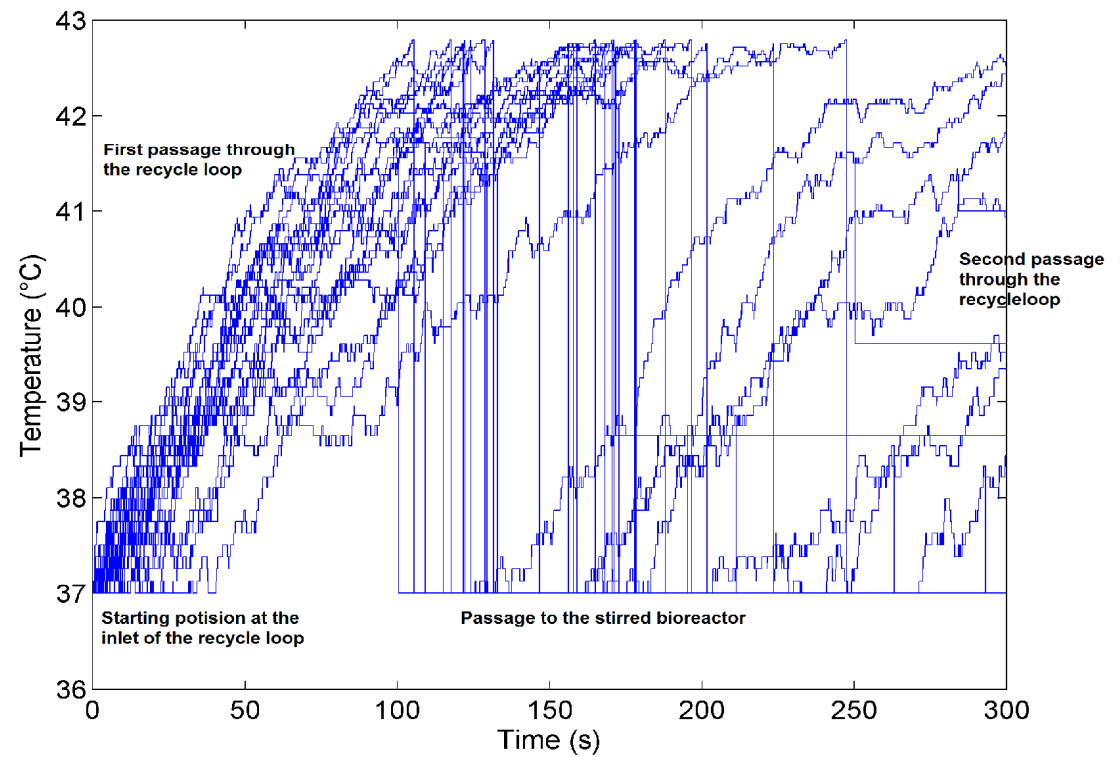
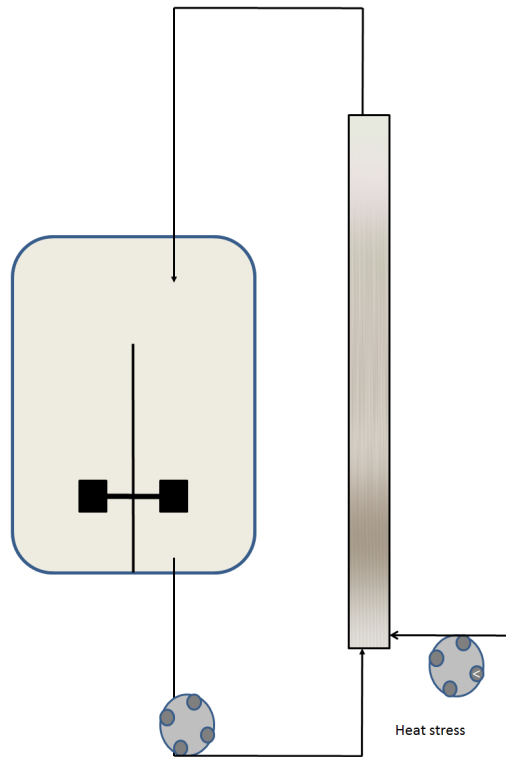
← C_L IN



Exemple 2

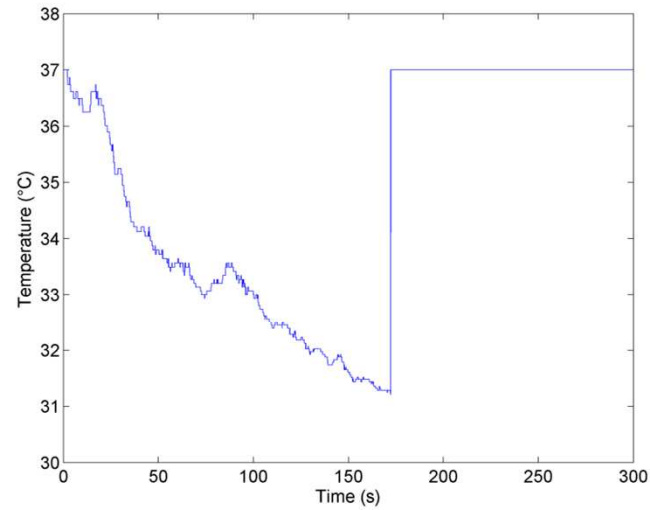
Choc thermique lors de la culture industrielle de *Bifidobacterium bifidum*

Nguyen, H.T., Razafindralambo, H., Blecker, C., N'Yapo, C., Thonart, P., Delvigne, F., (2014) Stochastic exposure to sub-lethal high temperature enhances exopolysaccharides (EPS) excretion and improves *Bifidobacterium bifidum* cell survival to freeze-drying. *Biochemical engineering journal* in press



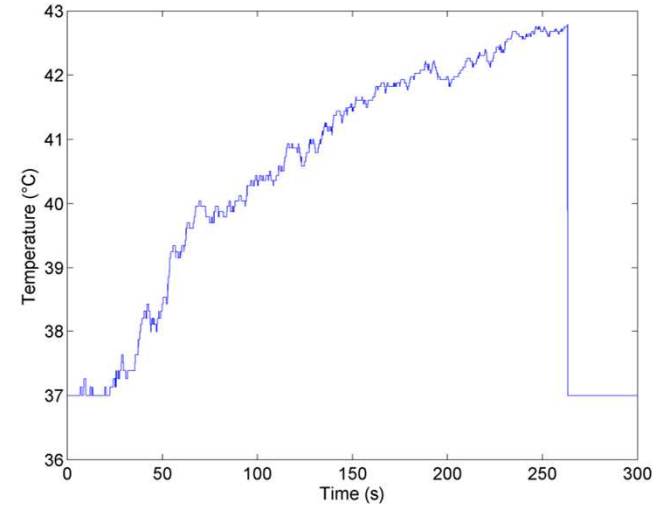
Choc « froid » 37°C → 32°C

A

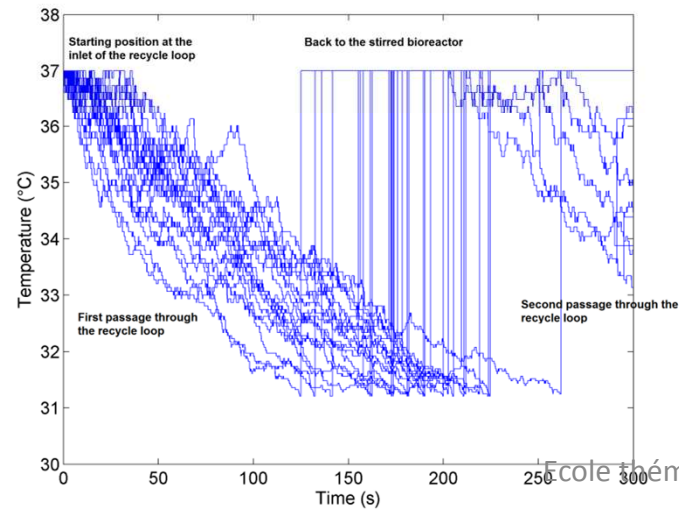


Choc « chaud » 37°C → 42°C

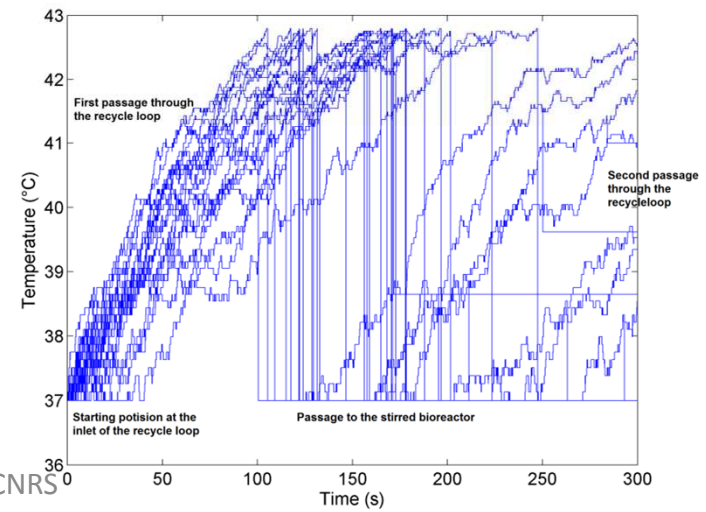
B



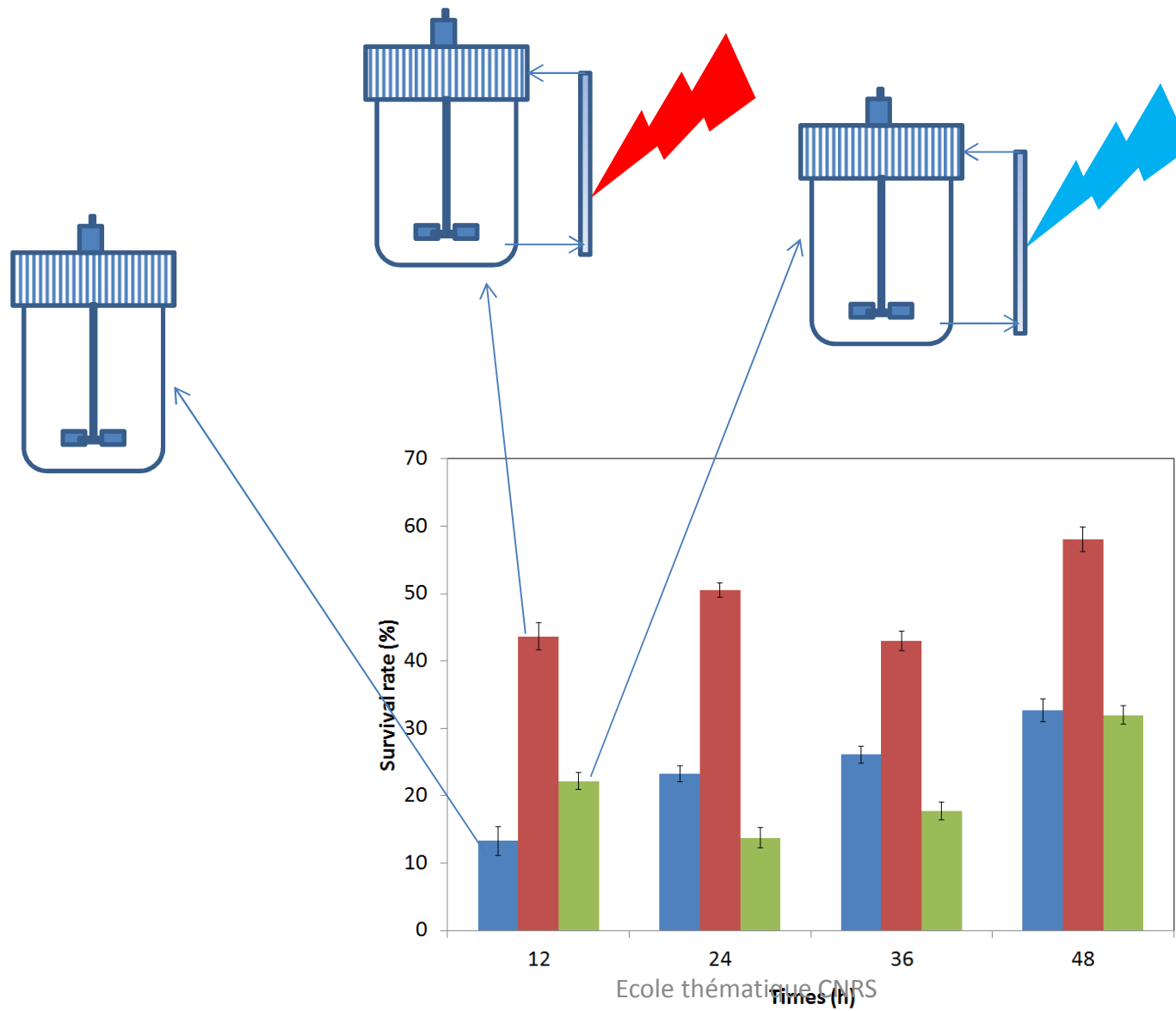
C

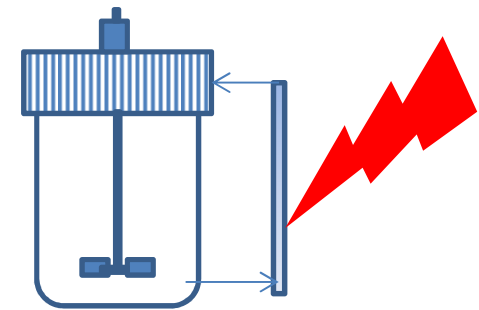
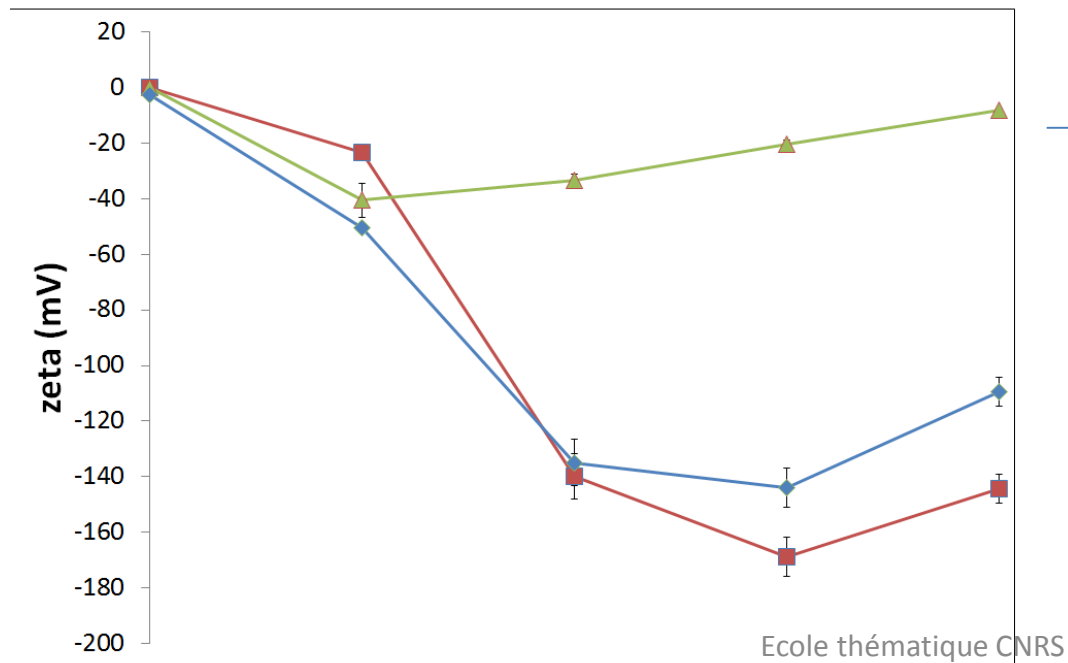
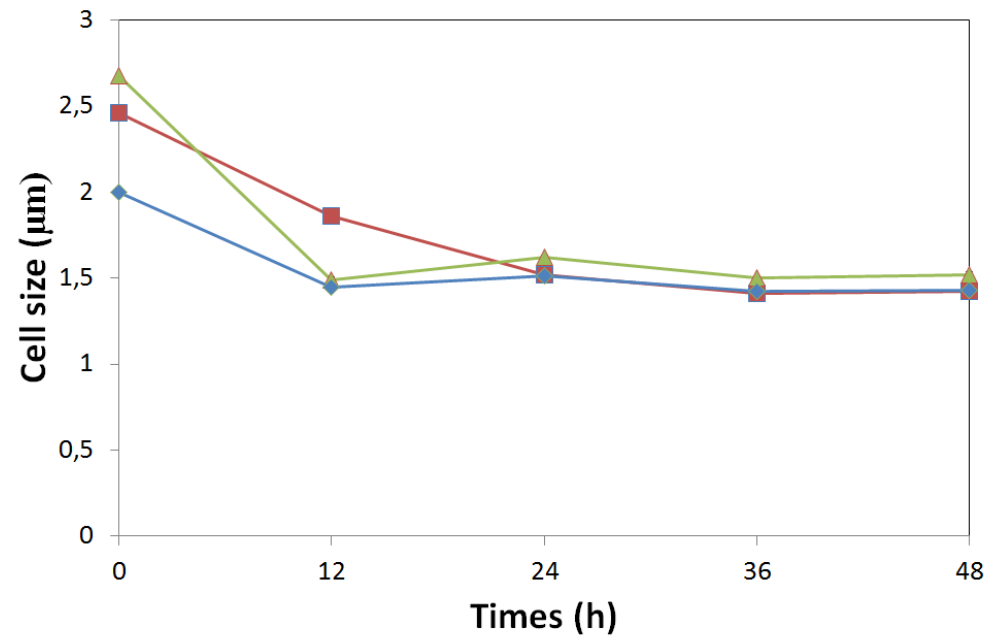


D



Taux de survie après lyophilisation





Physiologie microbienne ou génie des procédés ?

Réponse : ces deux aspects doivent être considérés de manière simultanée

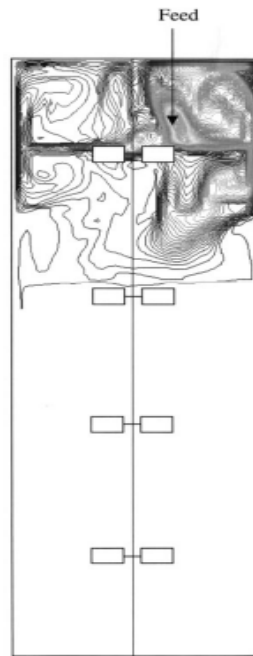
- Le génie des procédés donne une information sur l'environnement extracellulaire perçu par la cellule microbienne
- La physiologie microbienne donne la réactivité de cette cellule microbienne par rapport à sa biologie intrinsèque, mais également par rapport aux stimuli extracellulaire (stress) perçu au cours du procédé

Ces deux aspects sont indissociables pour une meilleure compréhension de la physiologie en conditions de procédé

2. Aspects du génie des procédés

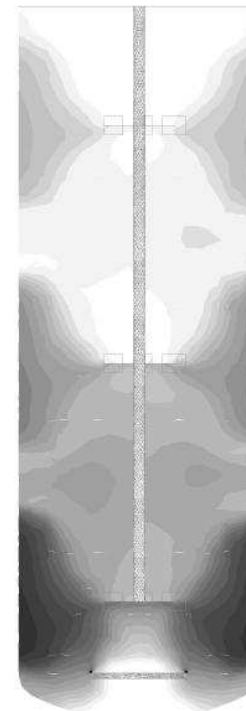
Problèmes associés au *scale-up*

Gradient de substrat dans les procédés *fed-batch*

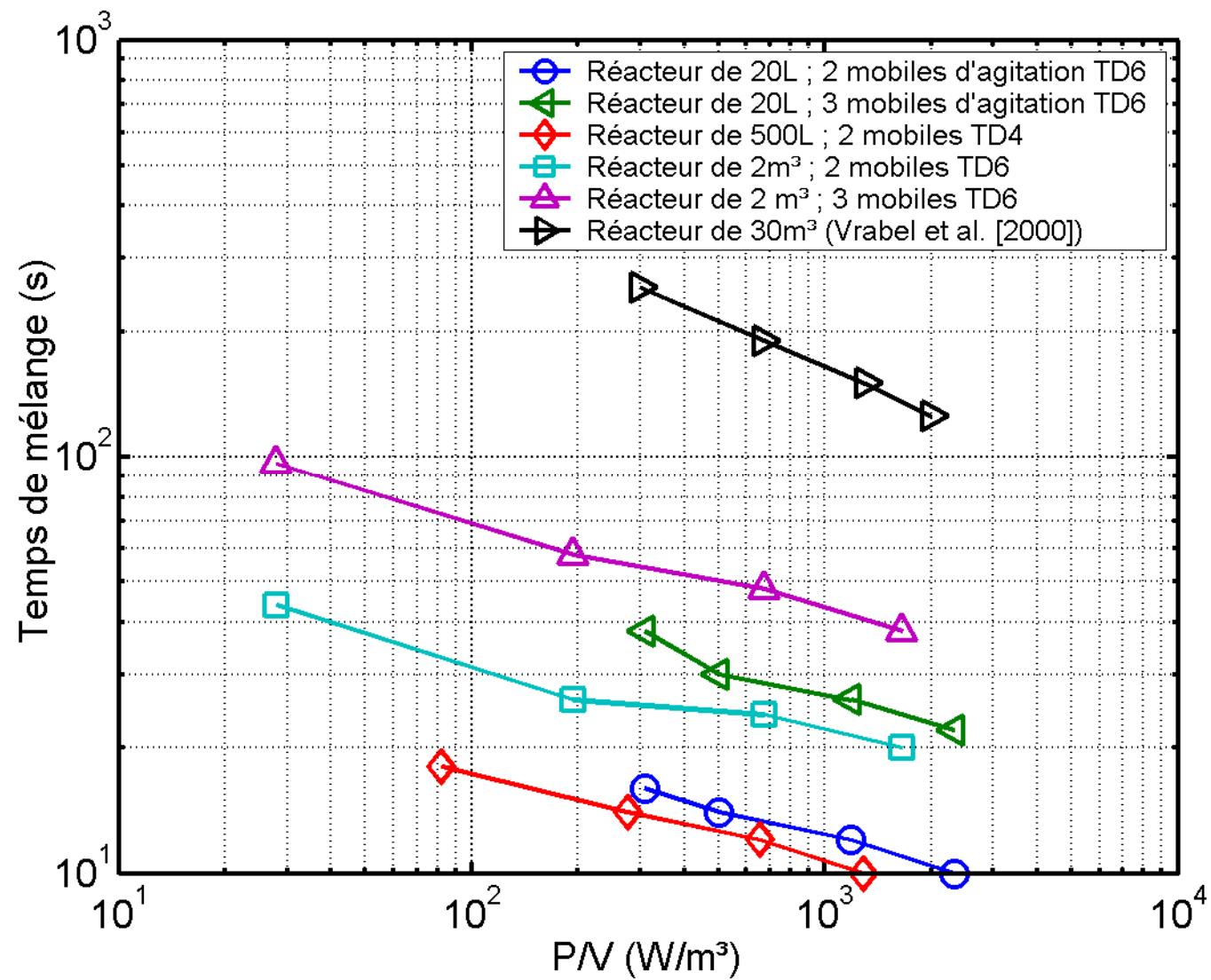


Enfors *et al.* [2001] Journal of biotechnology

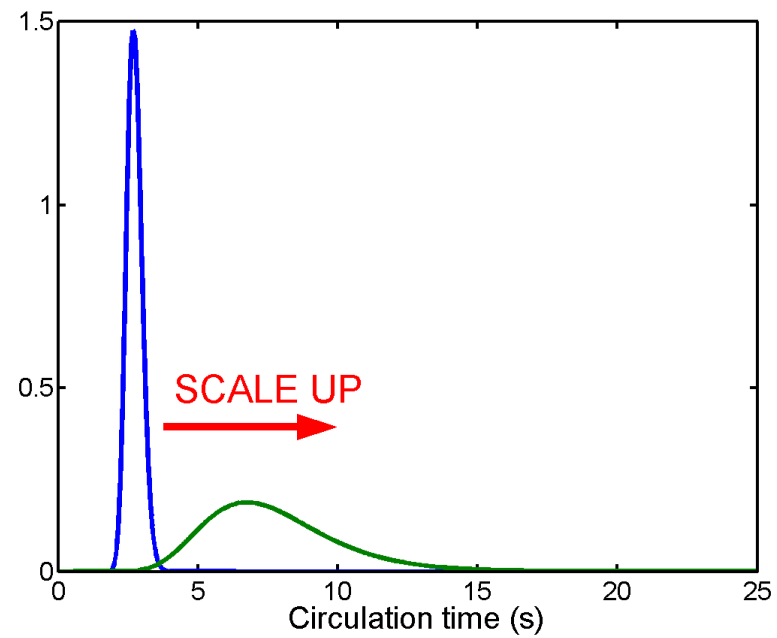
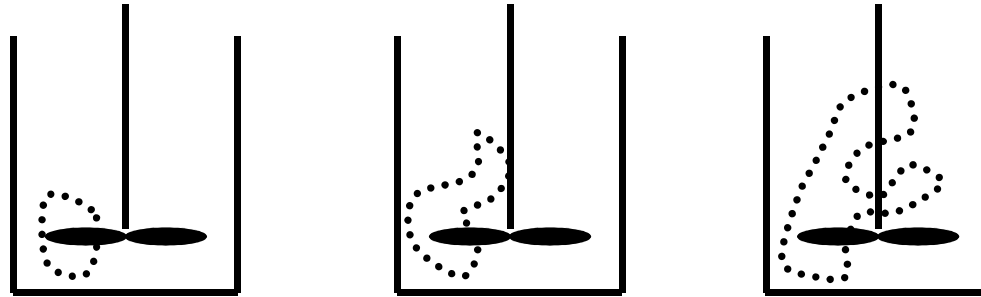
Gradient en oxygène dissous dans les procédés aérobies



Schütze *et al.* [2006] 12th European Conference on Mixing

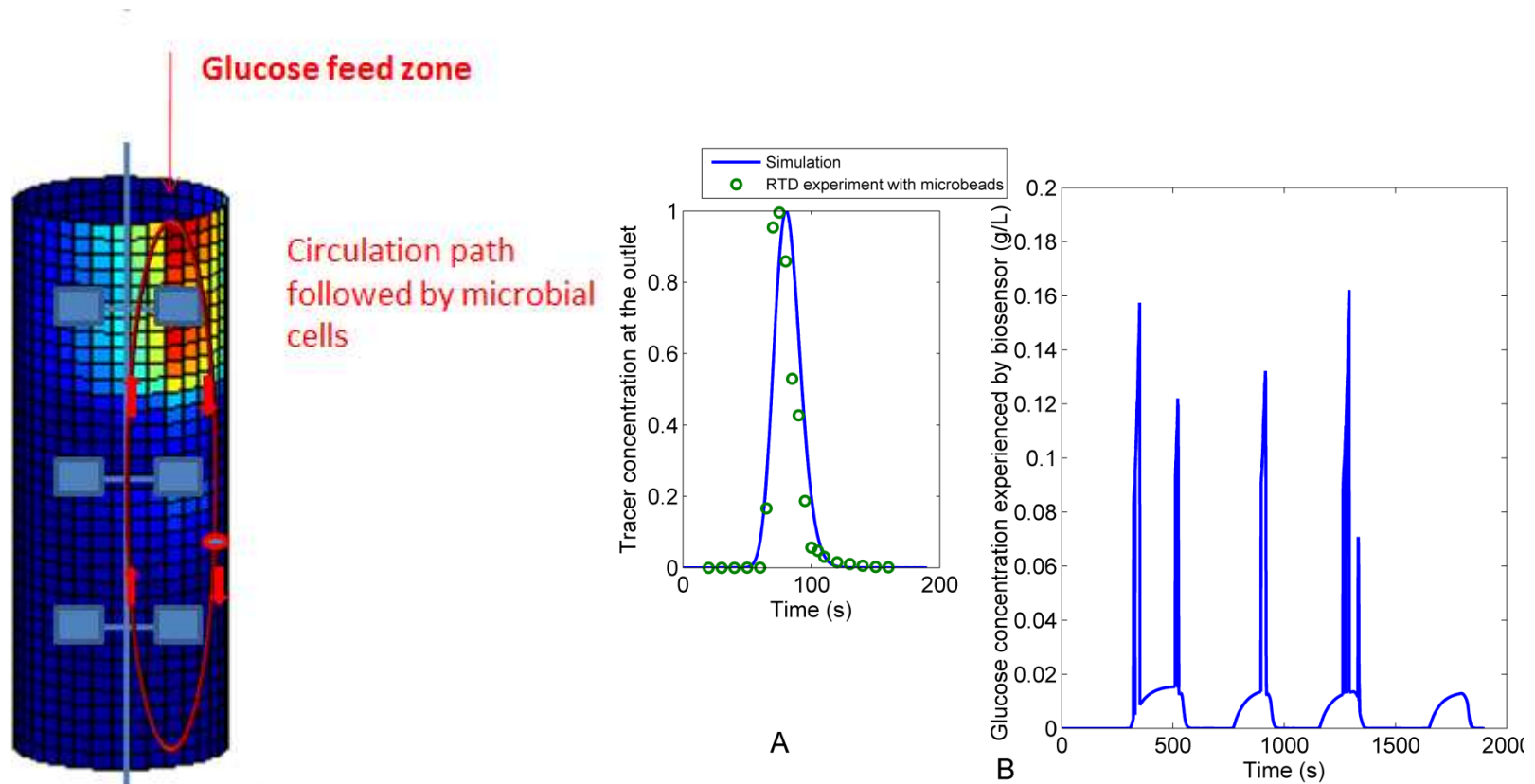


Apparition de facteurs aléatoires : stochasticité du déplacement des micro-organismes

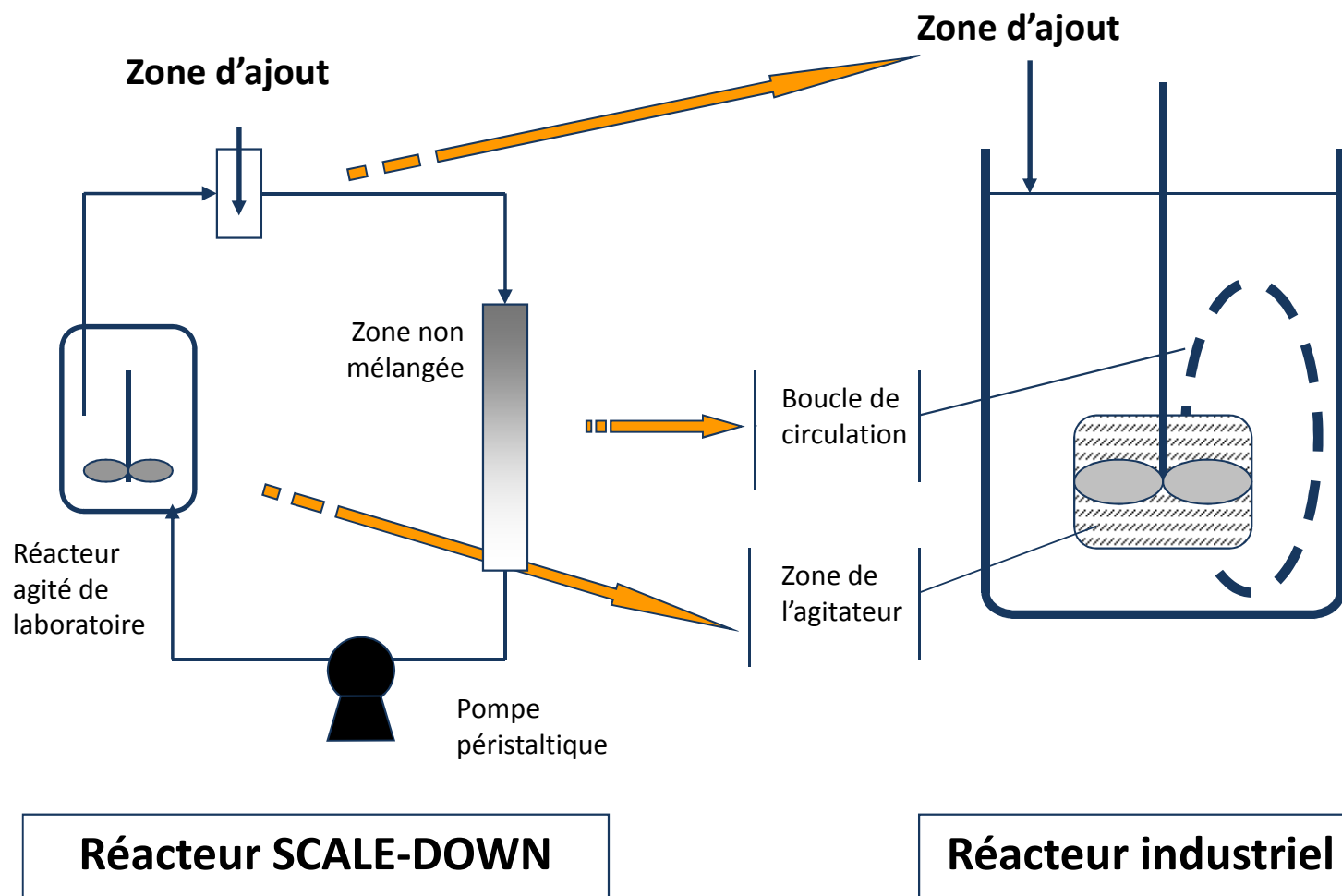


Deux phénomènes hydrodynamiques distincts (mais pouvant être traités par les mêmes équations) sont à prendre en compte :

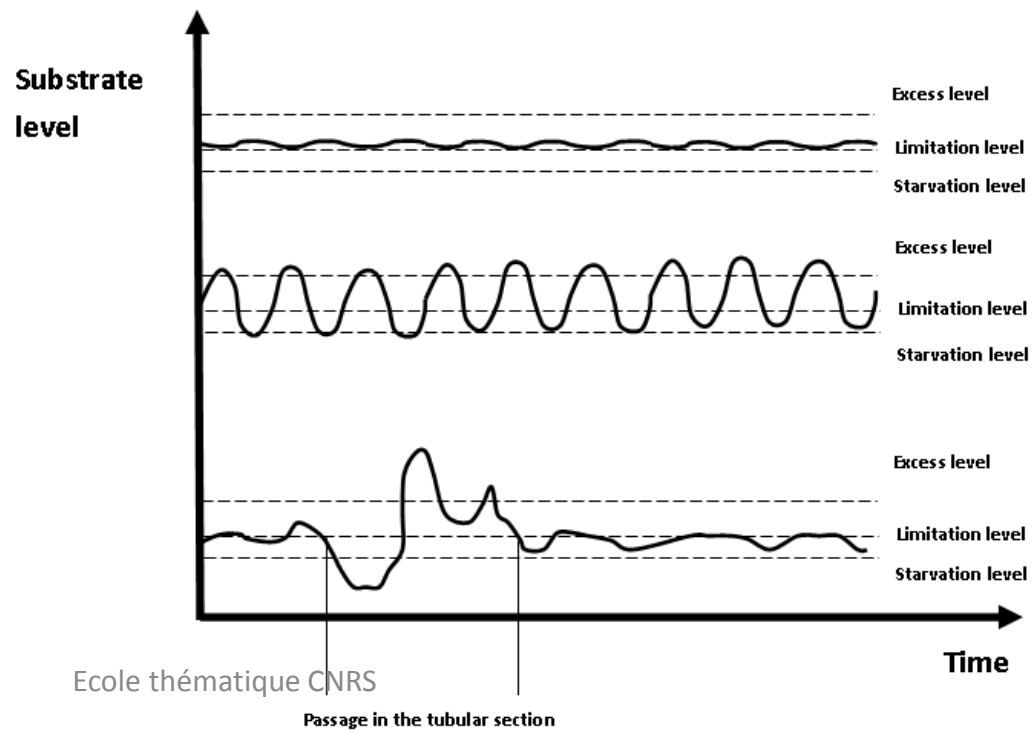
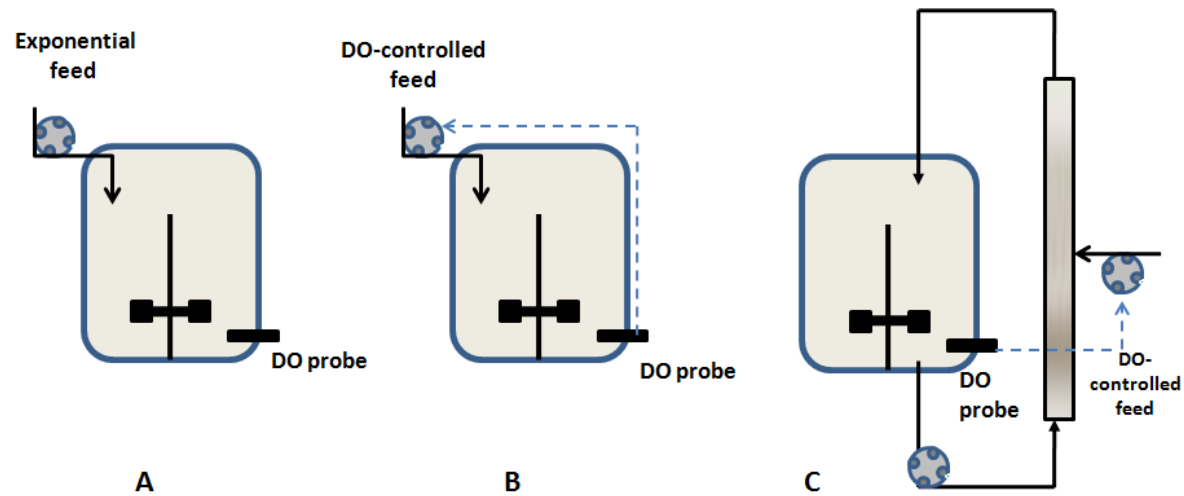
- Gradient de concentration (Performances d'homogénéisation du réacteur)
- Déplacement des micro-organismes (Débit de circulation)



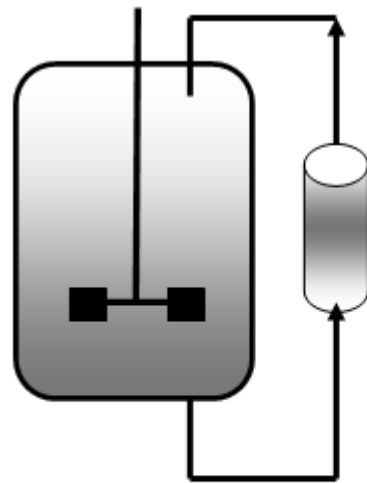
Approche *scale-down* :



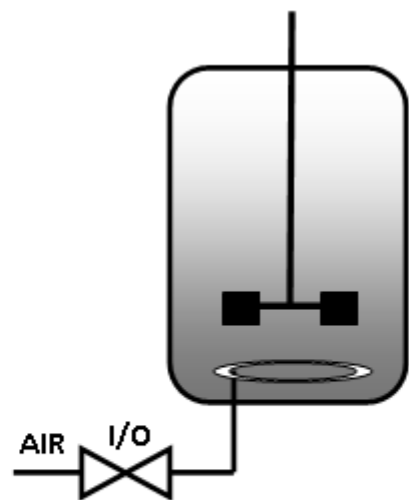
Concept du réacteur scale-down



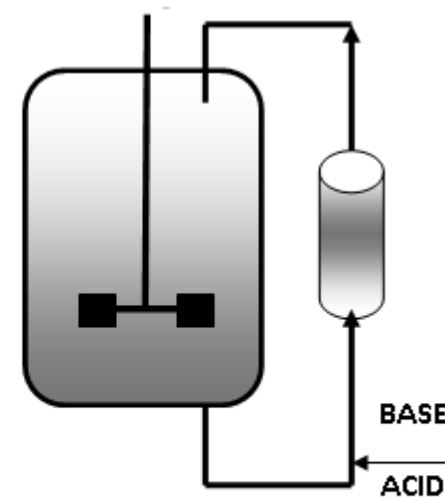
Différentes configurations sont possibles :



P-SDR



C-SDR for dissolved
oxygen fluctuation

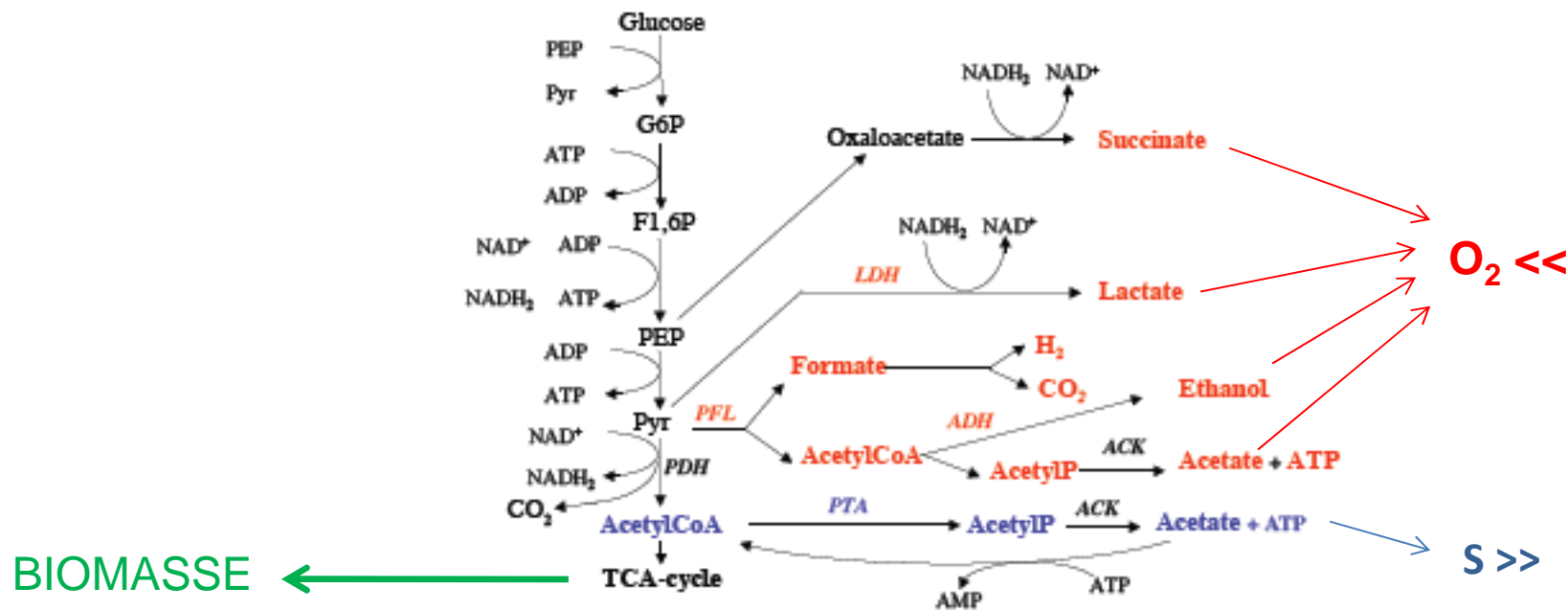


P-SDR with
pH gradient

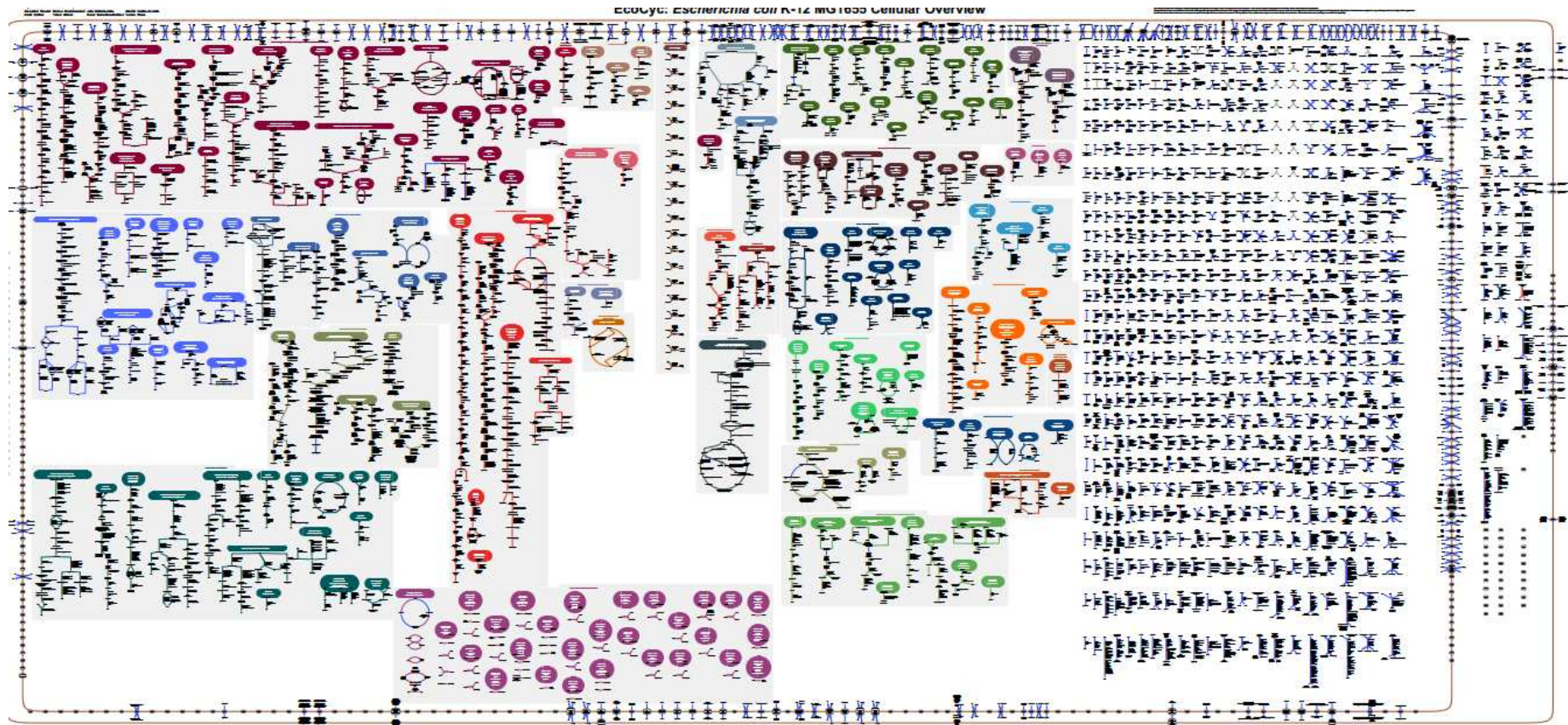
Influence de la concentration en substrat sur le métabolisme : exemple de *Escherichia coli*

Bascule métabolique déclenchée par :

- Excès de glucose
- Manque d'oxygène (voie des acides mixtes chez certains procaryotes)



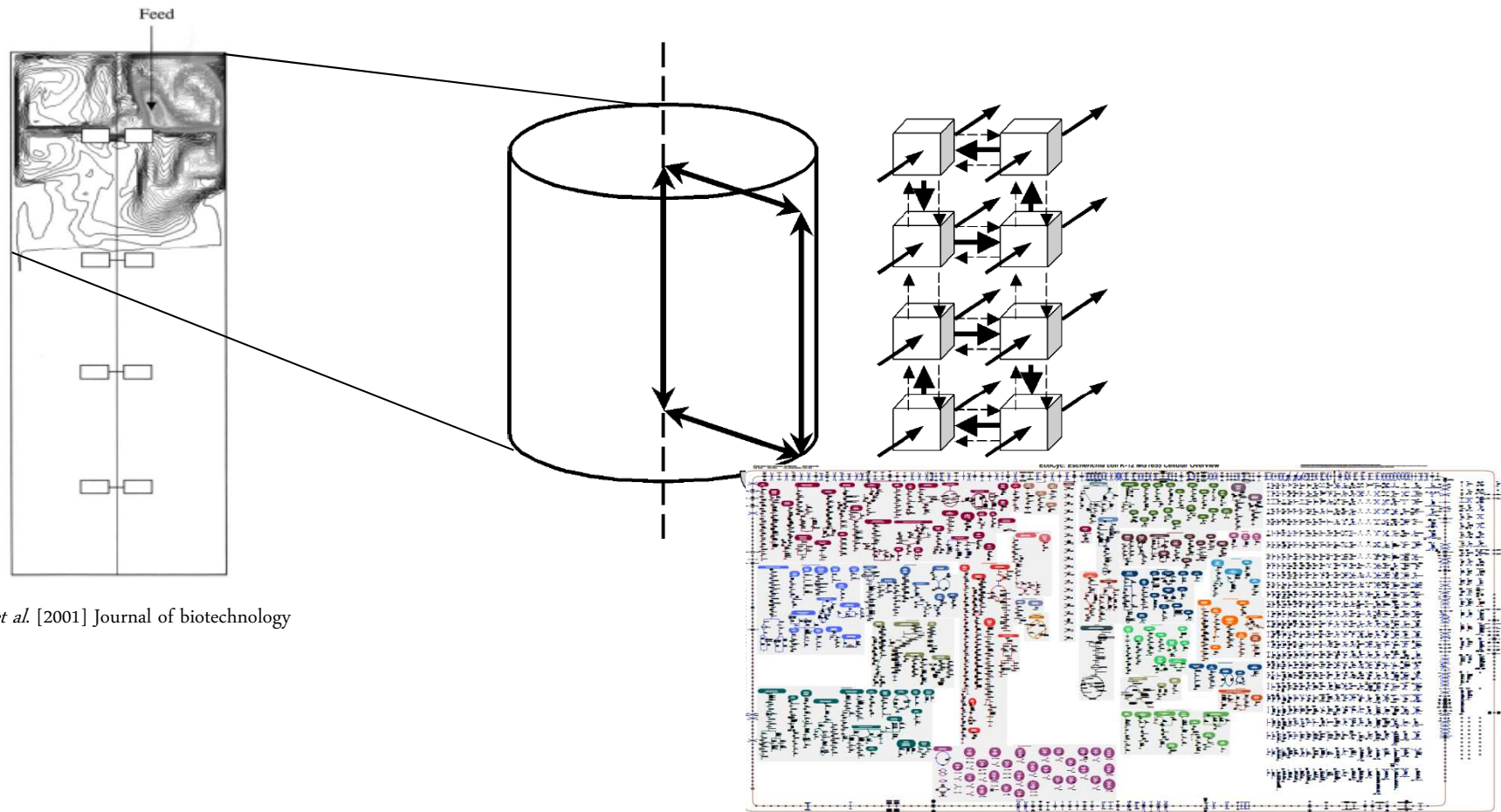
En réalité : réseau métabolique (« genome scale model »)



Source : <http://ecocyc.org/> Encyclopedia of Escherichia coli K12 genes and metabolism
Ecole thématique CNRS

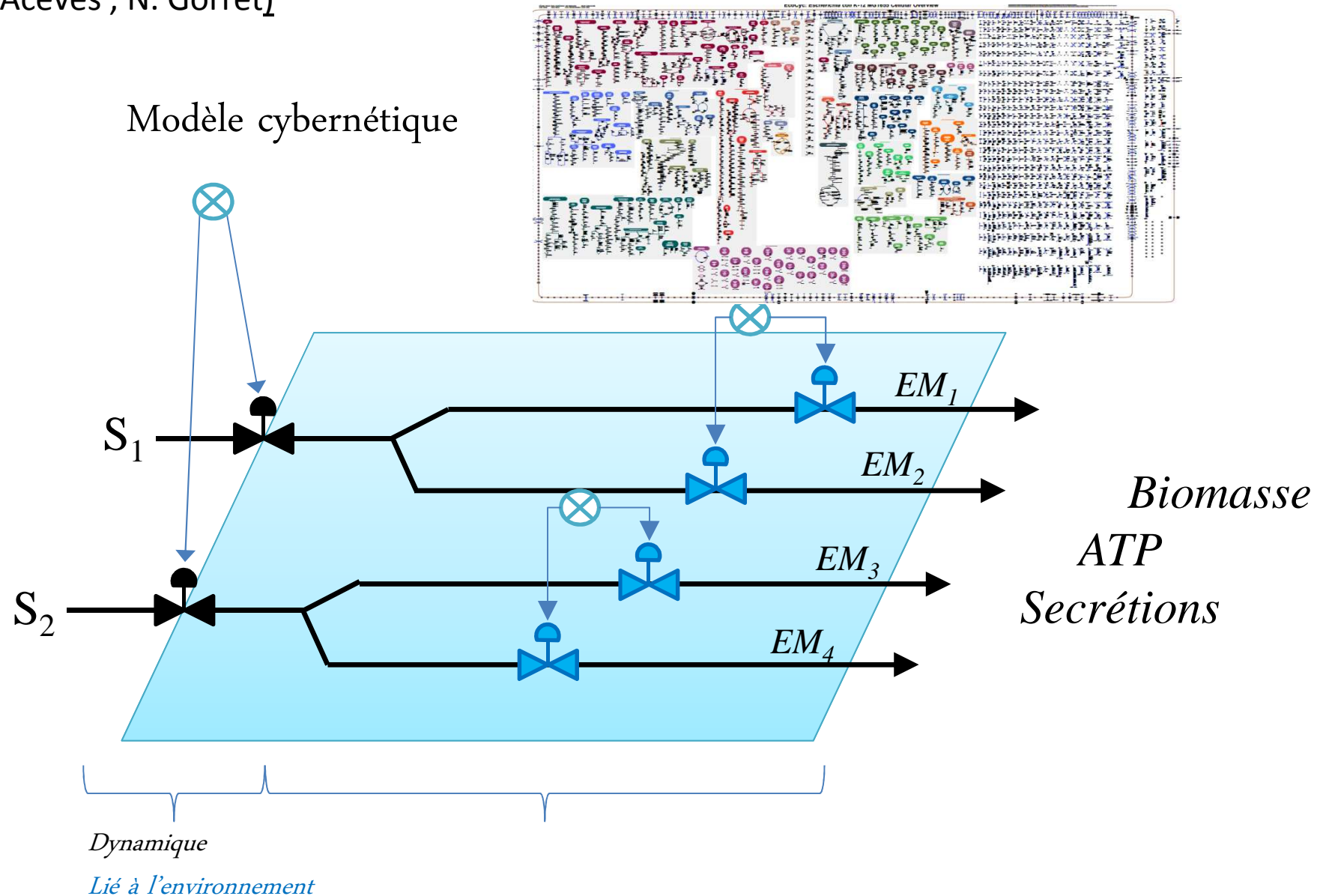
Couplage modèle hydro – modèle bio : 2 approches

1. Simplifier la partie biologique
2. Simplifier la partie hydrodynamique

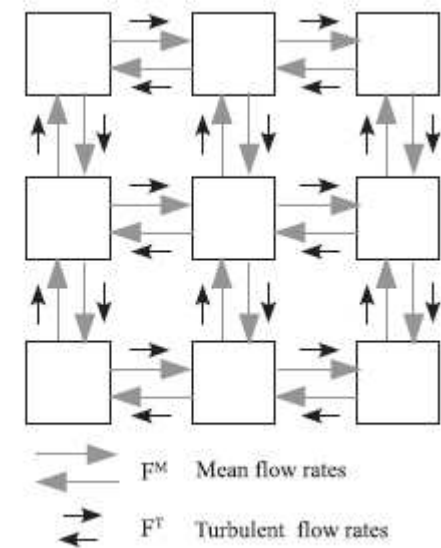
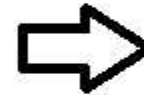
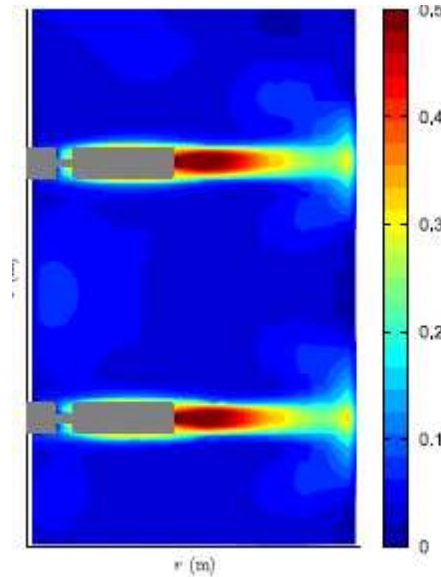
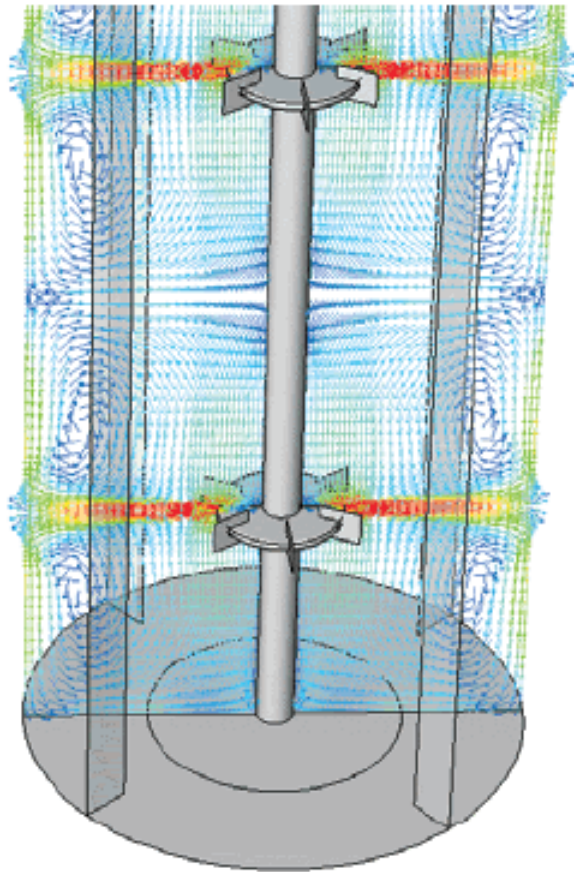


Enfors *et al.* [2001] *Journal of biotechnology*

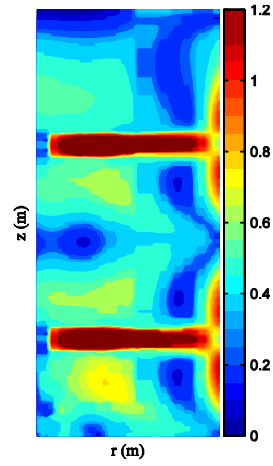
Modèle « bio » développé à l'INSA – LISBP (J. Günther, Bideaux, C. Molina-Jouve, C. Aceves, N. Gorret)



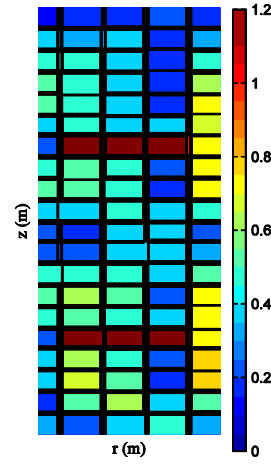
Modèle hydro simplifié (LGC Liège)



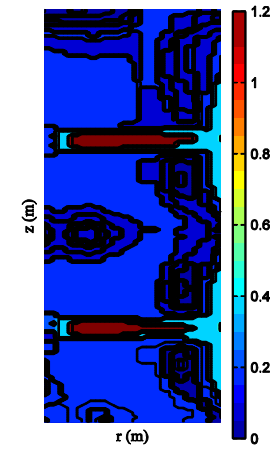
Delafosse, A., Collignon, M.L., Calvo, S., Delvigne, F., Crine, M., Thonart, P., Toye, D., (2014) CFD-based compartment model for description of mixing in bioreactors. *Chemical engineering science* 106, 76-85



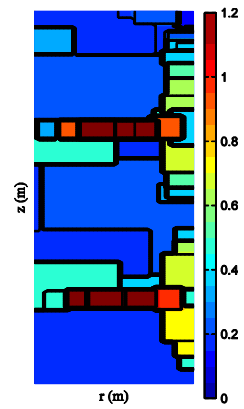
a. CFD



b. Manual zoning

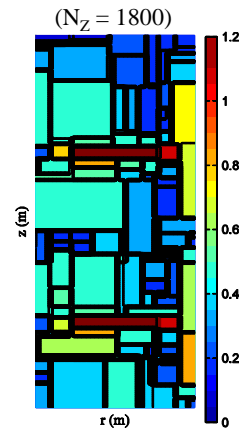


c. CBC zoning



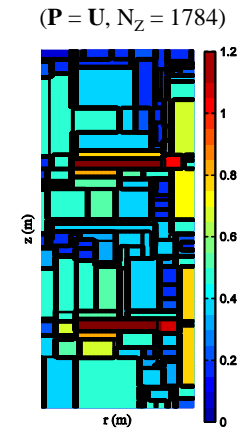
d. LBL1 zoning

($\mathbf{P} = \mathbf{U}$, $N_Z = 1023$)



e. LBL2 zoning

($\mathbf{P} = \mathbf{U}$, $N_Z = 3897$)



f. LBL2 zoning

($\mathbf{P} = \mathbf{U}_i$, $N_Z = 4300$)

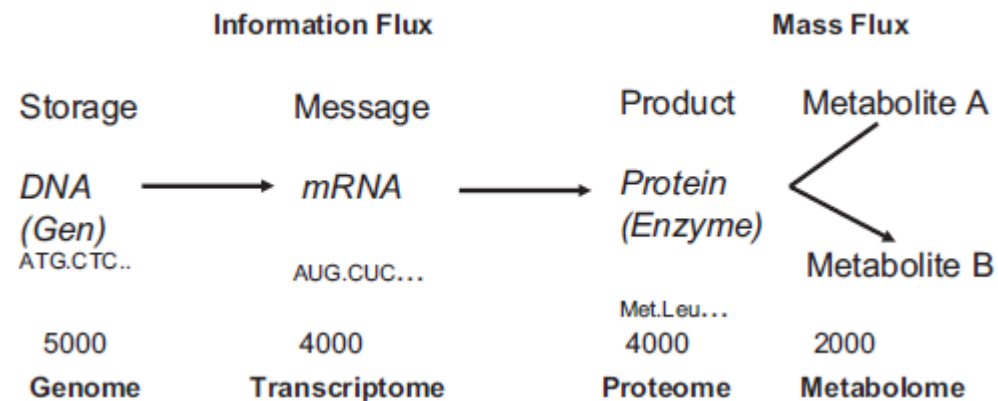
3. Aspects « physiologie microbienne »

Le micro-organisme : un système complexe

Plusieurs niveaux de complexité :

1. Organisation du flux d'informations suivants différents systèmes (biologie systémique)
2. Dynamique du flux d'information
3. Nombres de molécules impliquées dans les systèmes (approche « omique »)
4. Hétérogénéité phénotypique

3.1. Premier niveau de complexité : organisation du flux d'informations suivants différents systèmes (biologie systémique)



Basic molecular structure of biological processes. The information stored in DNA is transcribed in messenger molecules (mRNAs) which synthesize proteins at the ribosomes. Most of the produced proteins act as catalysts for the reactions of the metabolic network. The entire DNA information is the genome, for microorganisms it typically contains 5,000 identified genes. The proteome comprehends about 4,000 proteins, and roughly 2,000 metabolites could be identified.

Deckwer *et al.* [2006] Engineering in life sciences

3.2. Deuxième niveau de complexité : Dynamique du flux d'information à l'intérieur de la cellule

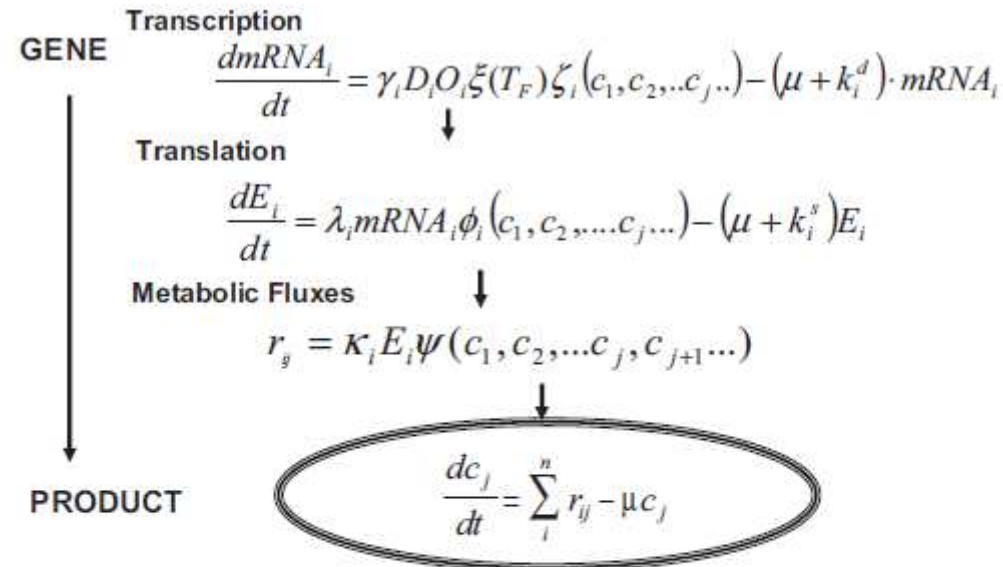
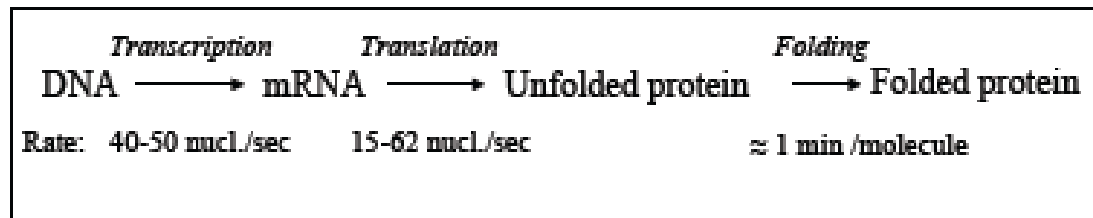


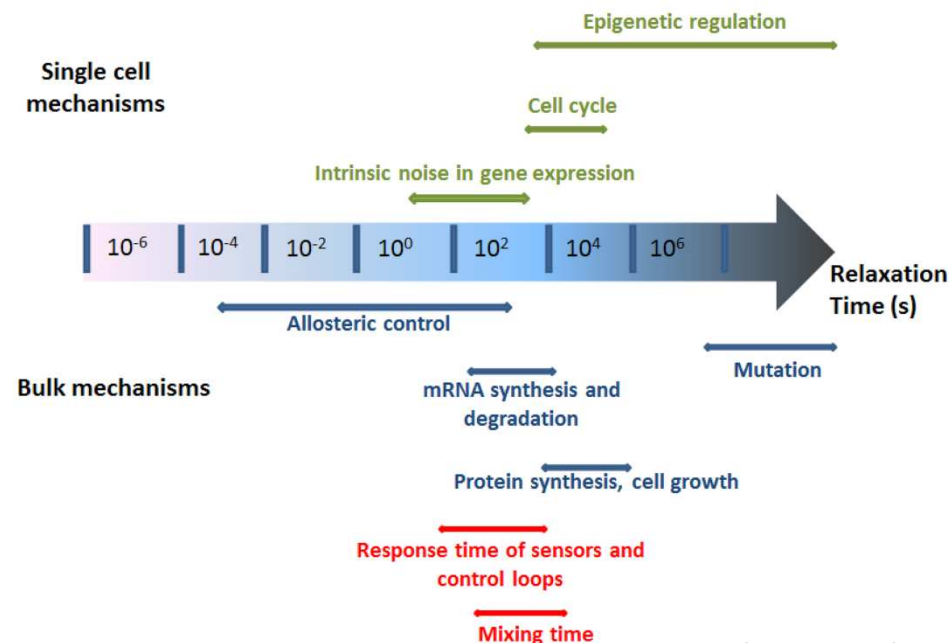
Figure 4. Differential equations governing the transcription, translation and metabolic fluxes of a cell. In simple cases such as the tryptophan synthesis balances for the metabolites (j) being involved in i metabolic reactions can be formulated which are linked to the kinetic relations of transcription, translation, inhibition, repression, growth (dilution) and degradation (of enzymes and mRNA). Thus, a (semi)-quantitative description can be achieved [4–6].

Exemple dans le cas de *E. coli*



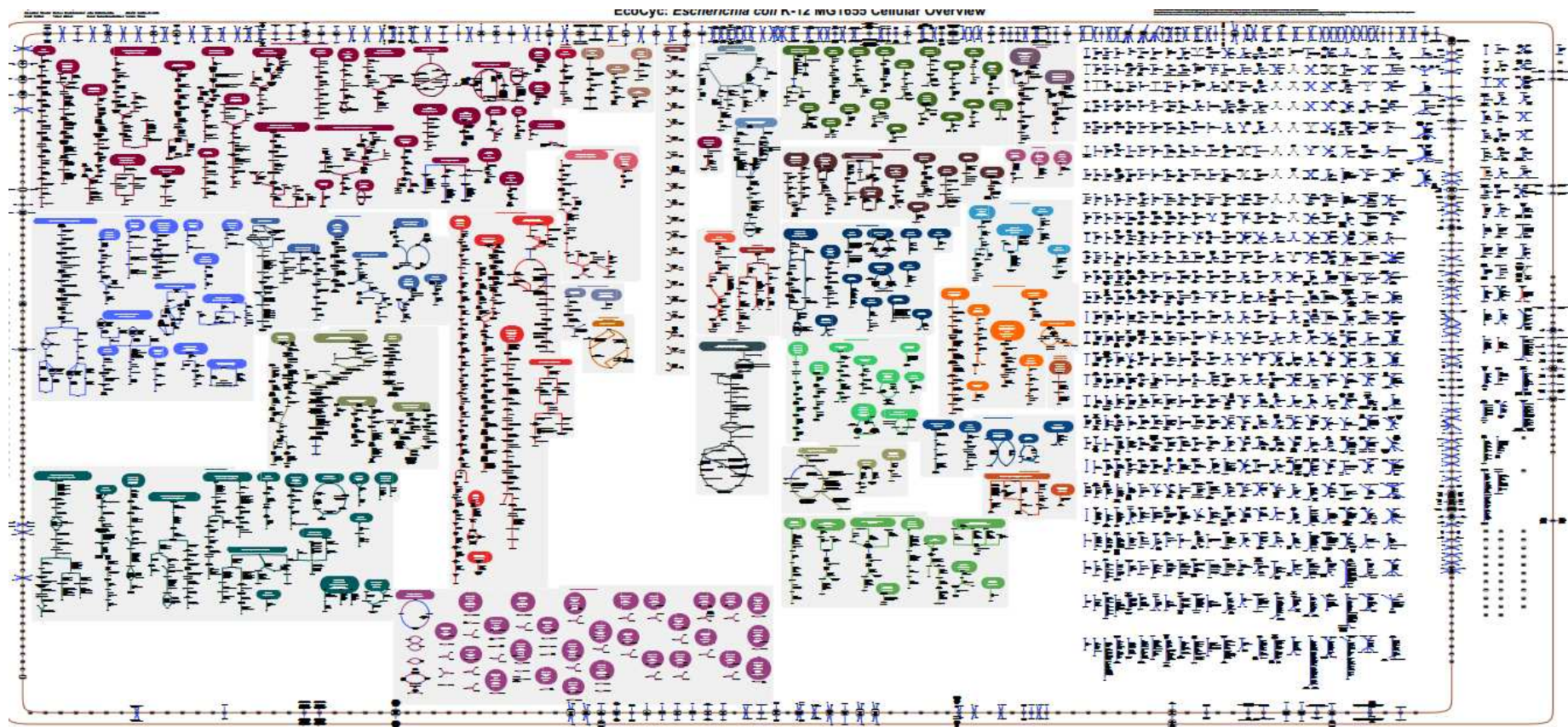
Enfors *et al.* [2001] Journal of biotechnology

Différents niveaux de réactions en fonction du temps caractéristiques de la physiologie (et du bioréacteur)

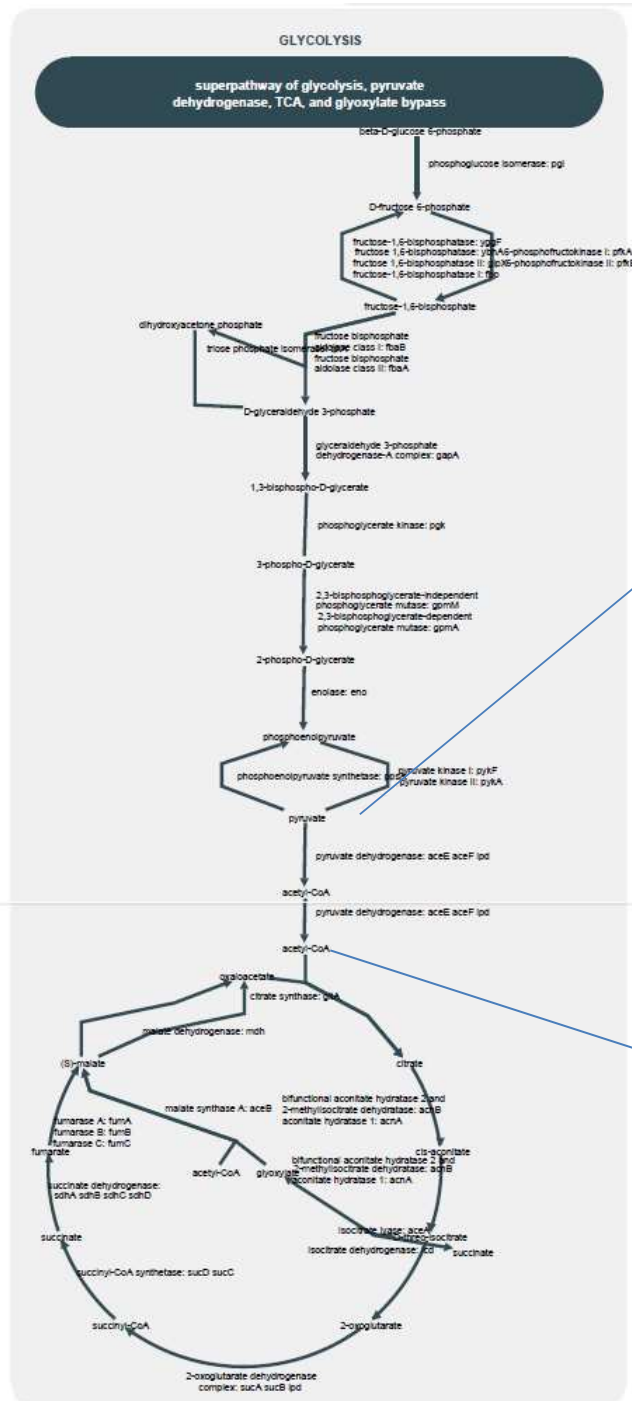


3.3. Troisième niveau de complexité : nombres de molécules impliquées dans les systèmes (approche « omique »)

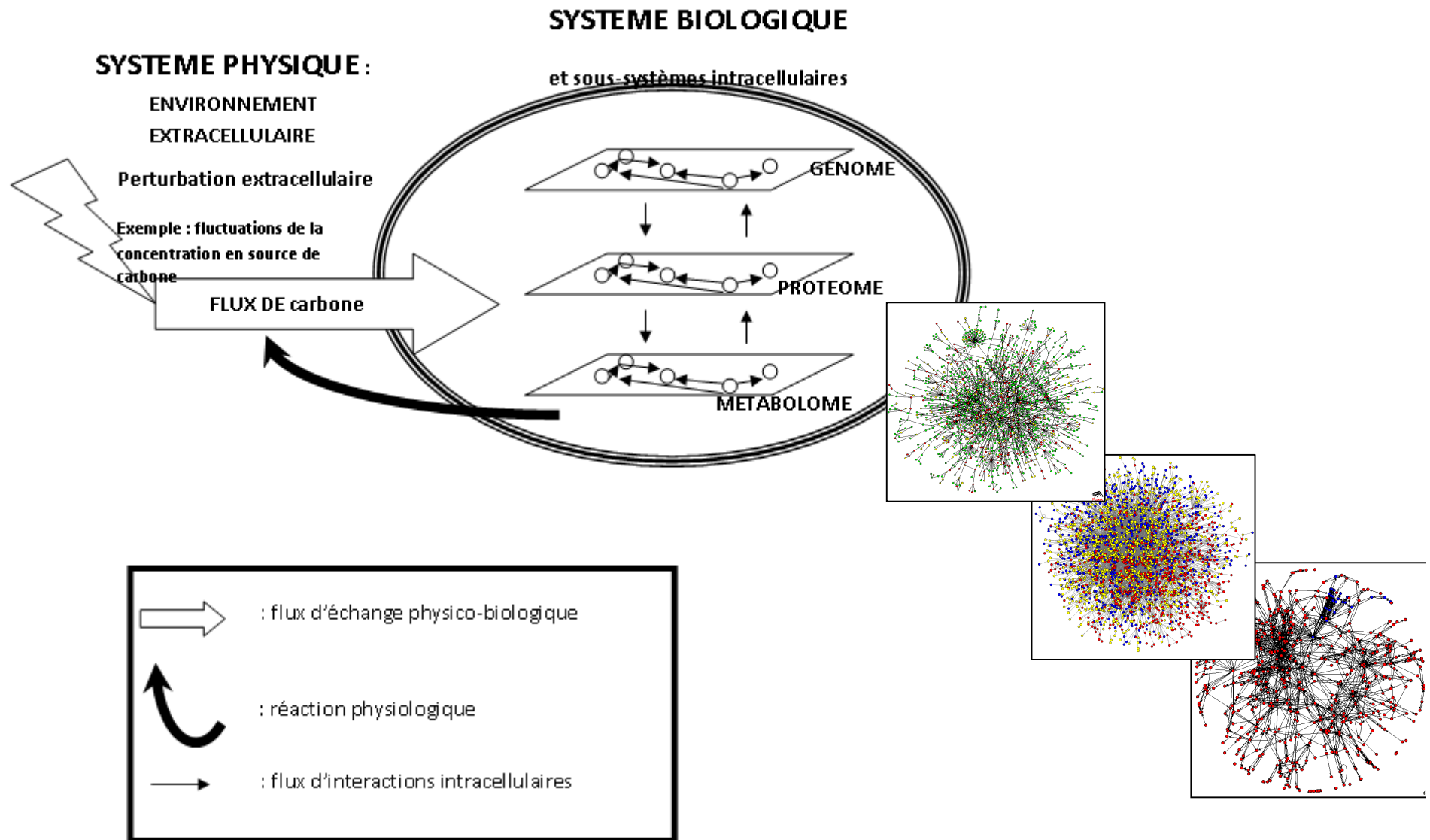
Exemple : réseau métabolique (« genome scale model »)

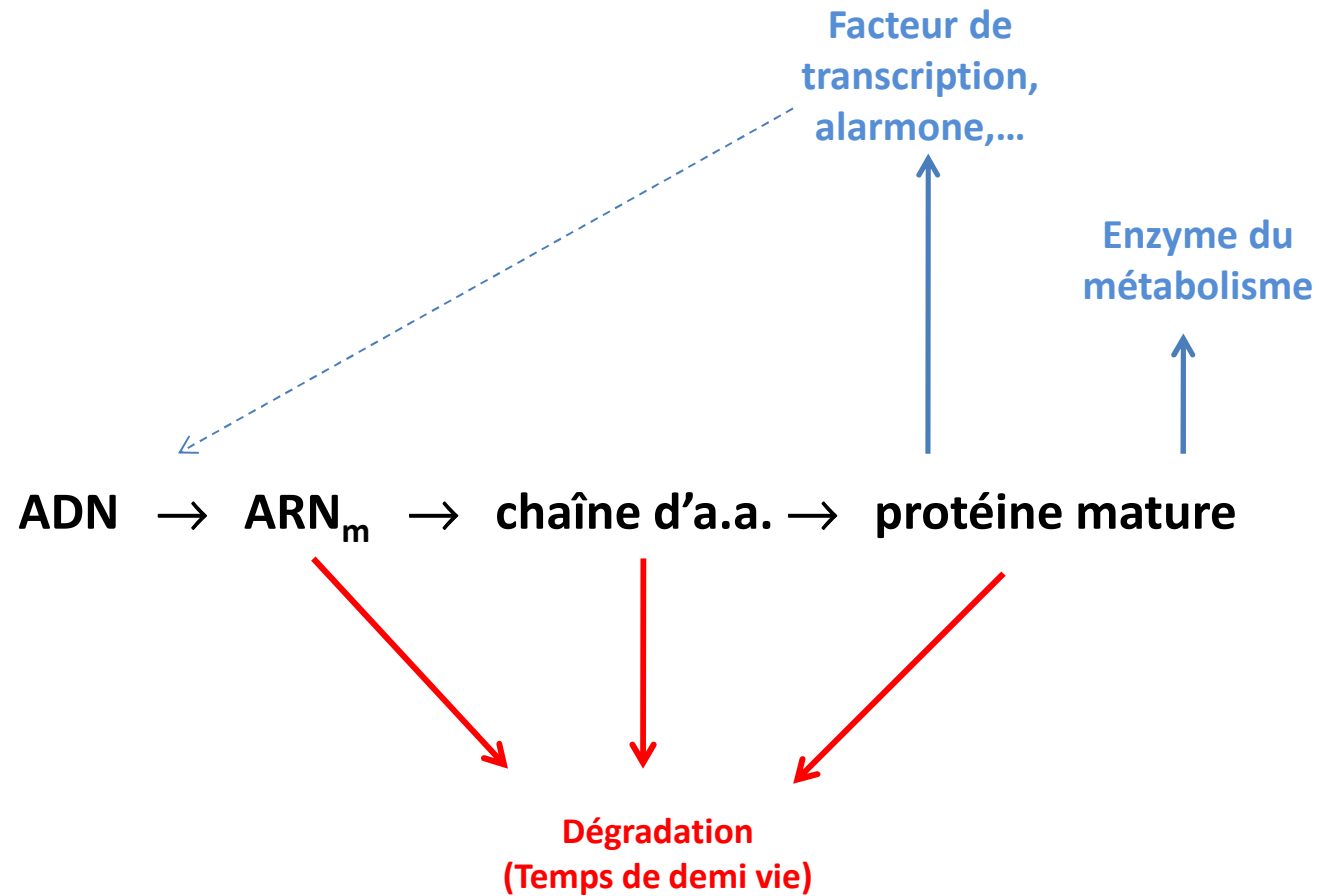


Source : <http://ecocyc.org/> Encyclopedia of Escherichia coli K12 genes and metabolism
Ecole thématique CNRS



acetate





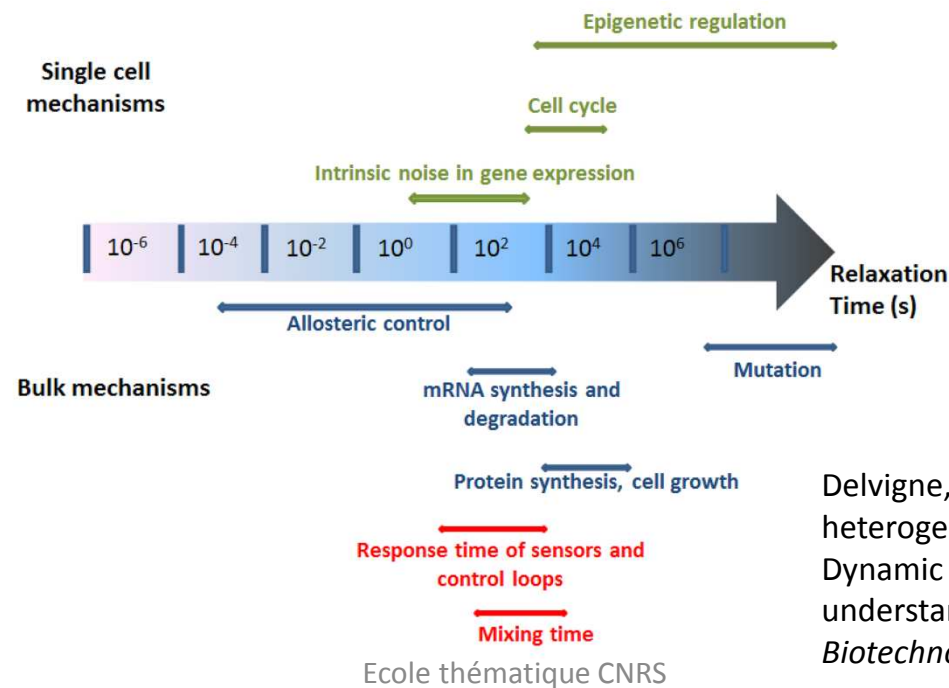
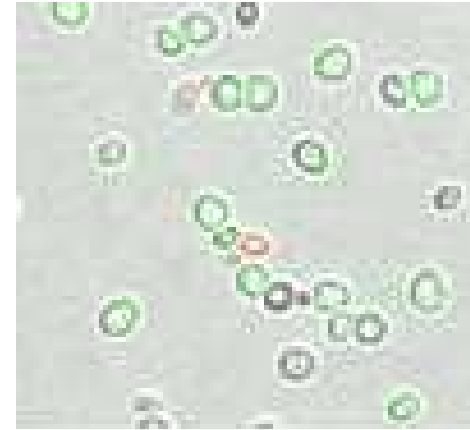
Exemple de cinétique d'expression pour *E. coli* :

<i>Transcription</i>	<i>Translation</i>	<i>Folding</i>
DNA → mRNA	mRNA → Unfolded protein	Unfolded protein → Folded protein
Rate: 40-50 nucl./sec	15-62 nucl./sec	≈ 1 min /molecule

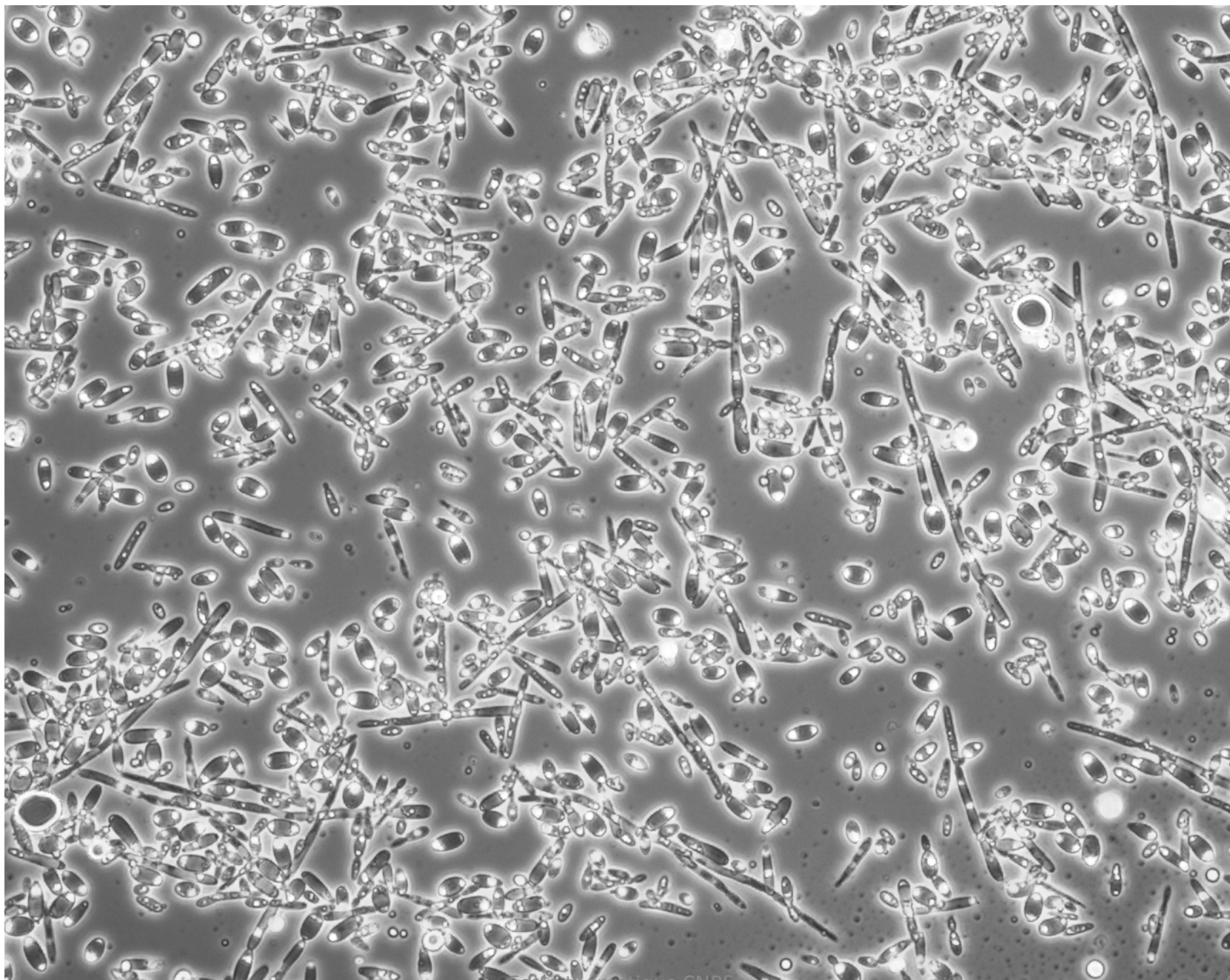
3.4. Quatrième niveau de complexité : Hétérogénéité phénotypique

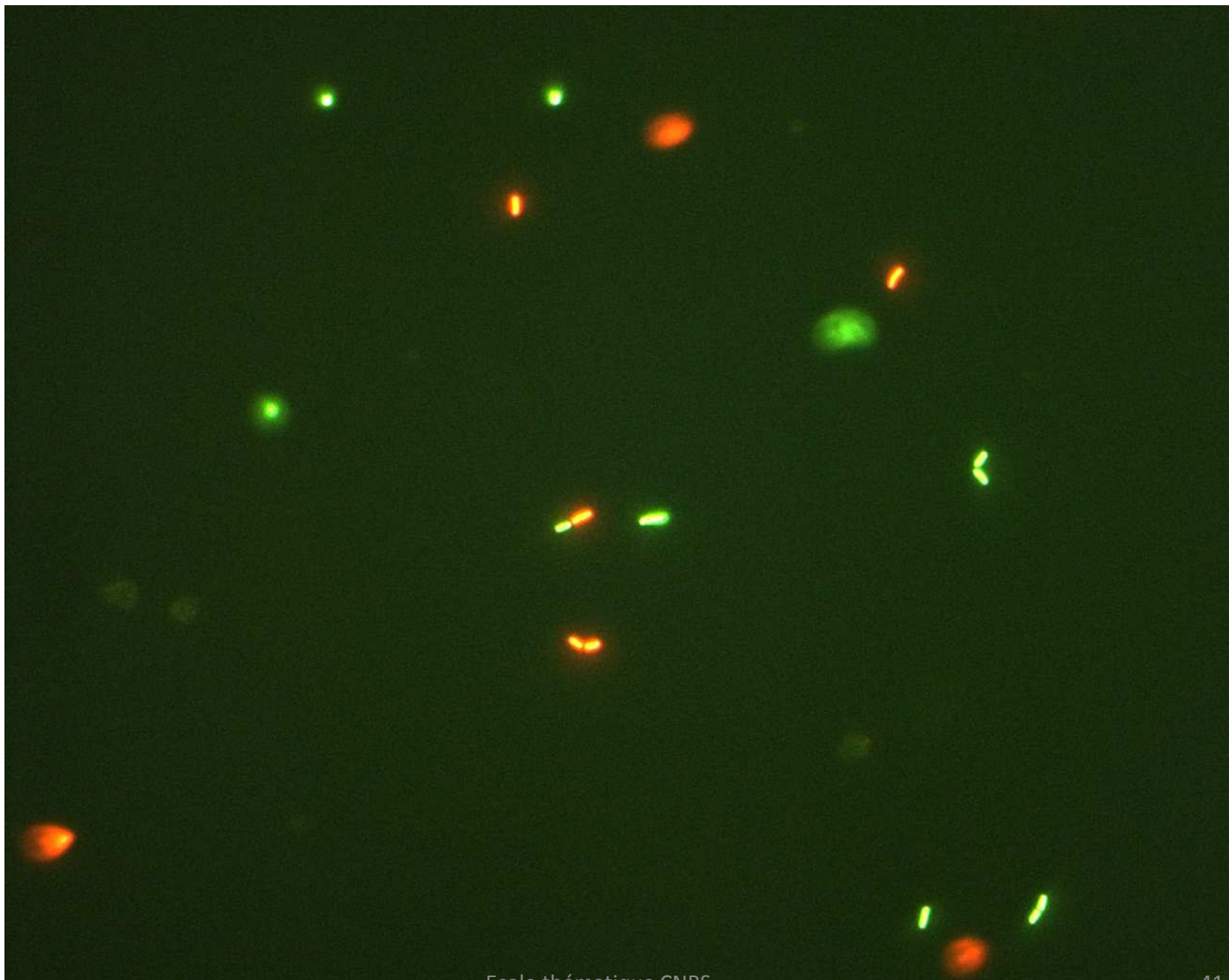
Hétérogénéité au niveau :

- Morphologie
- Viabilité
- Productivité
- Qualité du produit (protéine)

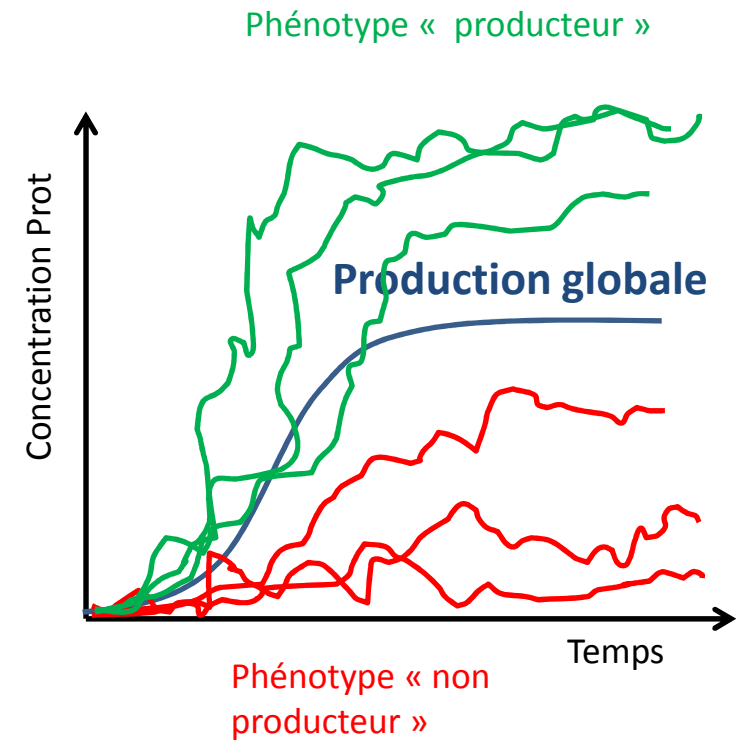
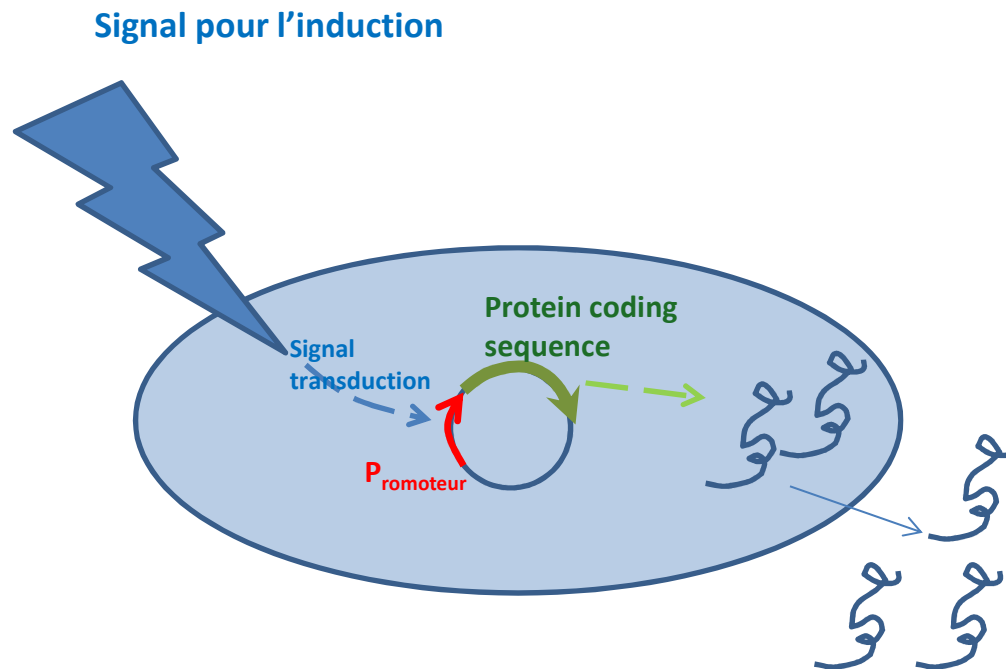


Delvigne, F., Goffin, P., (2014) Microbial heterogeneity affects bioprocess robustness: Dynamic single cell analysis contribute to understanding microbial populations. *Biotechnology journal* 9, 61-72





Hétérogénéité des systèmes recombinants



4. Développement d'outils d'étude adapté

Outils prenant en compte à la fois les aspects relatifs au génie des procédés et à la physiologie microbienne

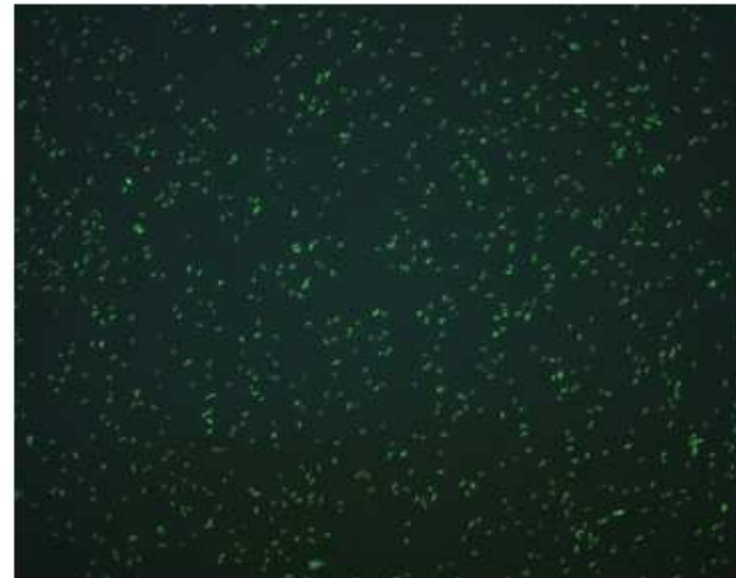
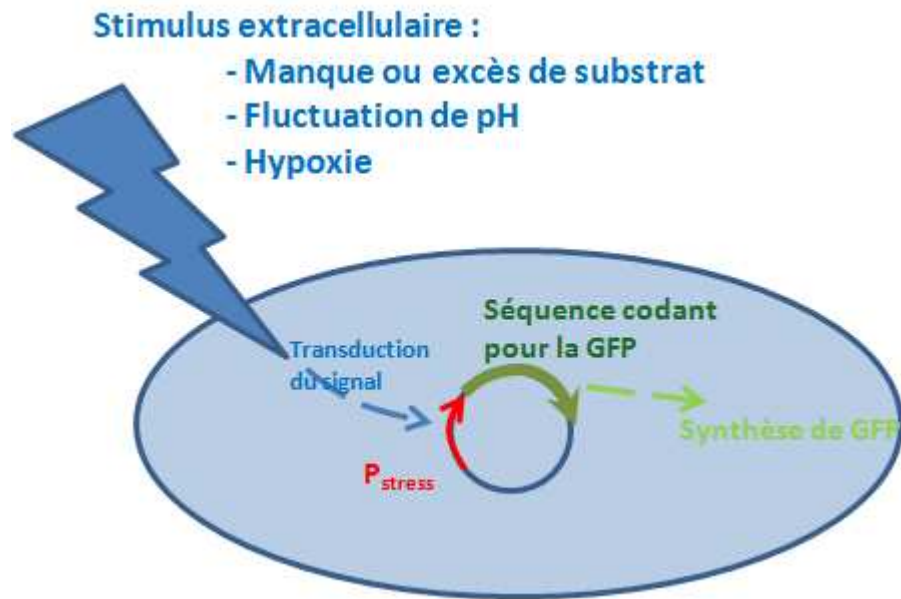
BUT : suivre la physiologie microbienne dans des conditions de procédés en prenant en compte :

1. **Organisation du flux d'informations suivants différents systèmes (biologie systémique)**
2. **Dynamique du flux d'information**
3. **Nombres de molécules impliquées dans les systèmes (approche « omique »)**
4. **Hétérogénéité phénotypique**

Suivi « single cell »

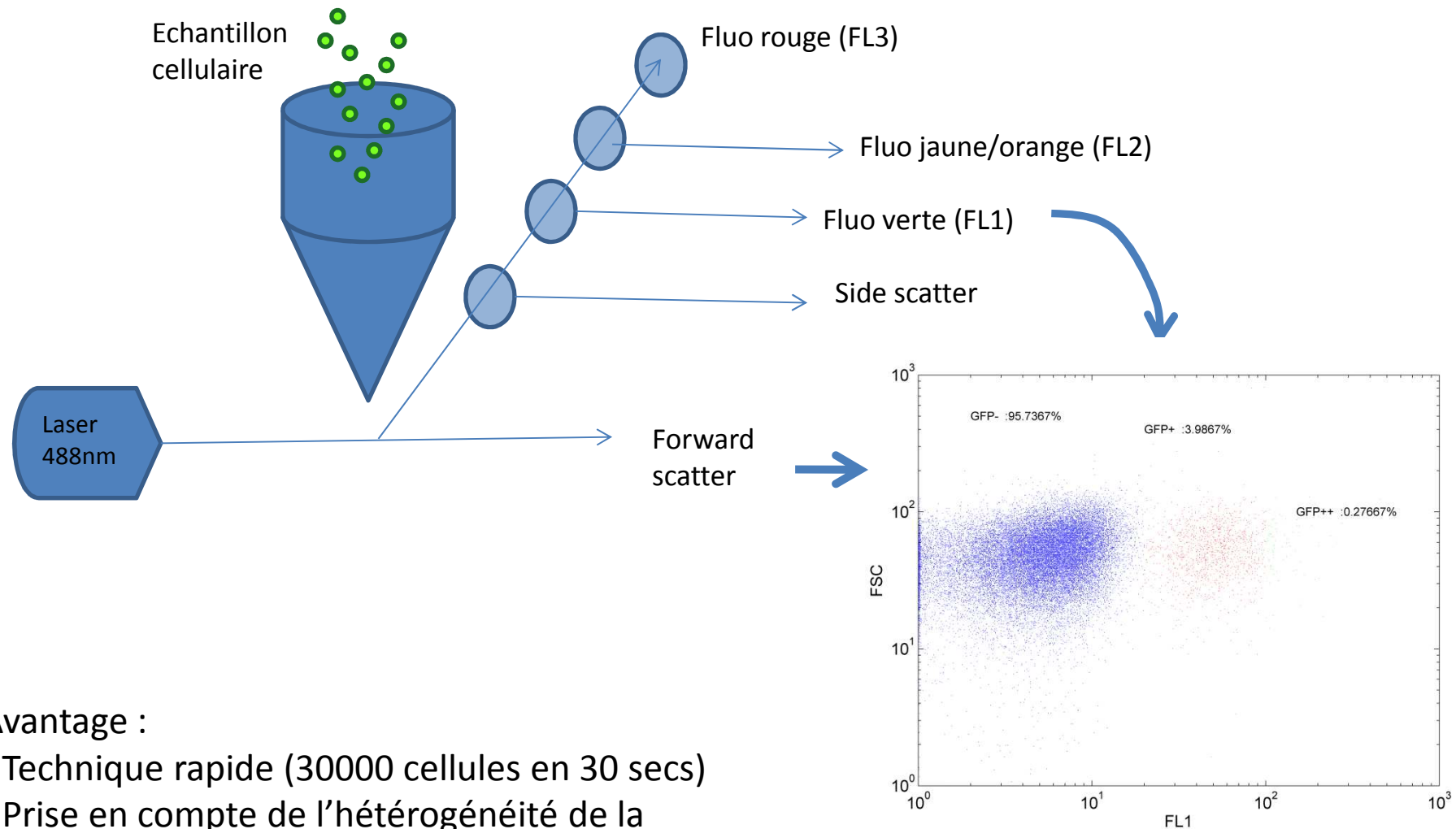
Concept de base : se servir de la population microbienne comme traceur des conditions environnementales rencontrées dans le réacteurs (et perçues au niveau physiologique)

Biocapteur GFP



Delvigne, F., Ingels, S., Thonart, P., (2010) Evaluation of a set of *E. coli* reporter strains as physiological tracer for estimating bioreactor hydrodynamic efficiency. *Process biochemistry* 45, 1769-1778

Analyse « single cell »



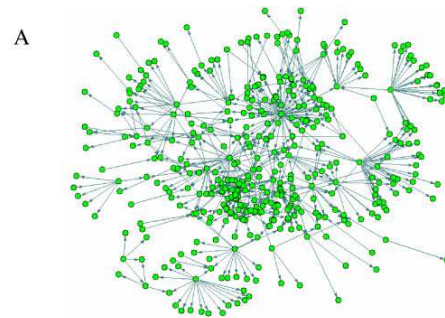
Avantage :

- Technique rapide (30000 cellules en 30 secs)
- Prise en compte de l'hétérogénéité de la population microbienne

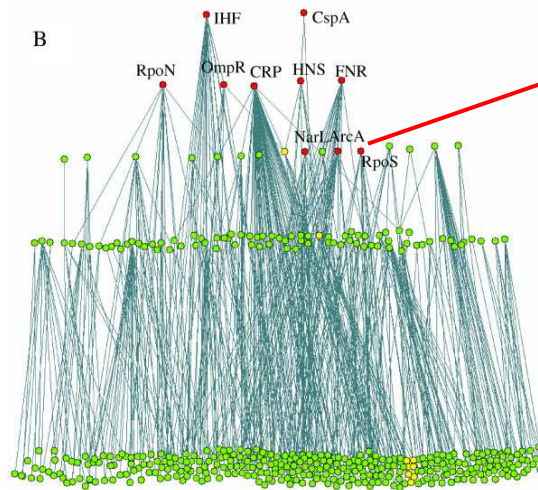
Sélection d'un promoteur de stress

E. coli : environ 4000 ORFs :

Réseau d'interaction



Réseau d'interaction
avec classification
hiérarchique

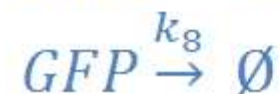
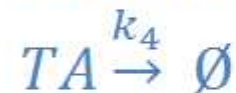
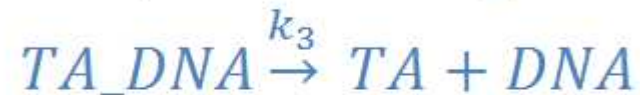
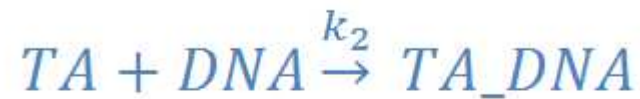
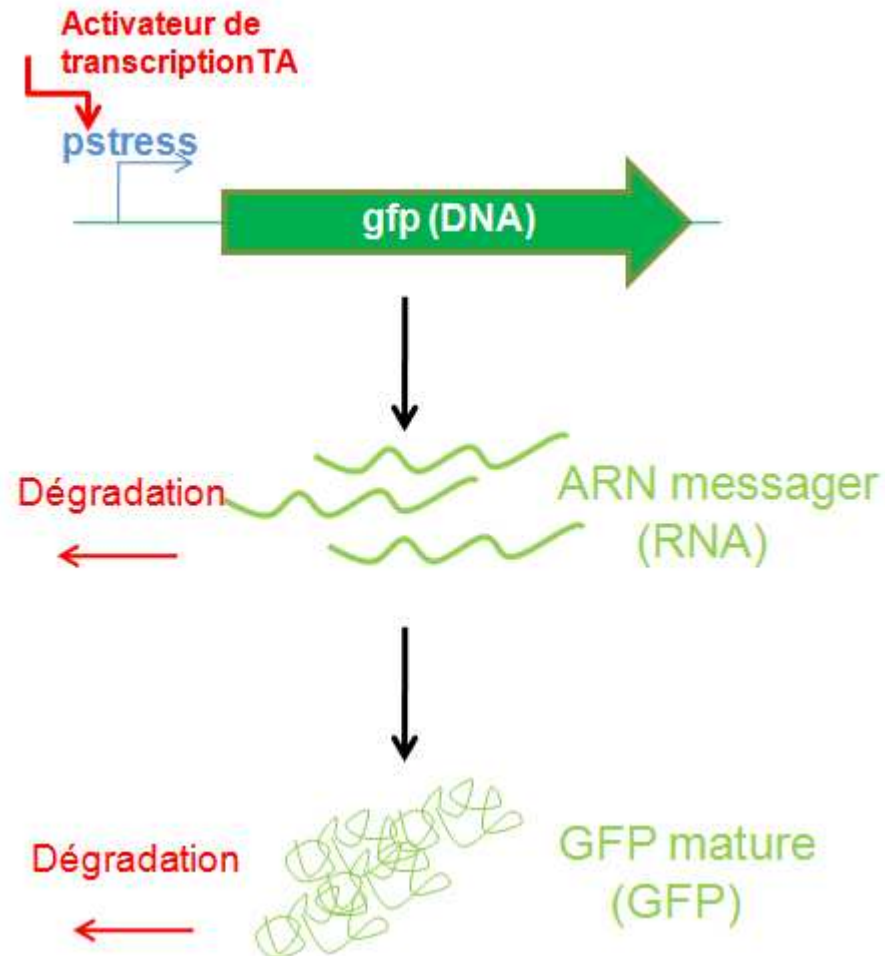


Promoteurs de stress
associés aux fluctuations de
concentration en substrat :

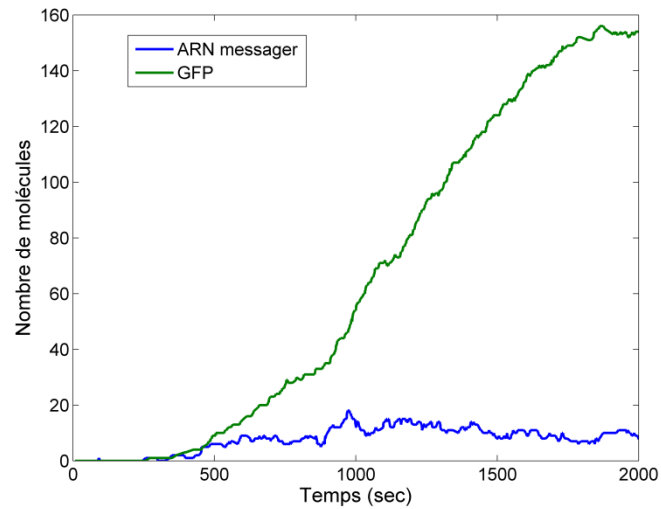
- **rpoS**
- crp
- rpoE
- rssB
- uspA
- csiE

Ma, H.W., Buër, J., Zeng, A.P., (2004) Hierarchical structure and modules in the Escherichia coli transcriptional regulatory network revealed by a new top-down approach. *BMC bioinformatics* 5, 199

Mécanismes moléculaires impliqués

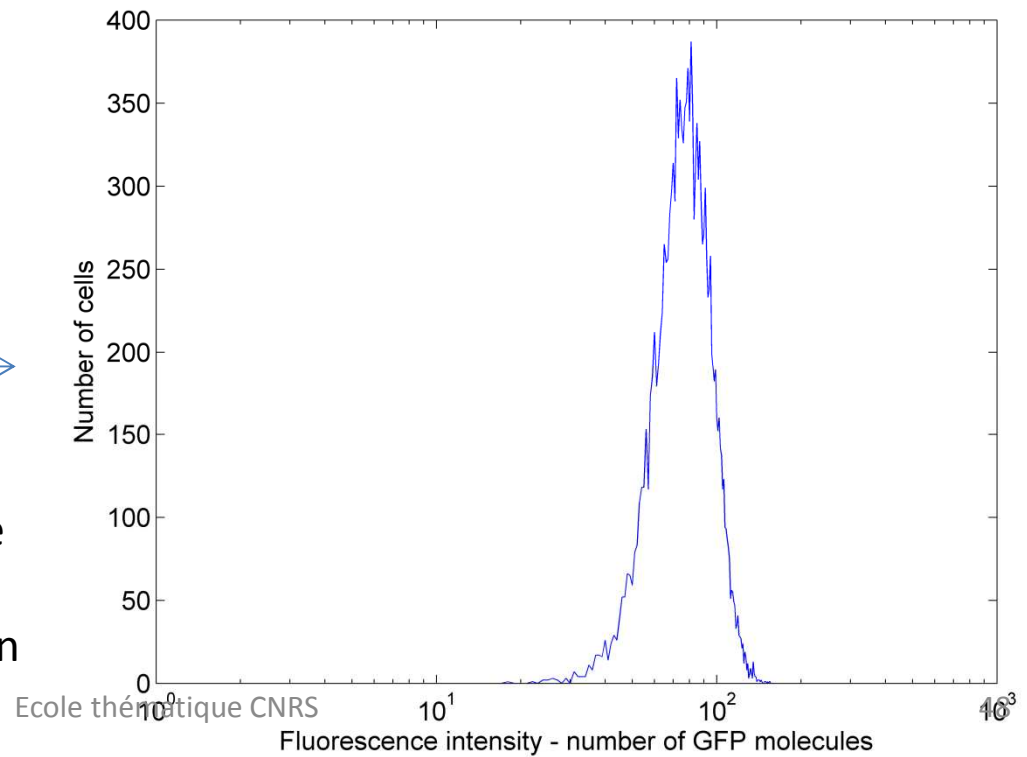


Effet stochastique : simulation sur une population de 30000 cellules microbiennes



Simulation à partir d'une cellule

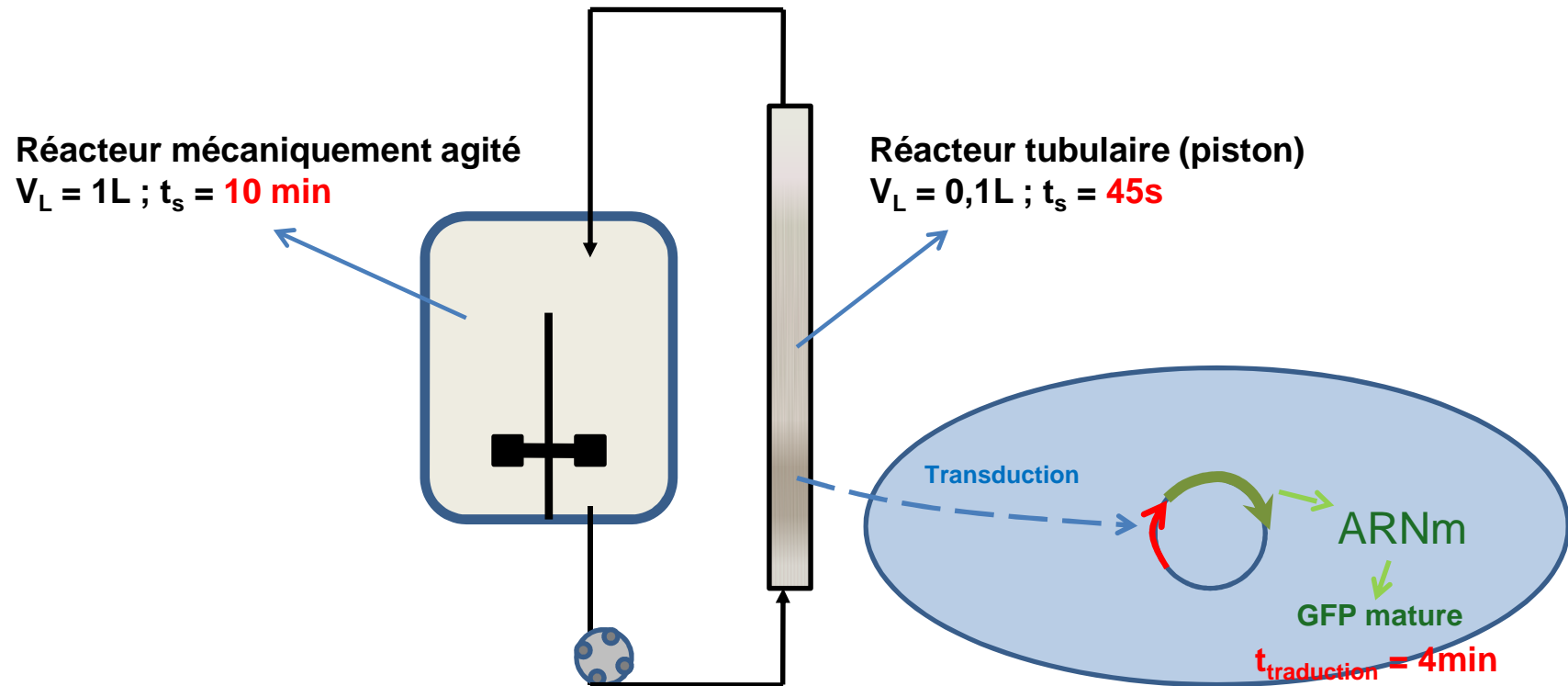
À l'échelle de la population



Ecole thématique CNRS

Comparaison des temps caractéristiques

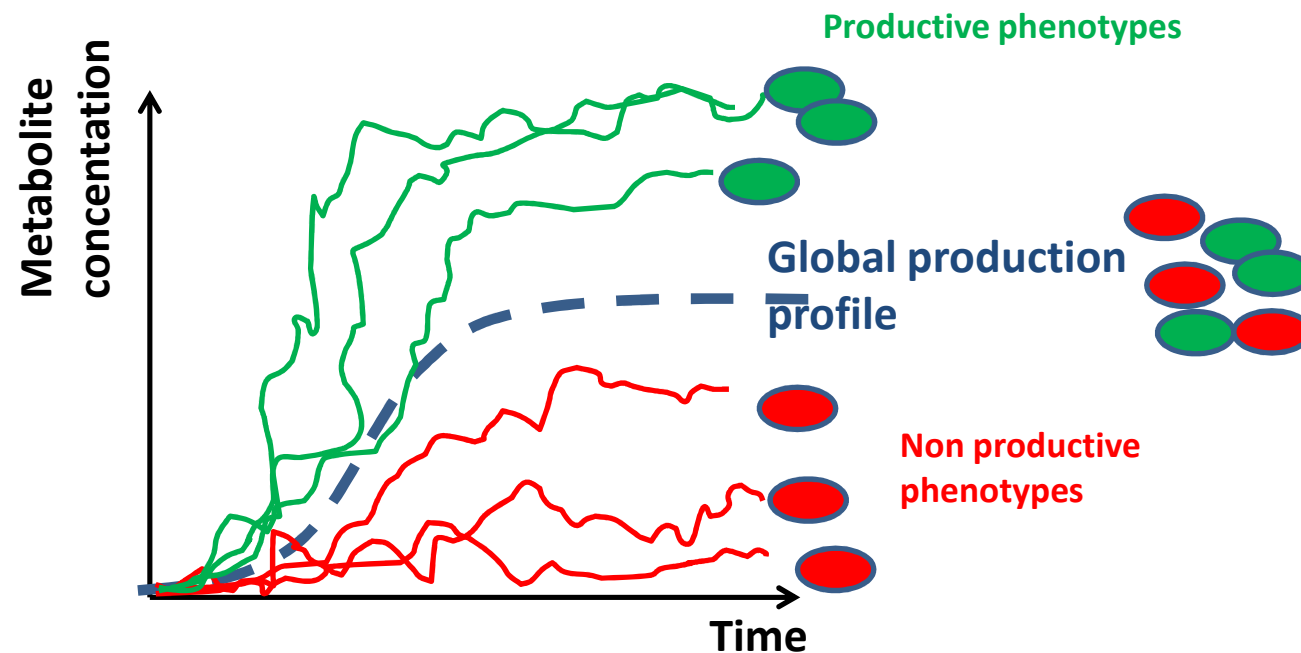
Réacteur *scale-down* configuré de manière à reproduire l'exposition des cellules à un excès local en glucose (temps d'exposition relativement court : 45 s)



4.1. Microbial phenotypic heterogeneity, two fields, two views

Impact of microbial phenotypic heterogeneity on process productivity

Accepted picture : only a fraction of the population (non productive phenotypes) affects the global production profile



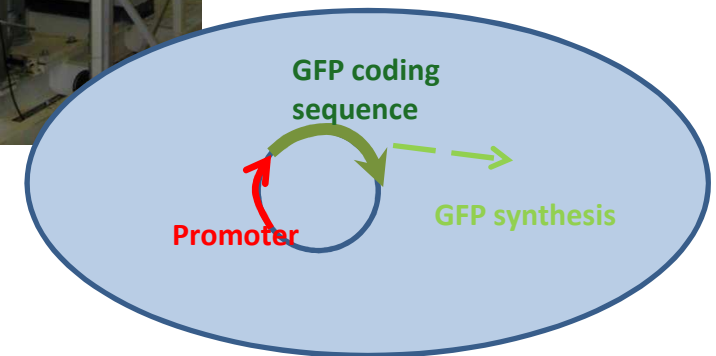
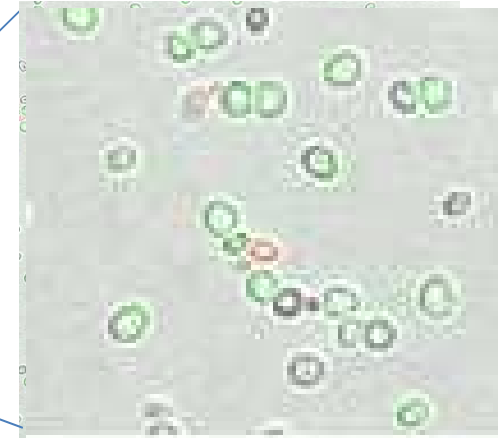
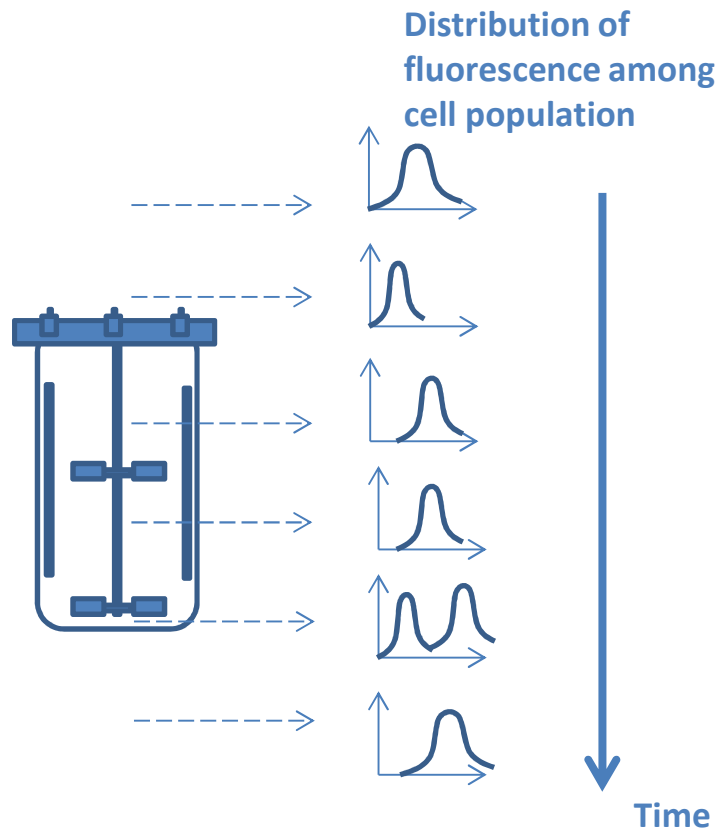
Single cell studies are of great importance both from a fundamental and from an applied perspective:

Two fields with two distinct perceptions of microbial phenotypic heterogeneity :

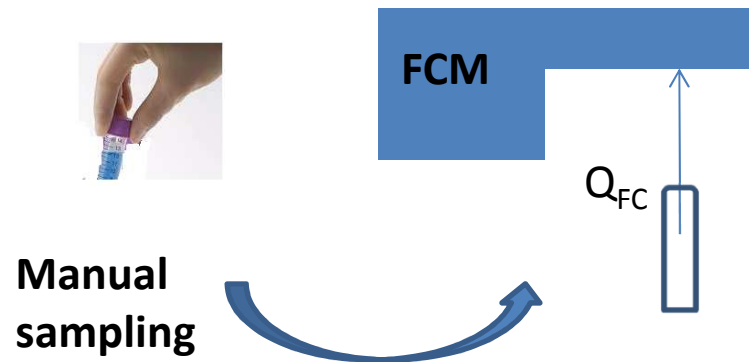
1. **Chemical engineering** : focused on the effect of external perturbations on biological noise
2. **System biology** : focused on the intrinsic and extrinsic source of noise

Each discipline integrates only a fraction of the knowledge and have evolved by considering distincts (cultivation and analytical) tools for the determination of microbial phenotypic heterogeneity

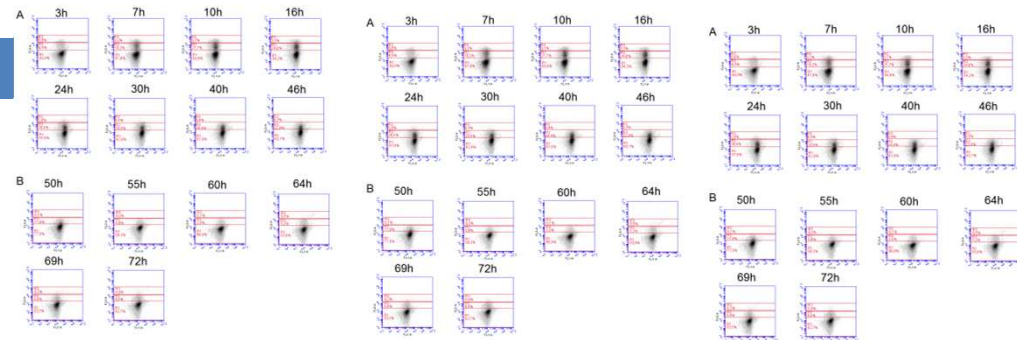
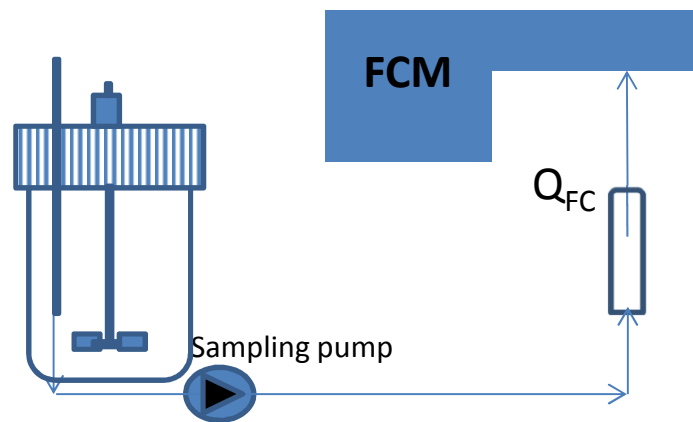
(Bio)chemical engineering approach :



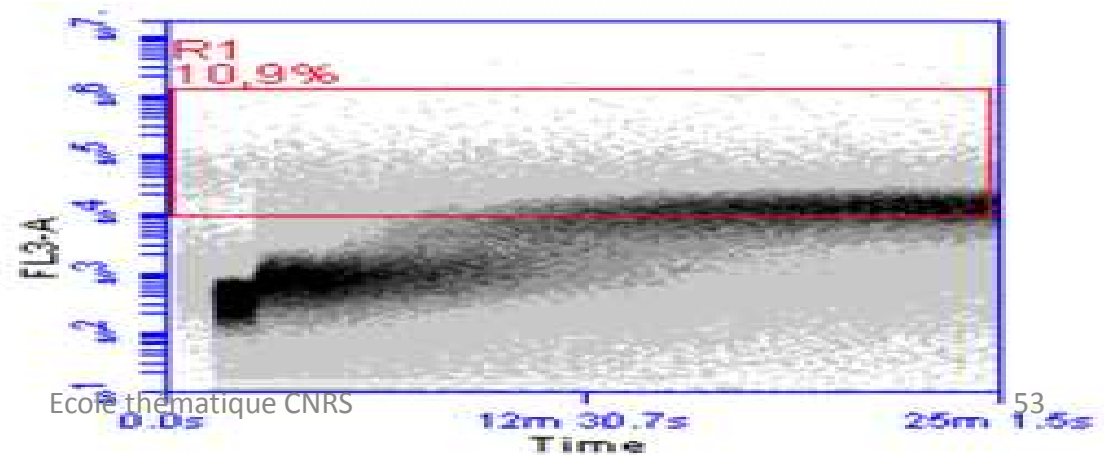
Flow cytometry (FCM) : different modes of operation



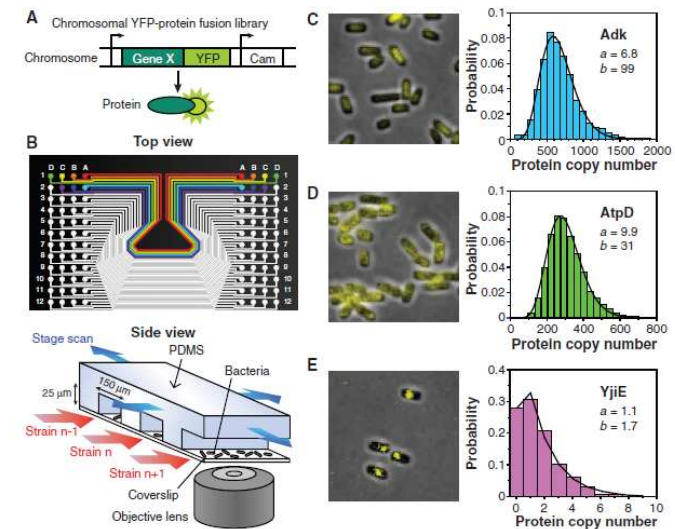
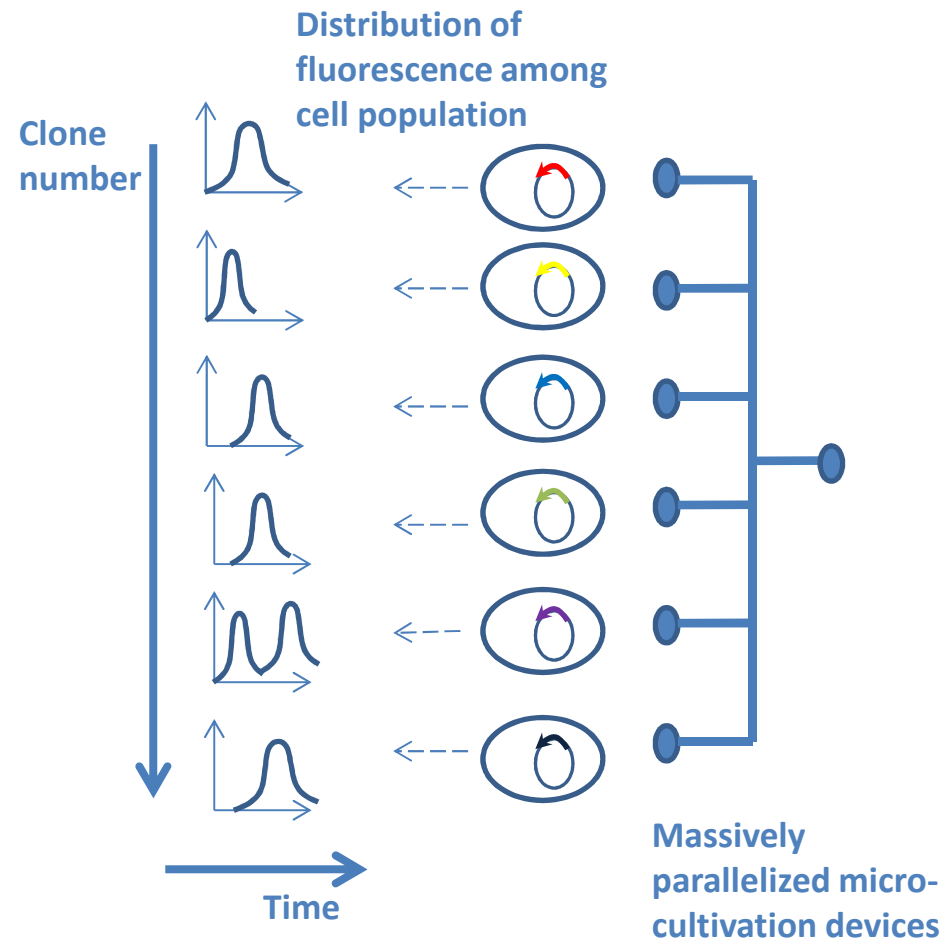
On-line



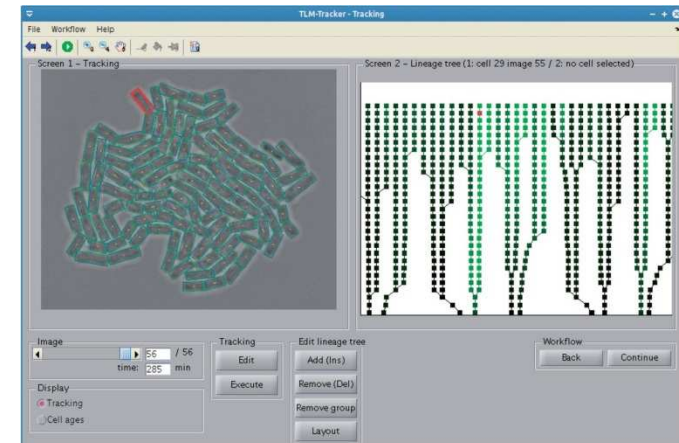
Real time



System biology approach :



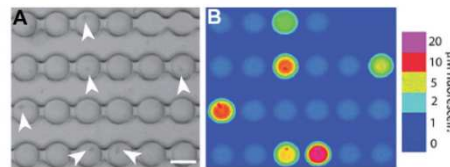
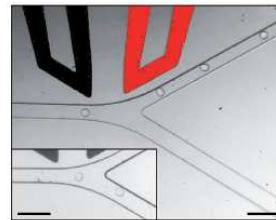
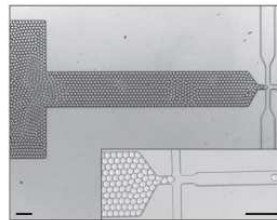
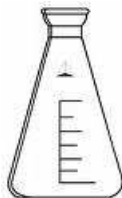
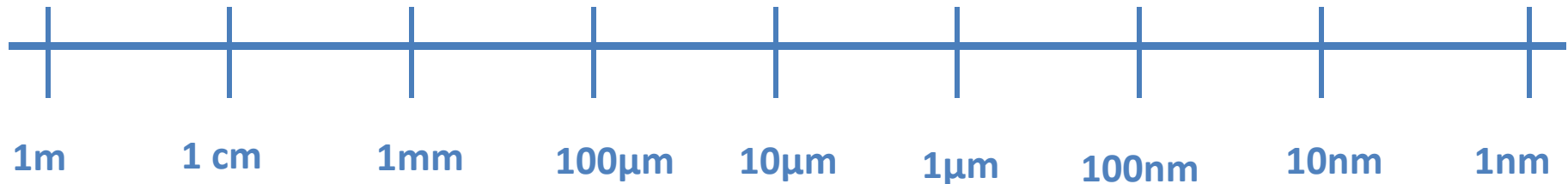
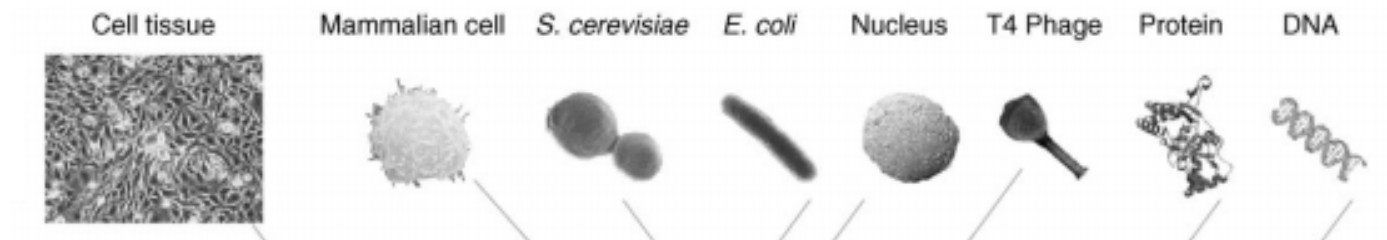
Taniguchi Y, Choi PJ, Li GW, Chen H, Babu M, Hearn J, Emili A and Xie XS, Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in single cells. *Science* **329**: 533-538 (2010).



Klein J, Leupold S, Biegler I, Biedendieck R, Munch R and Jahn D, TLM-Tracker: software for cell segmentation, tracking and lineage analysis in time-lapse microscopy movies. *Bioinformatics* **28**: 2276-2277 (2012).

Inter-scale comparison : biology vs chemical engineering

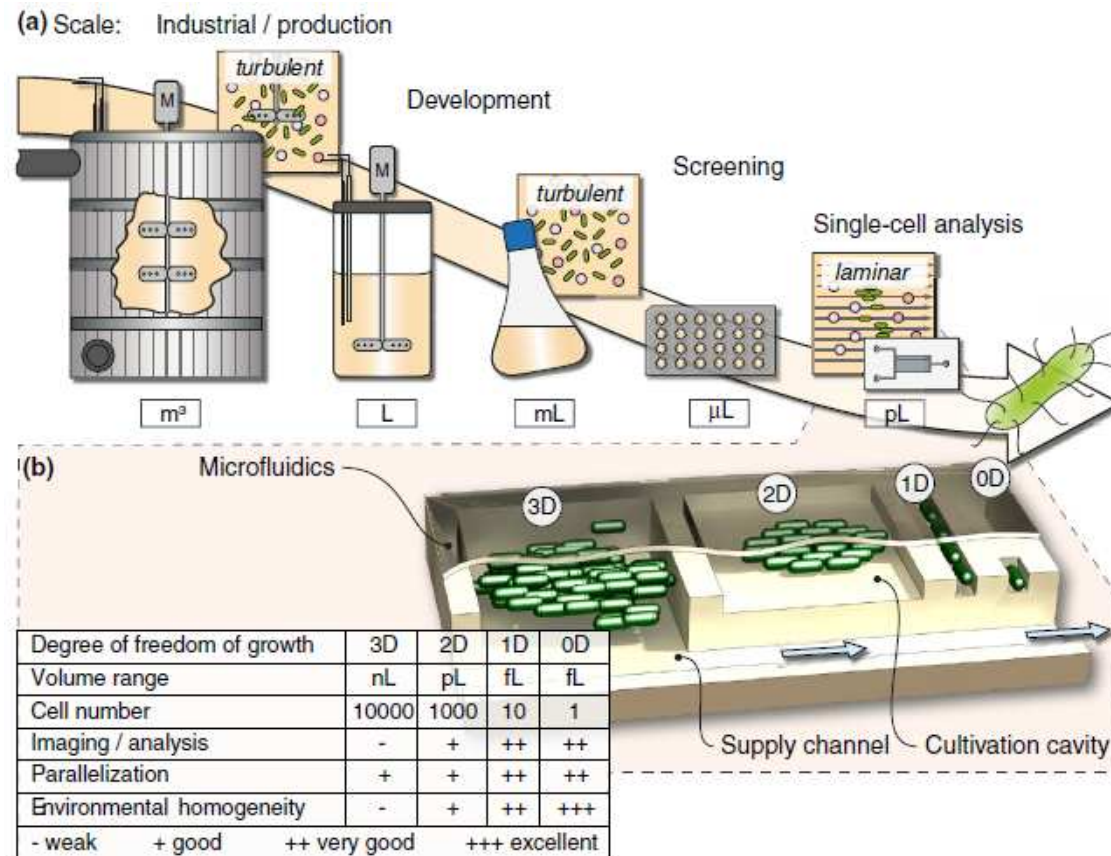
Biological systems...



... cultivation devices



Integrating microfluidics (and single cell microfluidics) in the scale-up/down loop ?



Grunberger A, Wiechert, W., Kohlheyer, D., Single-cell microfluidics: opportunity for bioprocess development. *Current opinion in biotechnology* **29**: 15-23 (2014).

BUT, flow regime is not the only difference between microfluidic-based micro-bioreactors and large-scale bioreactors

Some examples reported from the litterature :

- Cell density effect (Quorum sensing)

Boedicker J, Vincent, ME, Ismagilov, RF., Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. *Angewandte chemie* 48: 5908-5911 (2009)

- Competition for metabolically efficient phenotypes

Bachmann H, Fischlechner, M, Rabbers, I, Barfa, N, Branco dos Santos, F, Molenaar, D, Teusink, B.,, Availability of public goods shapes the evolution of competing metabolic strategies. *Proc Natl Acad Sci U S A* 110: 14302-14307 (2013)

- Differences in growth rate

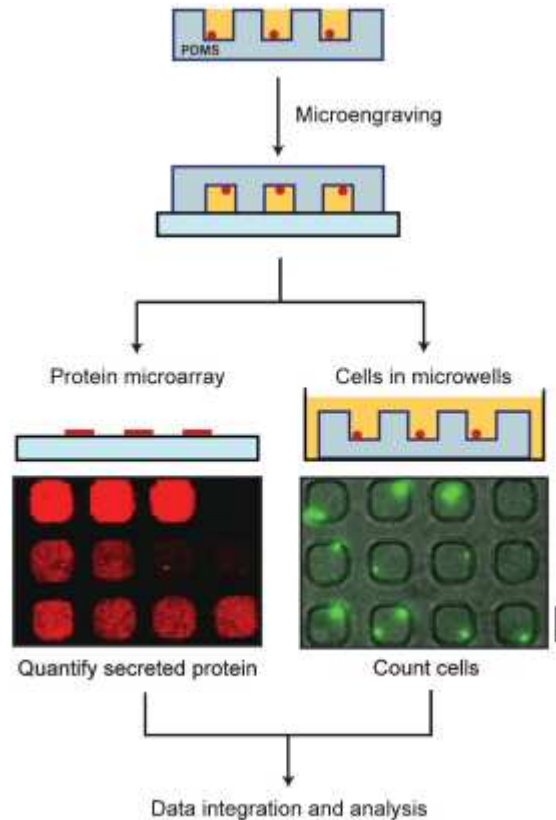
Dusny C, Fritzsche, F.S.O., Frick, O., Schmid, A., Isolated Microbial Single Cells and Resulting Micropopulations Grow Faster in Controlled Environments. *Applied and environmental microbiology* 78: 7132-7136 (2012).

- Transition to « solid-culture » phenotypes (biofilm, flocculation)

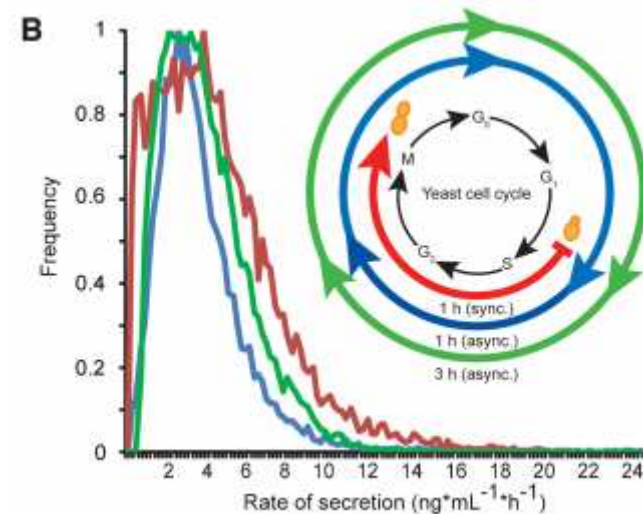
Kortmann H, Blank, LM, Schmid, A.,, Single cell analysis reveals unexpected growth phenotype of *S. cerevisiae*. *Cytometry Part A* 75: 130-139 (2009).

- ...

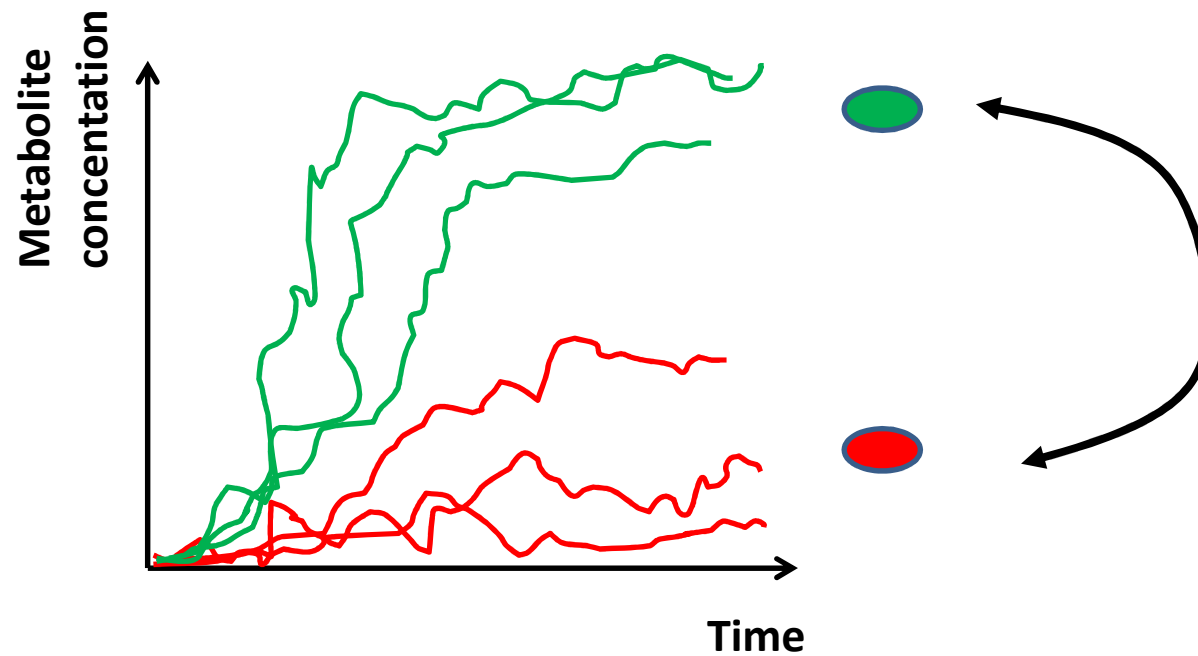
But gives useful information at the level of history dependent mechanisms



Recombinant protein secretion is cell-cycle dependent (switch between producer/non producer state)



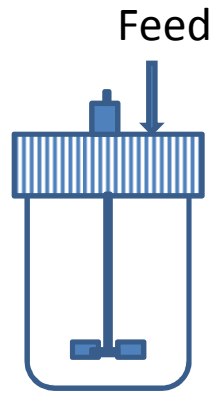
Love K, Panagiotou, V, Jiang, B, Stadheim, TA, Love, JC., Integrated single-cell analysis shows *Pichia pastoris* secretes protein stochastically. *Biotechnology and bioengineering* **106**: 319-325 (2010)



Impossible to point out this phenomenon in process conditions (history independent techniques)

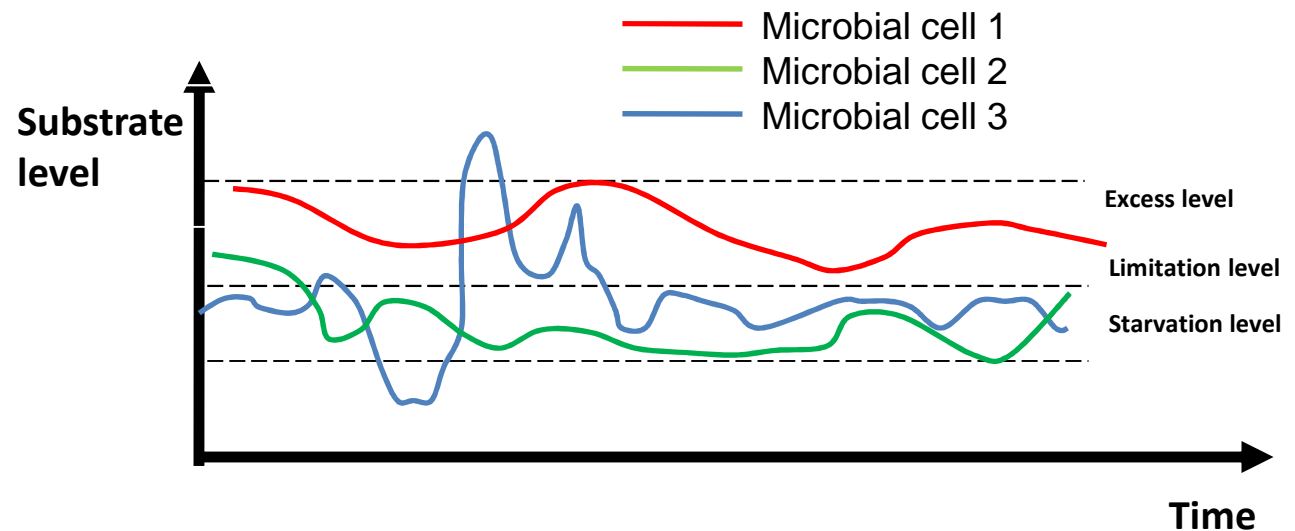
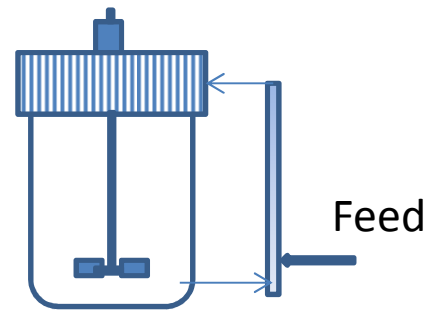
4.2. Single cell analysis in process conditions : *rpos* system

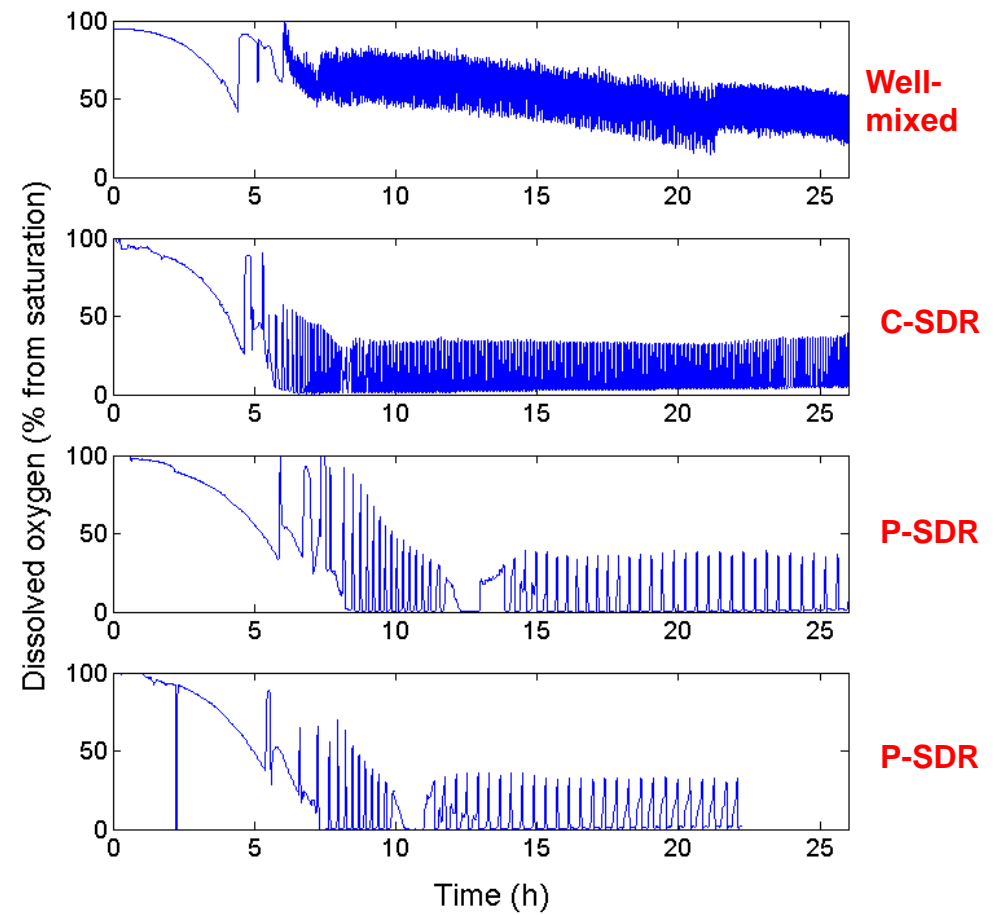
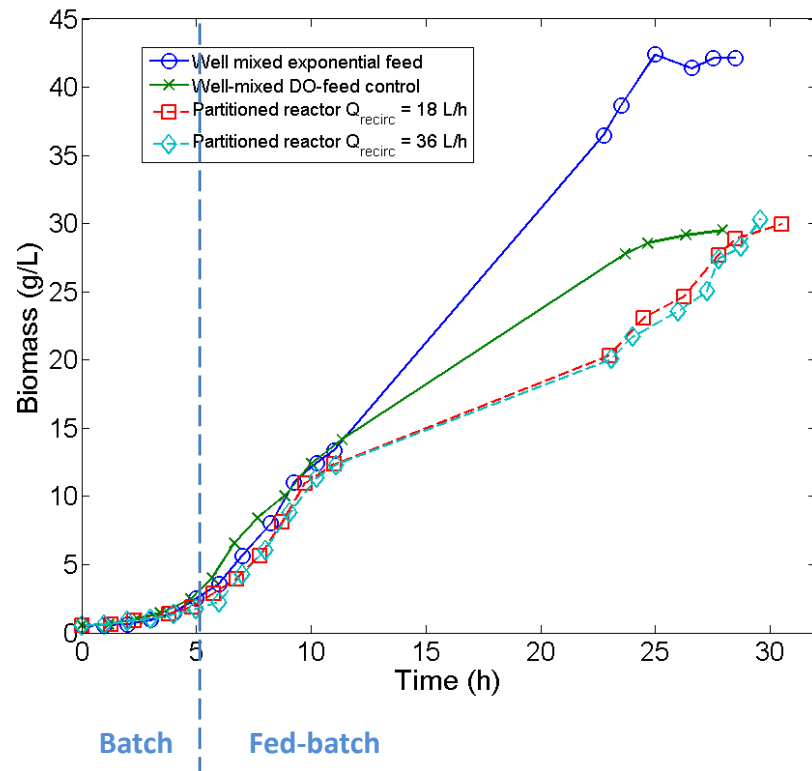
Well-mixed



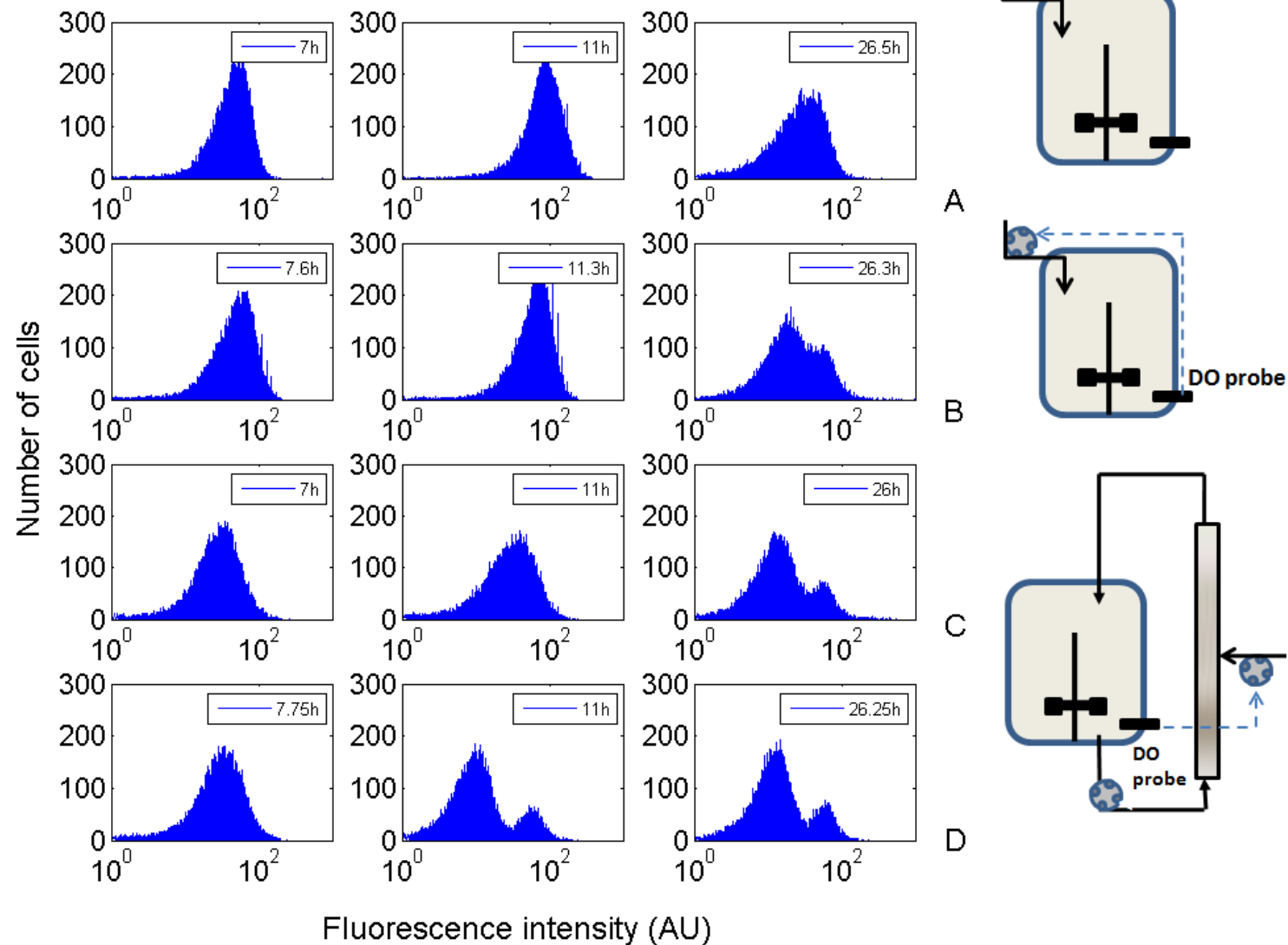
vs

Scale-down





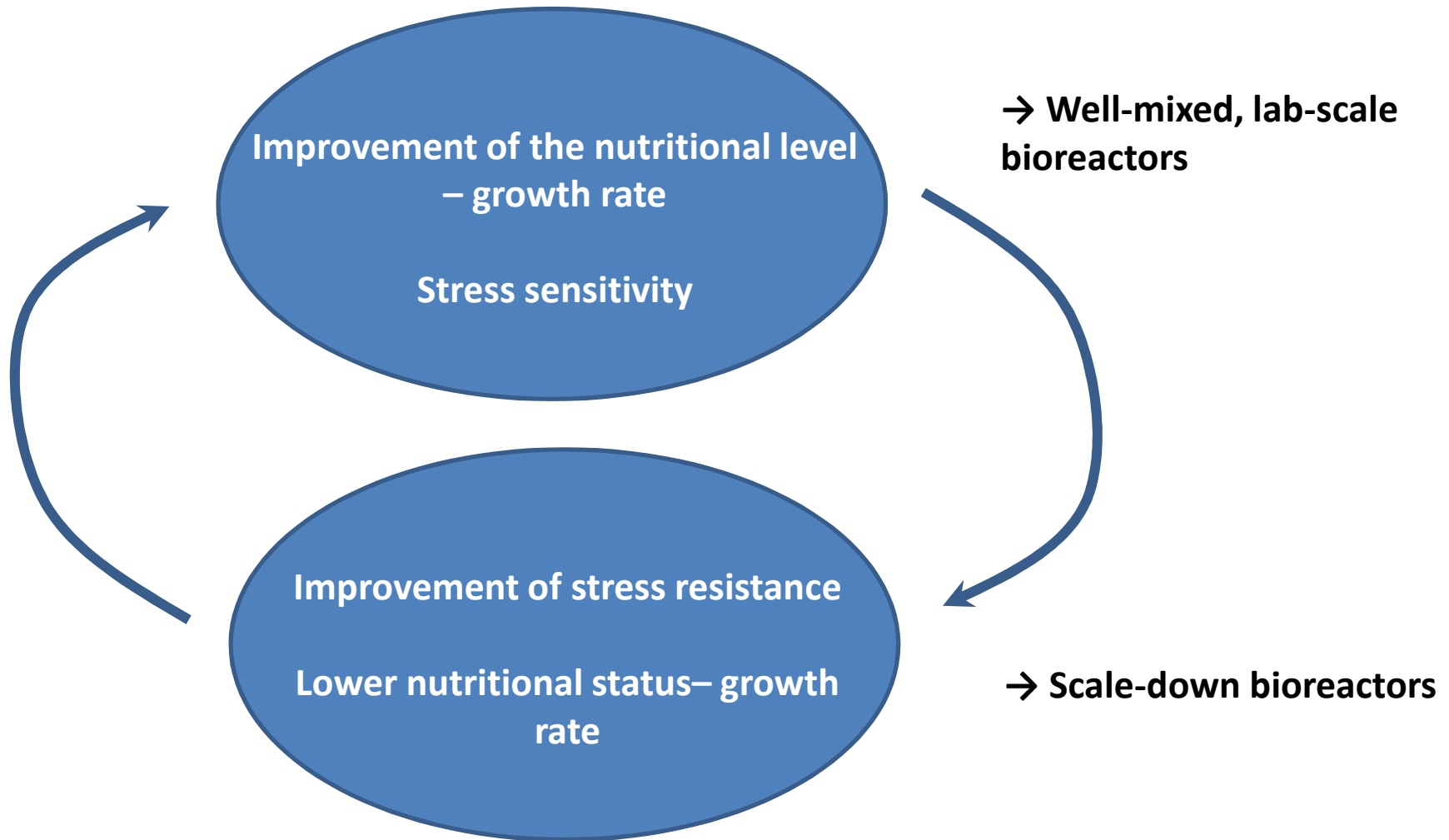
prpoS::gfpmut2



Delvigne F, Boxus, M., Ingels, S., Thonart, P., Bioreactor mixing efficiency modulates the activity of a *prpoS::GFP* reporter gene in *E. coli*. *Microbial cell factories* 8: 15 (2009)

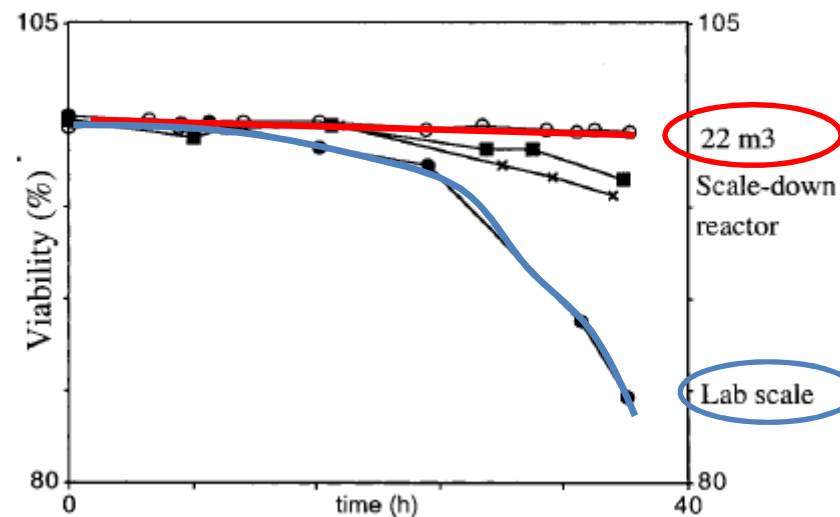
Link between mixing quality and population segregation at the level of the *rpoS* response :

Trade-off effect - microbial cells have to choose between :



4.3. Single cell analysis in process conditions : membrane permeability

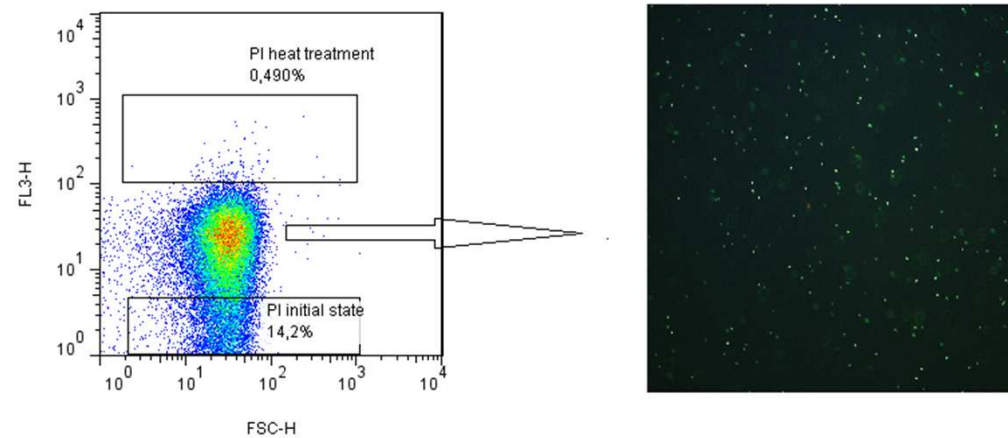
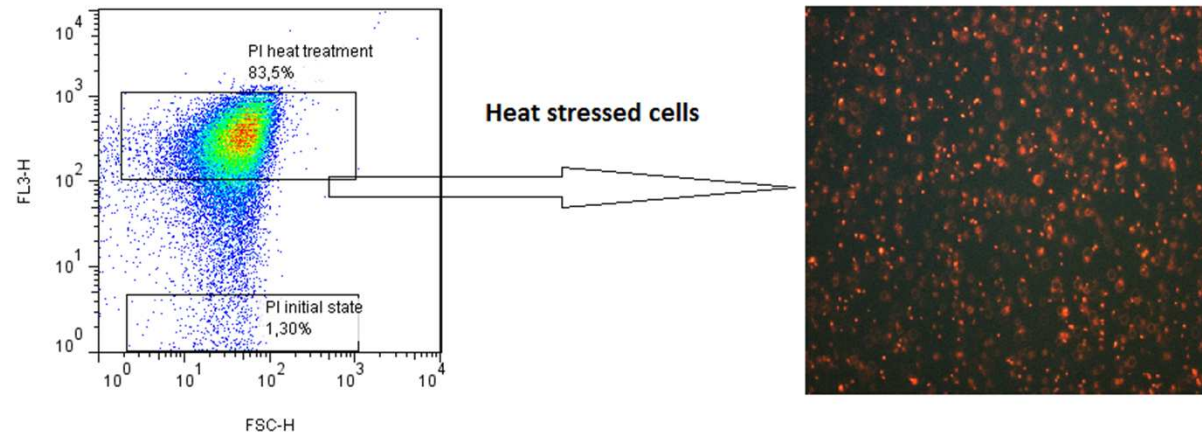
Extracellular perturbations increases cell viability



Enfors SO, *et al.* Physiological responses to mixing in large scale bioreactors. *Journal of biotechnology* **85**: 175-185 (2001)

Analysis performed by flow cytometry with propidium iodide exclusion test and thus related to cell membrane permeability

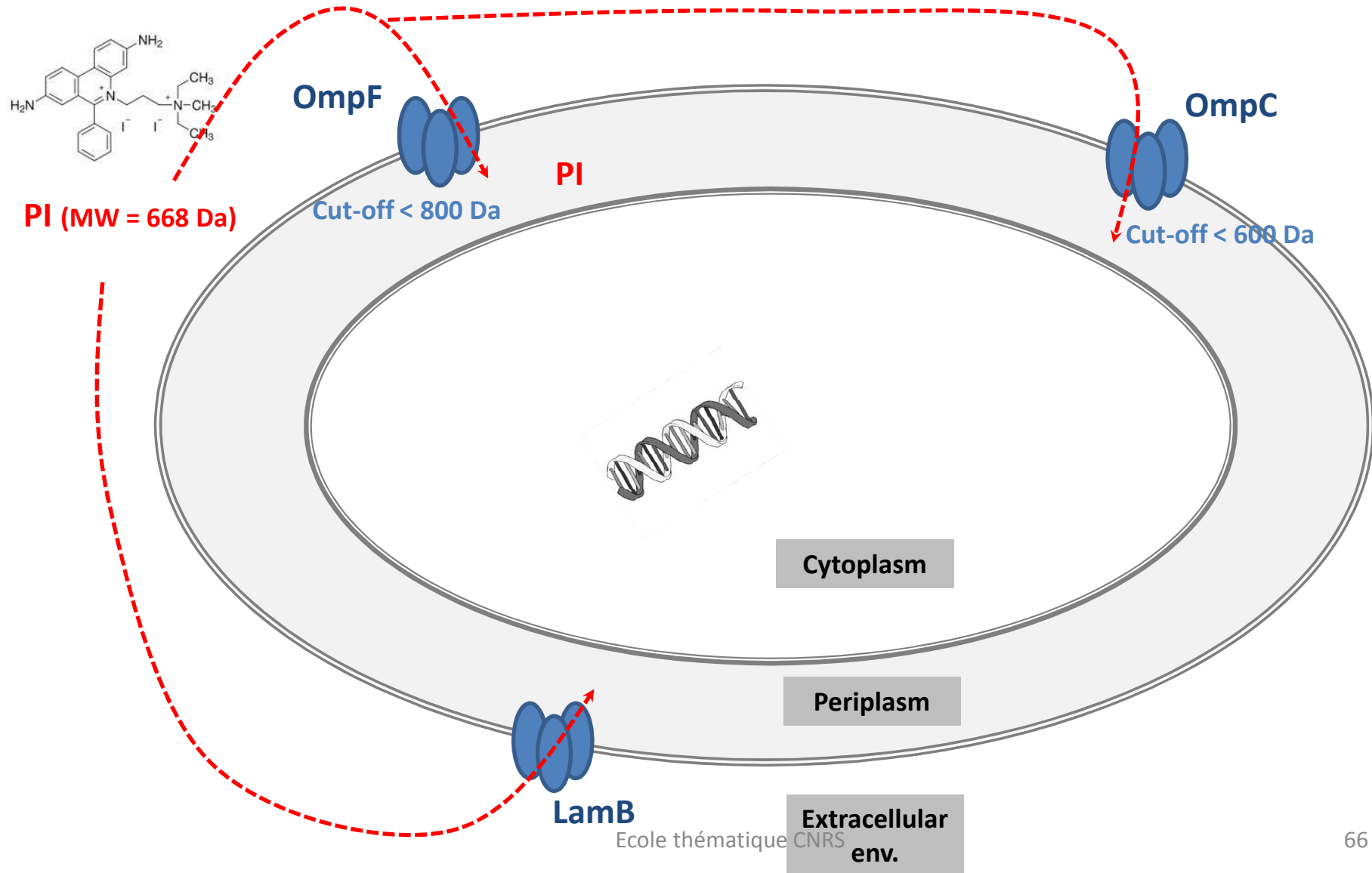
Intermediate PI uptake :



Fed-batch, well-mixed reactor

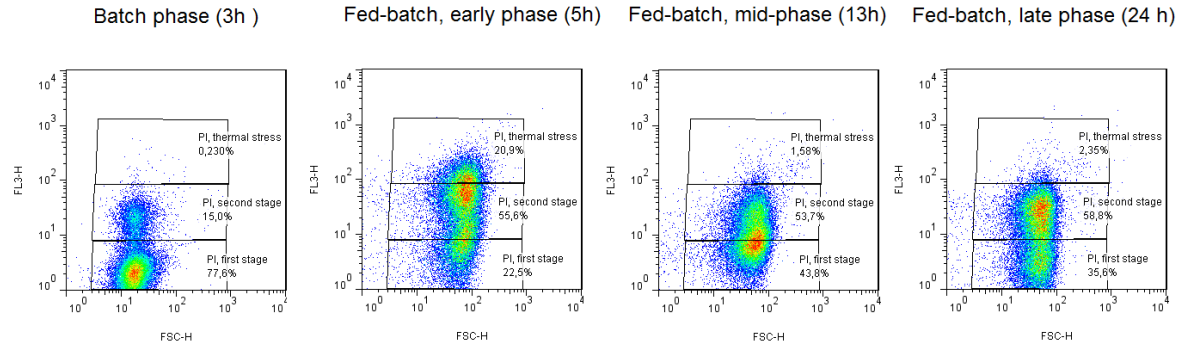
Propidium iodide (PI) uptake and appearance of an intermediate subpopulation with reduced red fluorescence

Hypothesis : accumulation of PI in the periplasm when cells are exposed to substrate limitation (increased expression of porins)



PI exclusion test

Ref. bioreactor

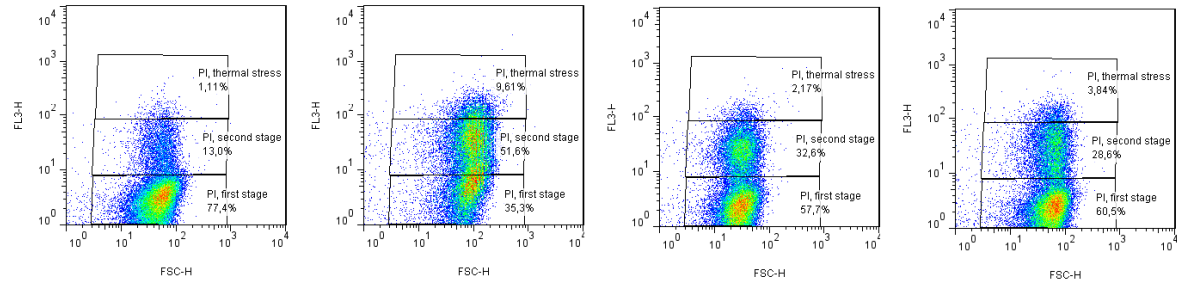


A

SDR bioreactor



$t_R = 38s$

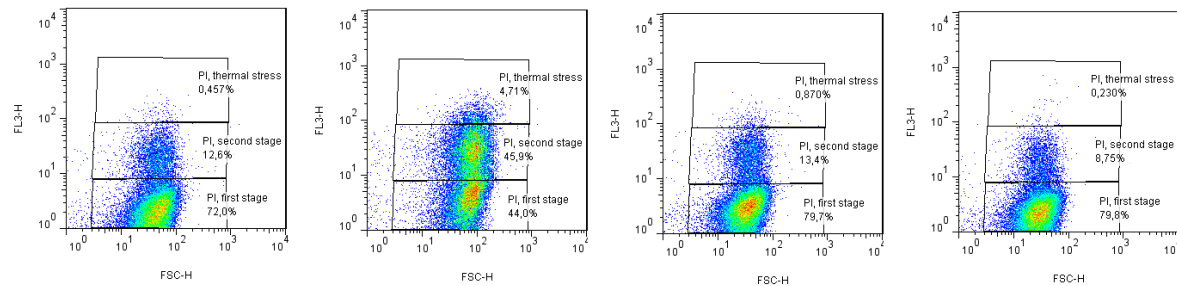


B

SDR bioreactor



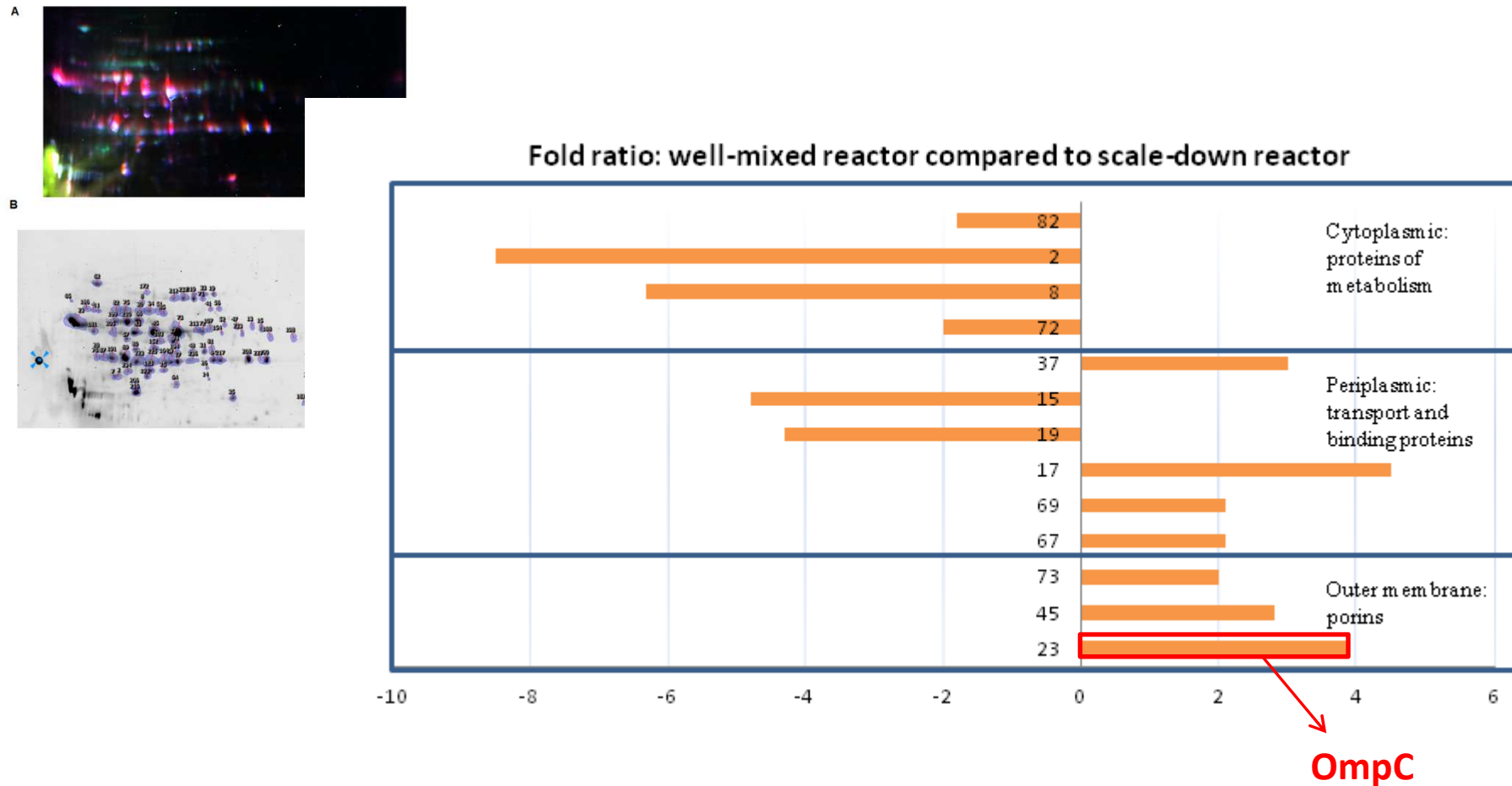
$t_R = 79s$



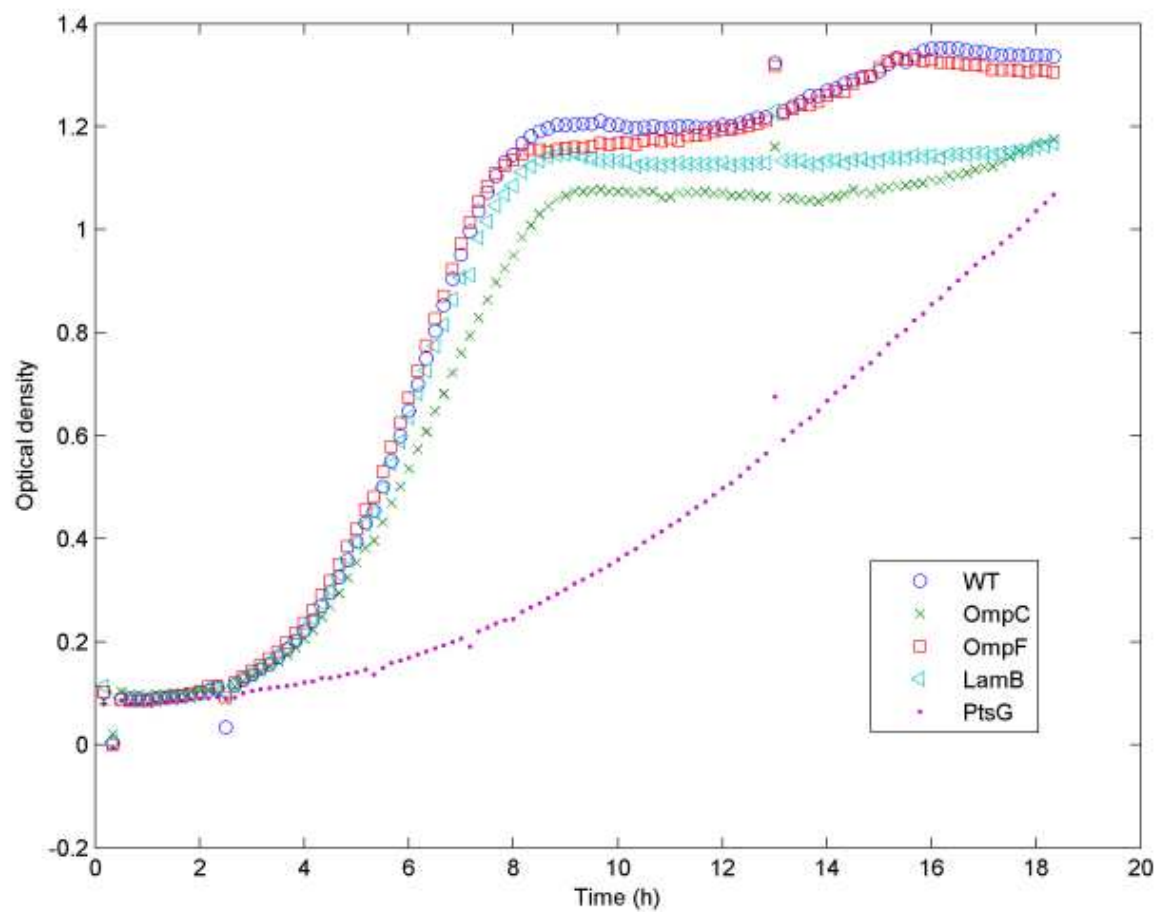
C

Extracellular proteome analysis of the impact of bioreactor performances

Well-mixed/scale-down comparative analysis : 2-D differential in-gel electrophoresis

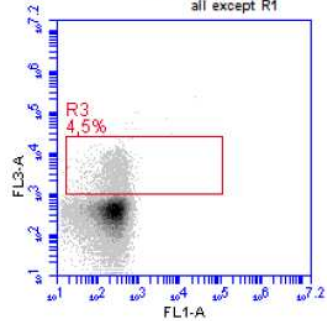


Brognaux A, Francis F, Twizere JC, Thonart P and Delvigne F, Scale-down effect on the extracellular proteome of *Escherichia coli*: correlation with membrane permeability and modulation according to substrate heterogeneities. *Bioprocess Biosyst Eng* **14**: 14 (2014)



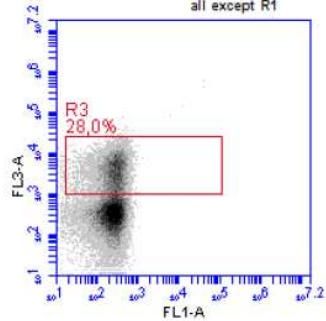
WT

Plot 4: A01 wt 10g/L
all except R1



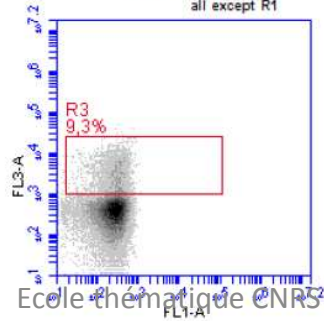
Δ ompC

Plot 4: A02 ompc 10gl
all except R1



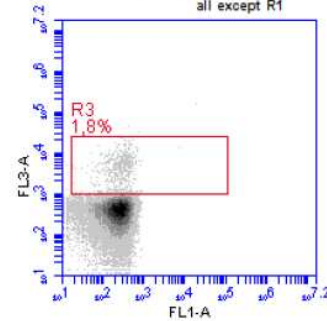
Δ ompF

Plot 4: A03 ompf 10gl
all except R1



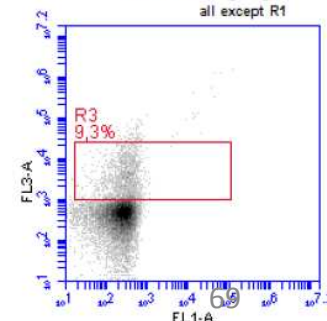
Δ lamB

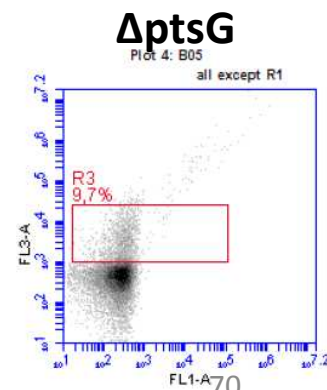
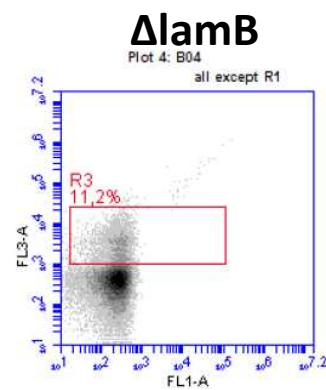
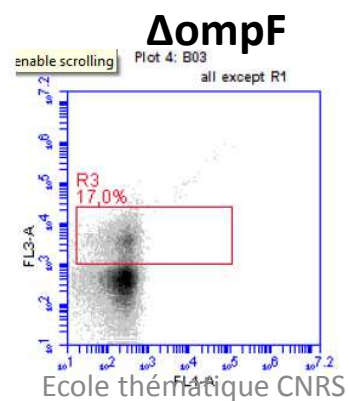
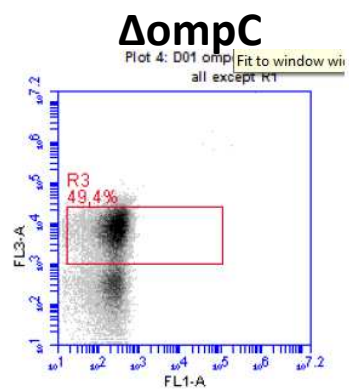
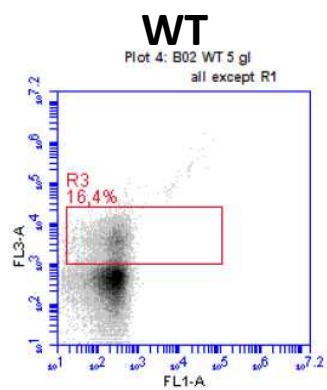
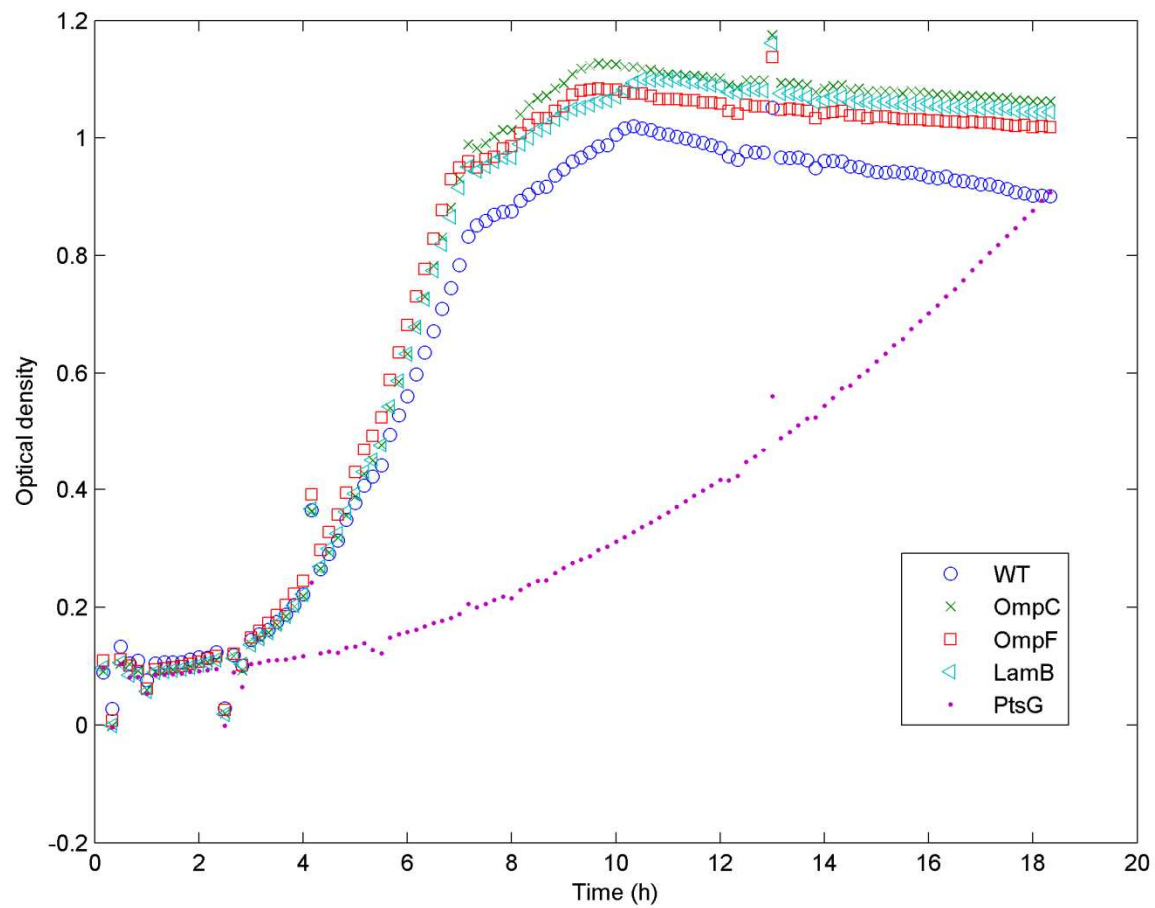
Plot 4: A04 lamb 10 gl
all except R1



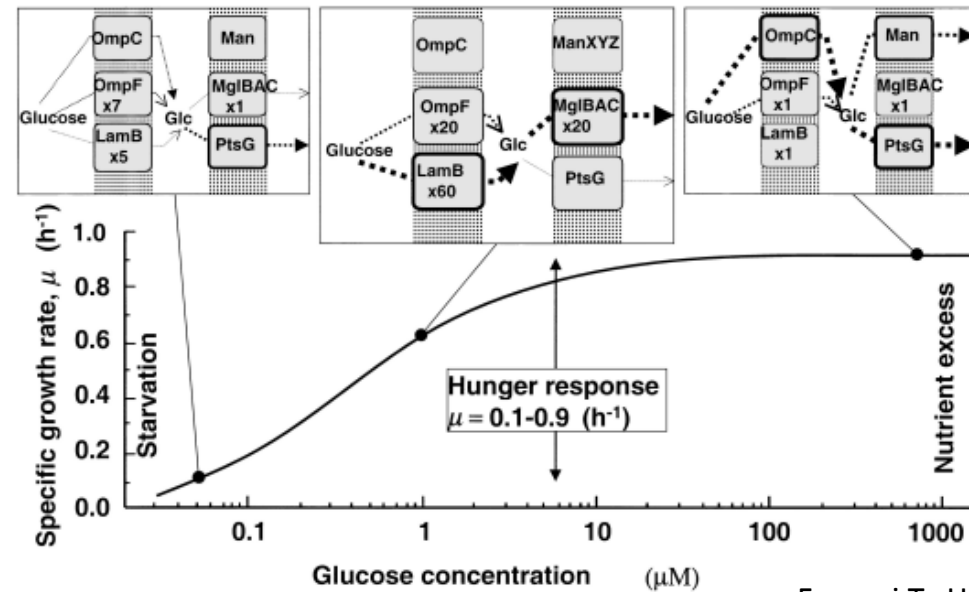
Δ ptsG

Plot 4: A05 ptsg 10 gl
all except R1



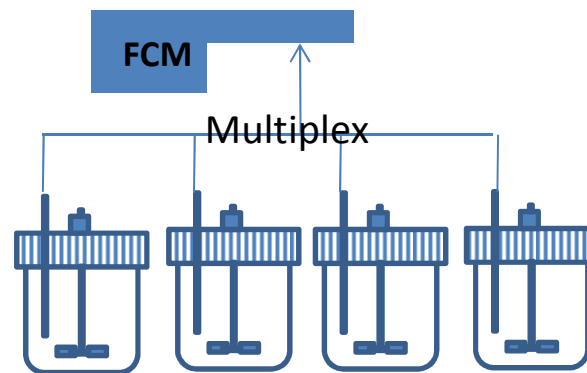


Interesting results but without any control at the level of the substrate limitation and growth (batch)

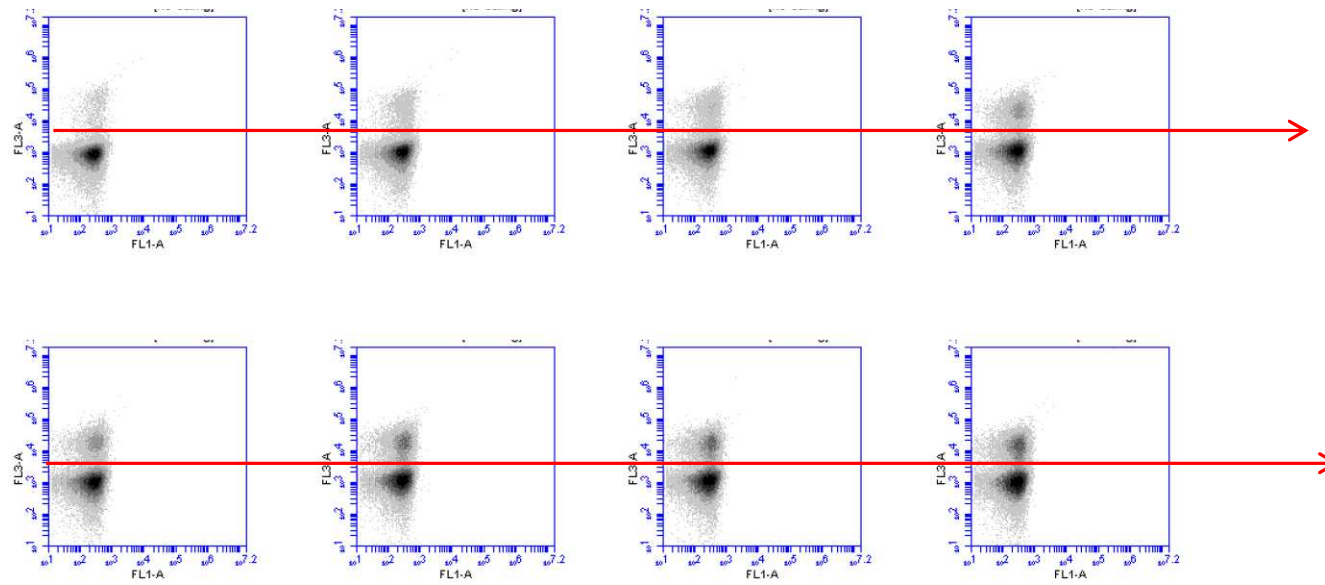


Validation is needed (chemostat)

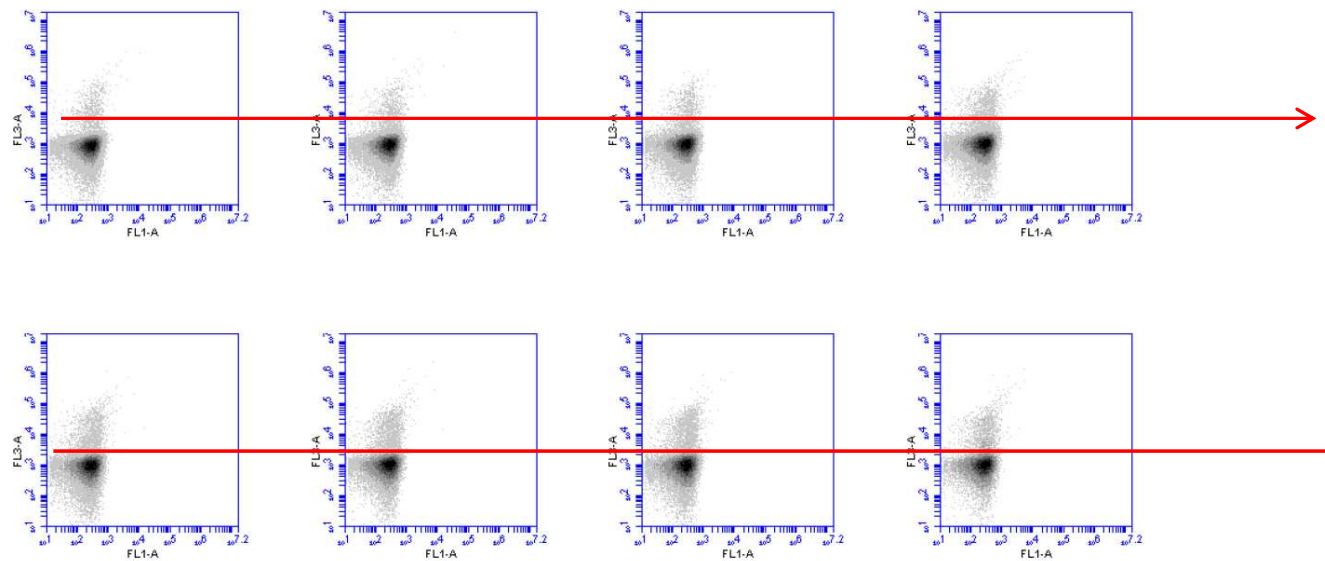
Ferenci T., Hungry bacteria - definition and properties of a nutritional state.
Environmental microbiology **3**: 605-611 (2001)



$\Delta ompC$



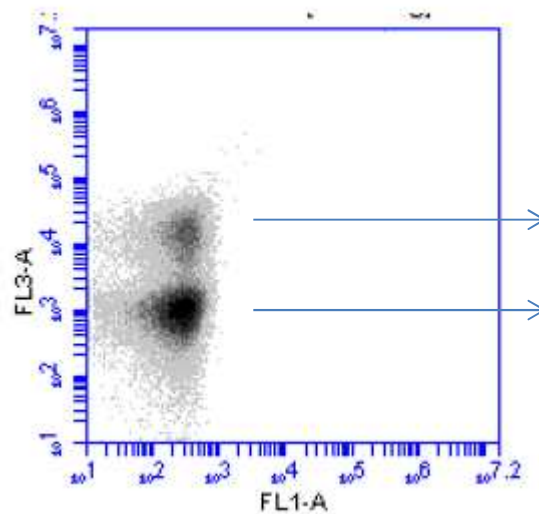
$\Delta lamB$



4.4. Further developments:

Limitation of the actual fluorescent reporter system : global view of physiology. There is a need to be closer to the metabolism :

- By appropriately choosing promoter sequences
- By using new generation of promoter independent fluorescent reporter
- Subpopulations omics



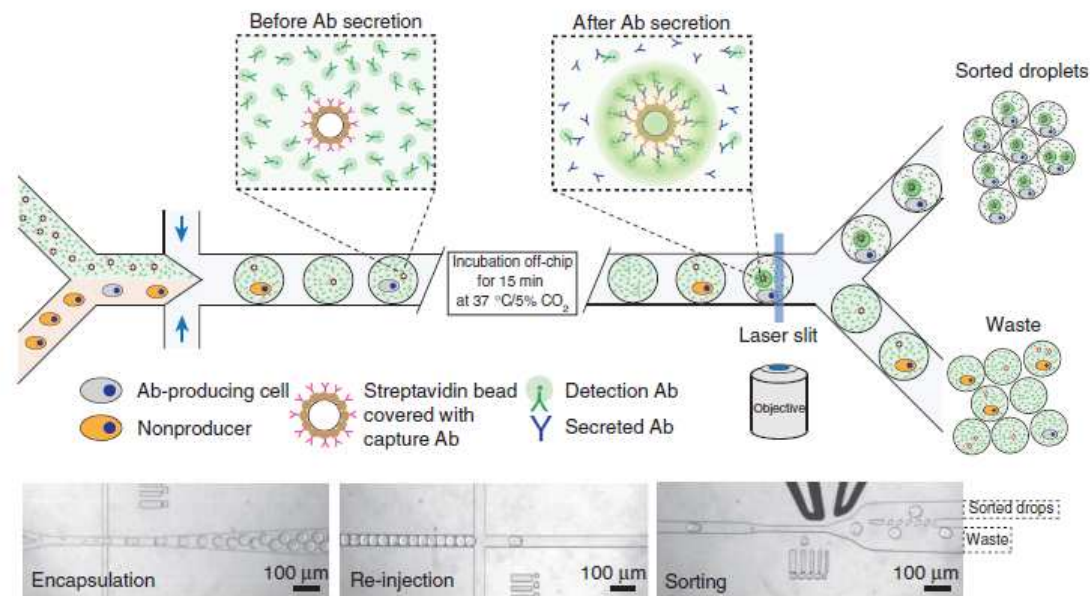
Subpopulations → Metabolically relevant ?
Switch between subpopulations ?

Challenges :

Going beyond the need of fluorescent tags : single cell omics technologies

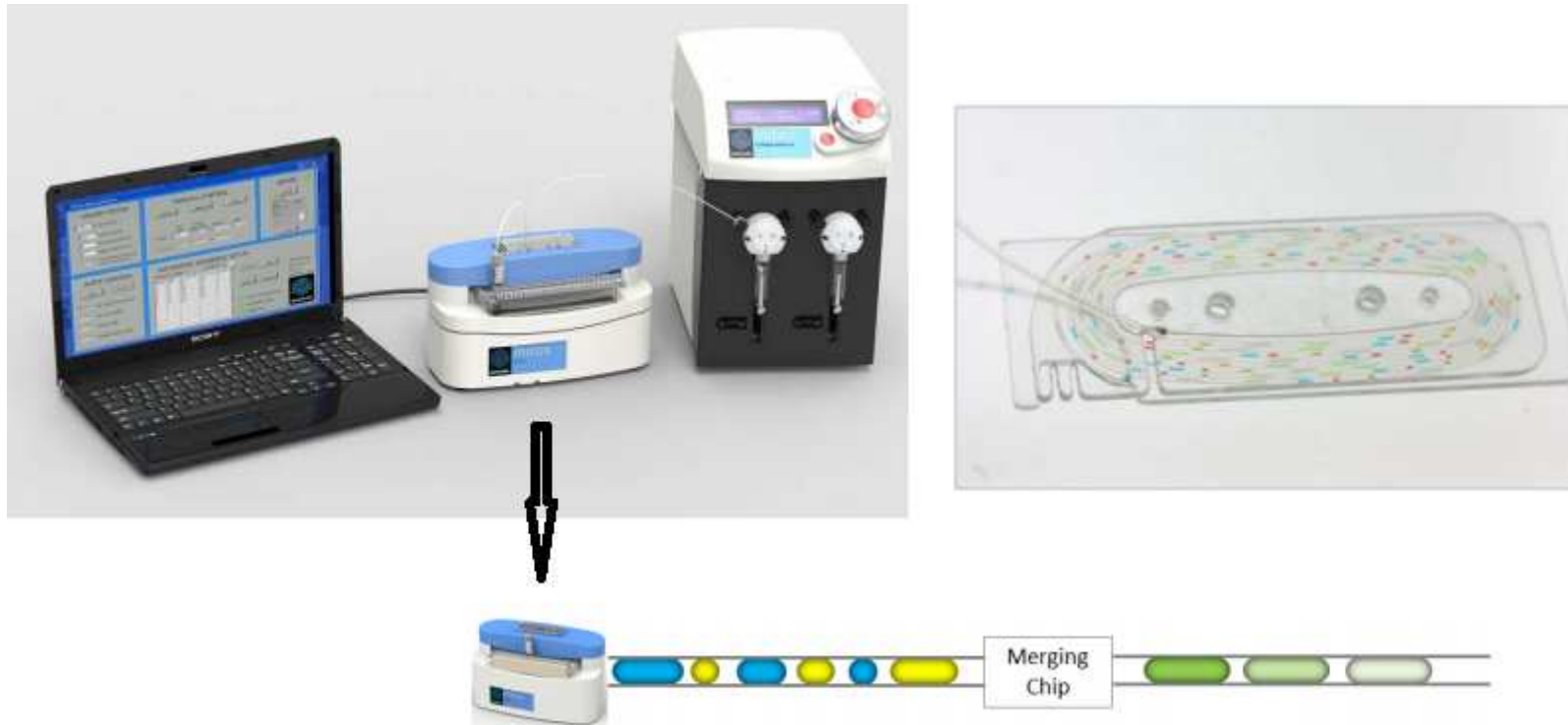
Metabolic engineering aiming at controlling microbial phenotypic heterogeneity

Insertion of microfluidics bioreactor in the scaling-up/down loop



Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD and Heyman JA, Single-cell analysis and sorting using droplet-based microfluidics. *Nat Protoc* **8**: 870-891 (2013)

Système intégré (Mitos dropix)



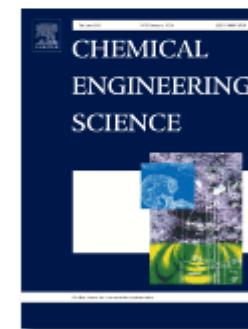
5. Conclusions

- Certaines prédictions effectuées sur base de concepts de génie des procédés peuvent s'avérer obsolètes vu la complexité de la réponse biologique
- Néanmoins, le génie des procédés est essentiel pour comprendre l'environnement extracellulaire dans lequel se développe la population microbienne

→ Besoin d'équipes multidisciplinaires comprenant des spécialistes de chaque domaine

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in collaboration with ACS
Lille, September 7-10, 2014, France**