Dr Friederike Hoffmann fhoffman@mpi-bremen.de
Max Planck Institute for Marine Microbiology, Celsiusstr. 1,
28359 Bremen, Germany

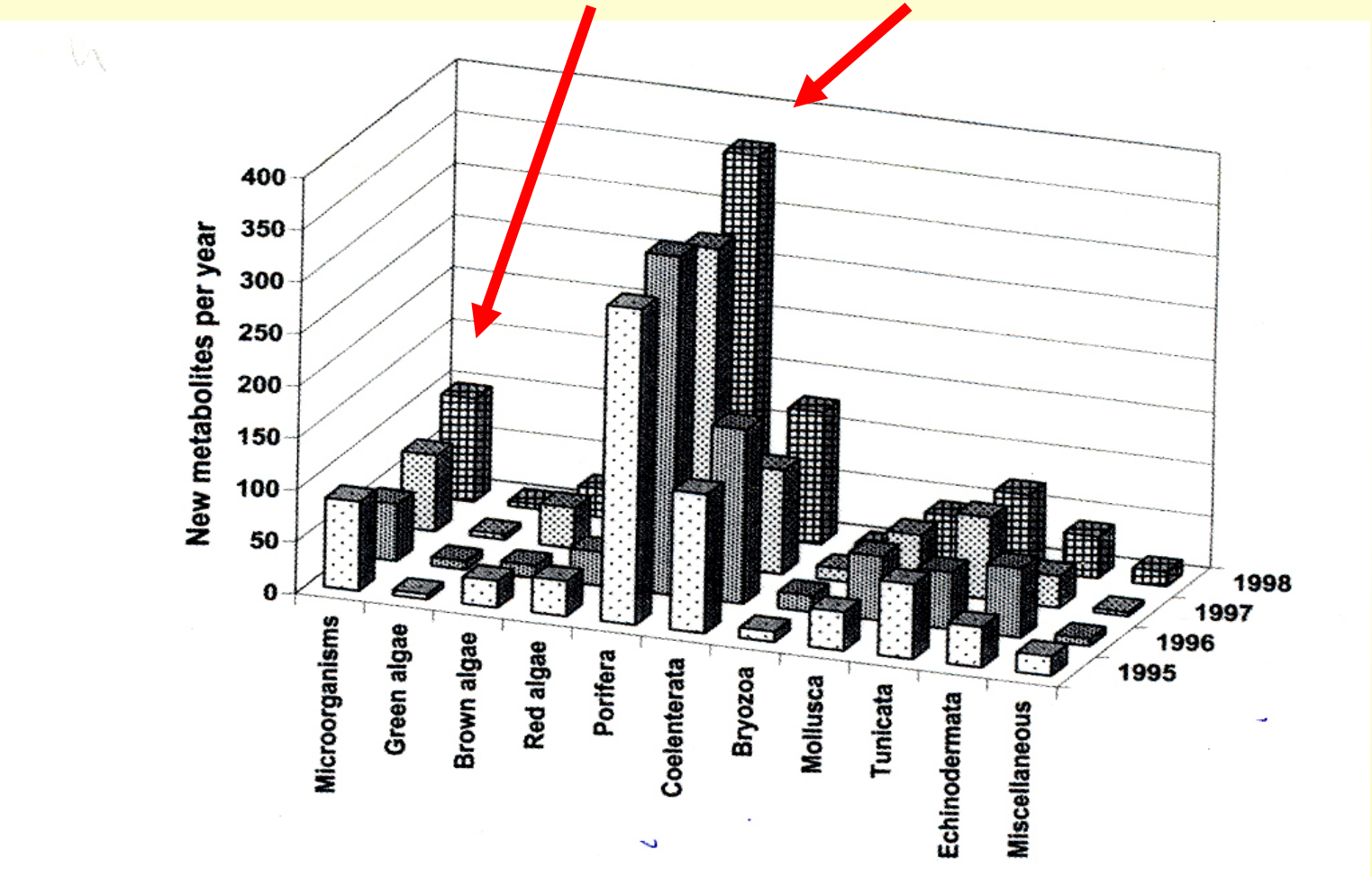
High density and high diversity of prokaryotes

Challenge and chance for chemical ecology

Focus on functional aspects

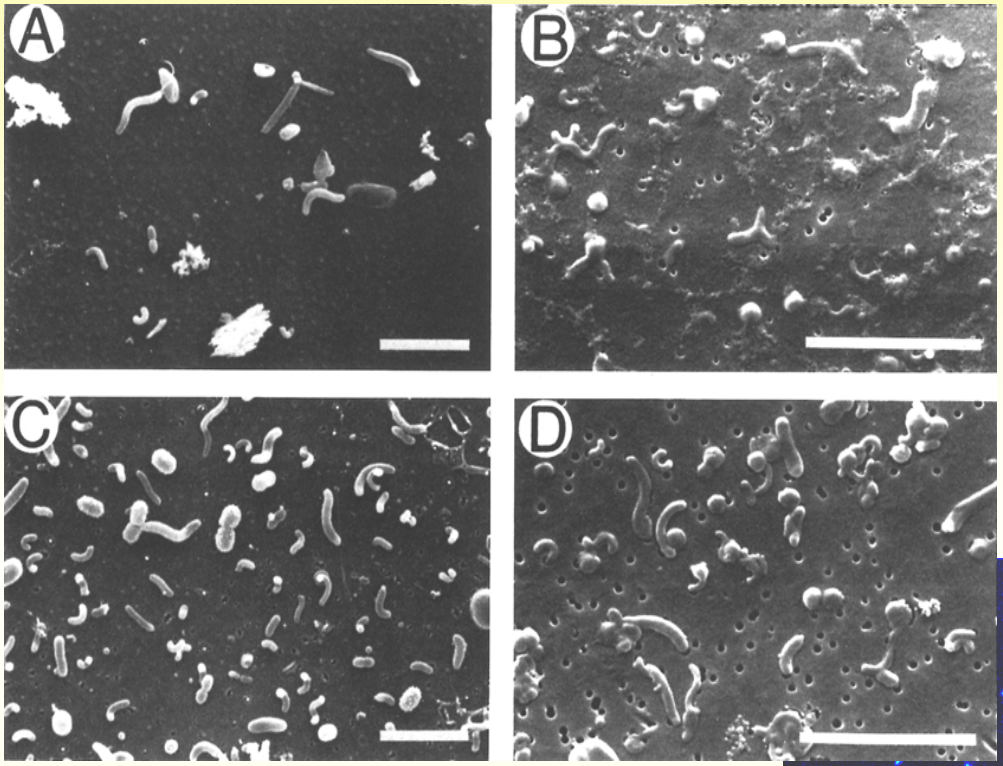
1. principles of cultivation and isolation
2. chemical communication between bacteria
3. considerations on a bioluminescent bioassay
4. cultivation-independent molecular studies
    - 4.1 Exploring diversity (who is out there?)
    - 4.2 Exploring function (what are they doing?)
5. porifera-microbe interactions

Main producers of marine natural products

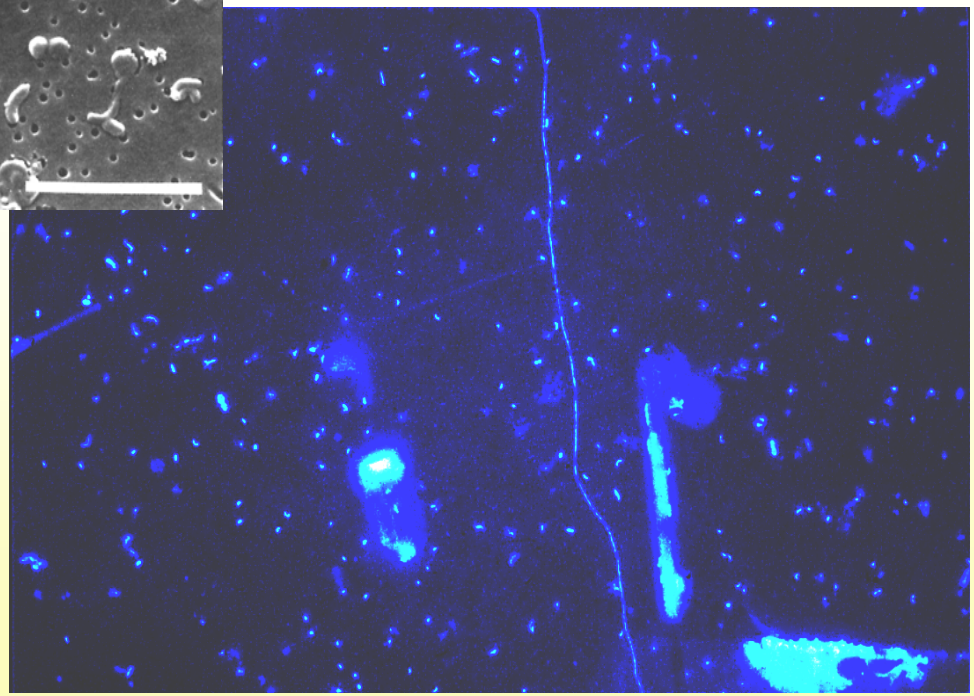


Nickel et al (2001) J Biotechnol 92: 169-178

4.1. Exploring diversity: Who is out there?



They have a restricted morphological diversity

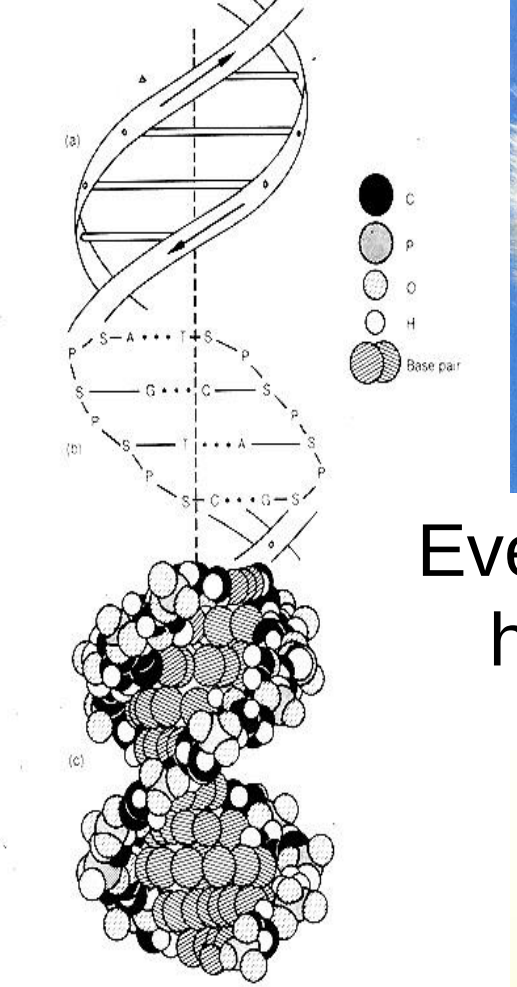


They do all look the same!

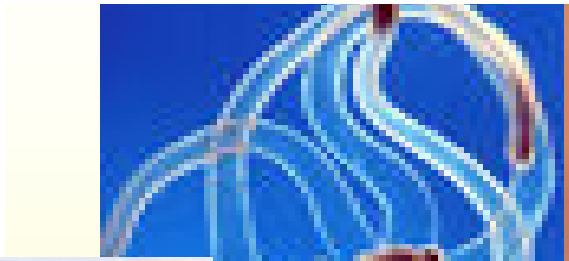
Many environmental bacteria are notoriously difficult to isolate....

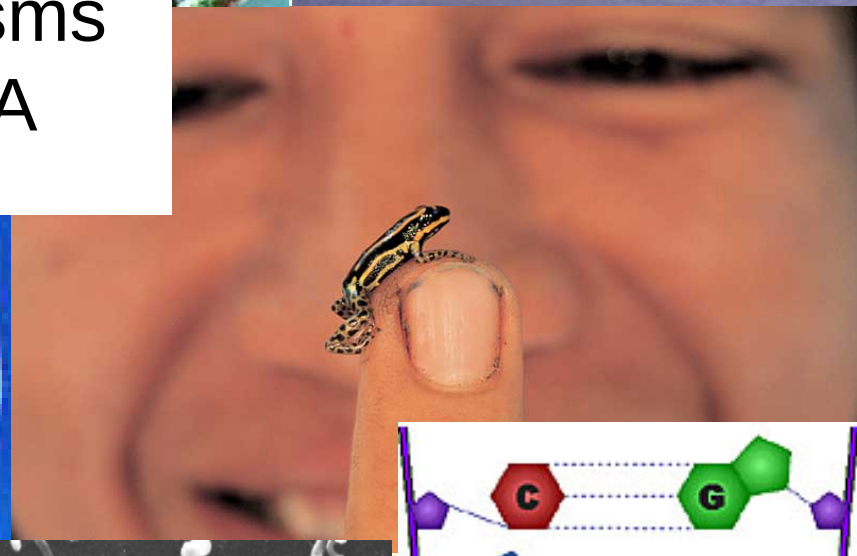
Habitat	Cultivation efficiency [%]	Reference
Marine water	0.001 to 0.1	Ferguson *et al.* 1984
Lake water	0.1 to 1	Staley & Konopka 1985
Estuary	0.1 to 3	Ferguson *et al.* 1984
Activated sludge	1 to 15	Wagner *et al.* 1993
Sediment	0.25	Jones 1977
Soil	0.3	Torsvik *et al.* 1990

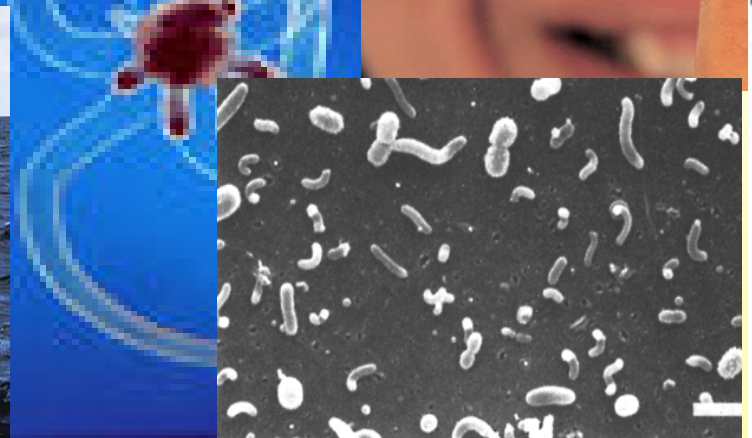
.... <1000 species of marine bacteria have been validly described!
Does that mean that marine microbial diversity is low?

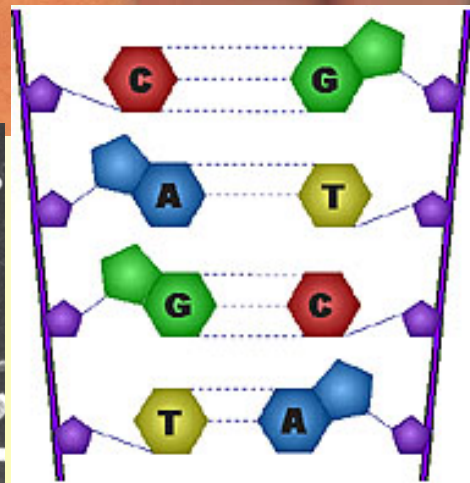


Every living organisms has a unique DNA

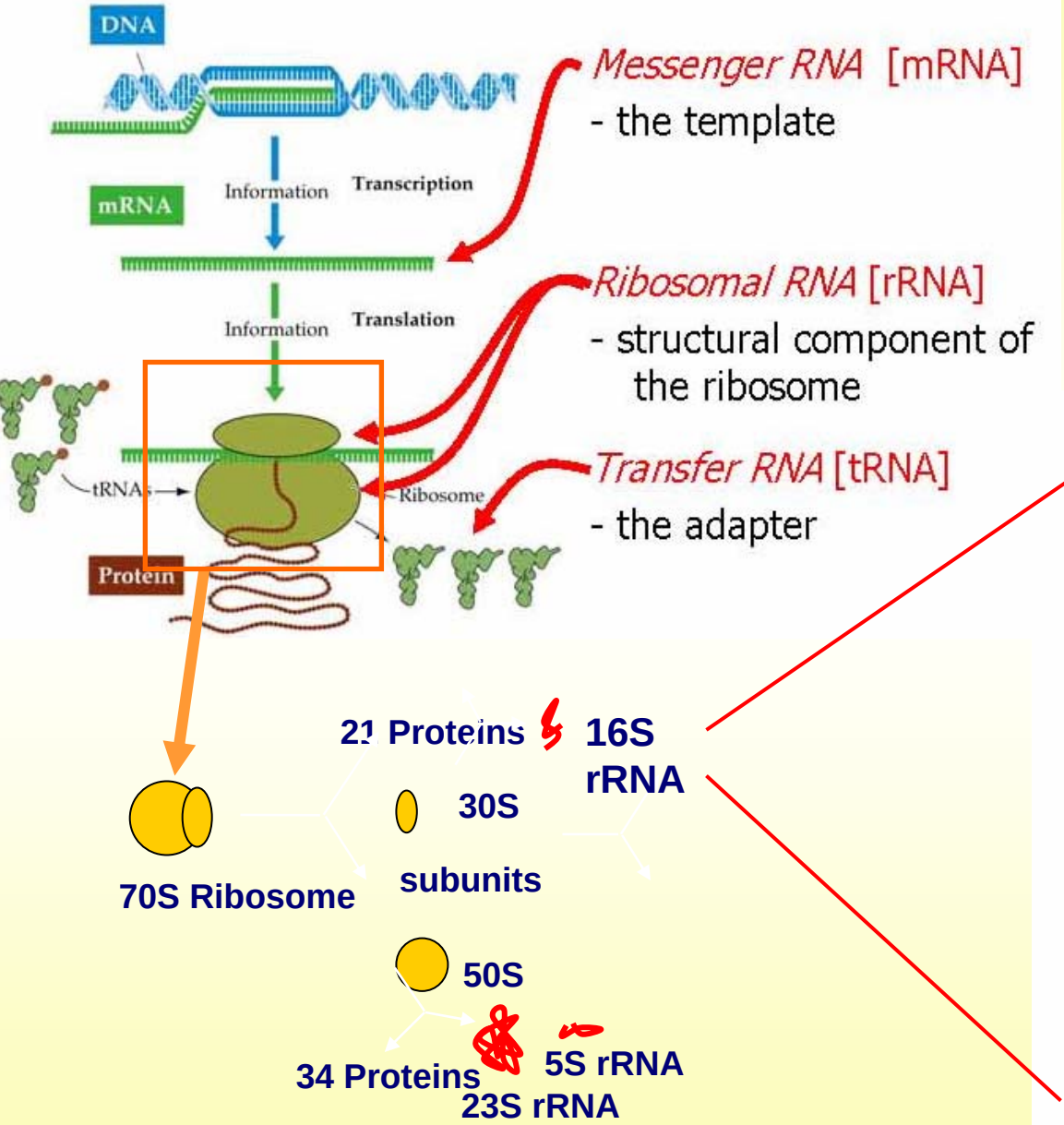




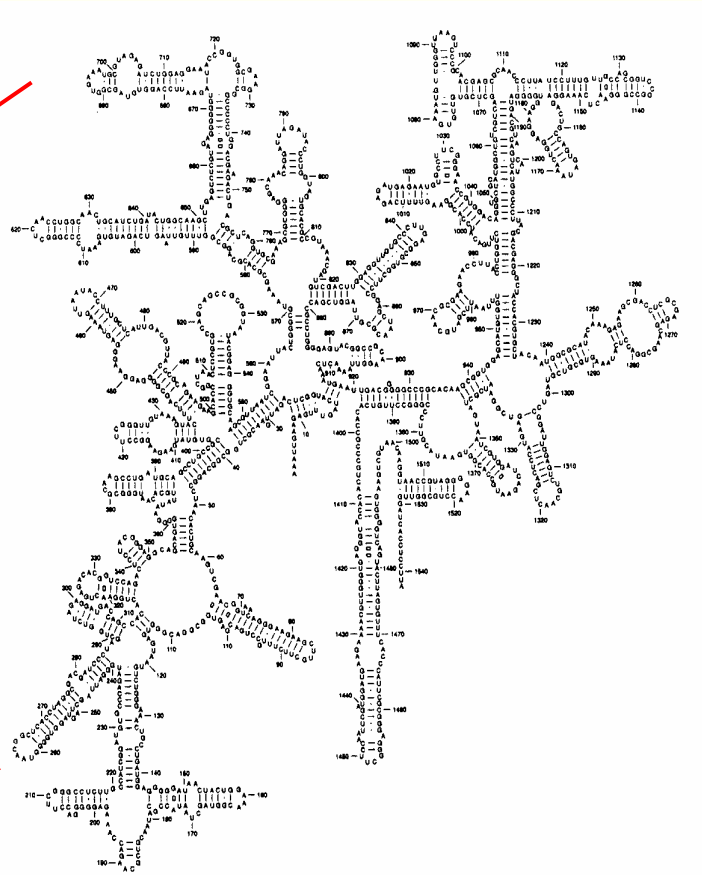




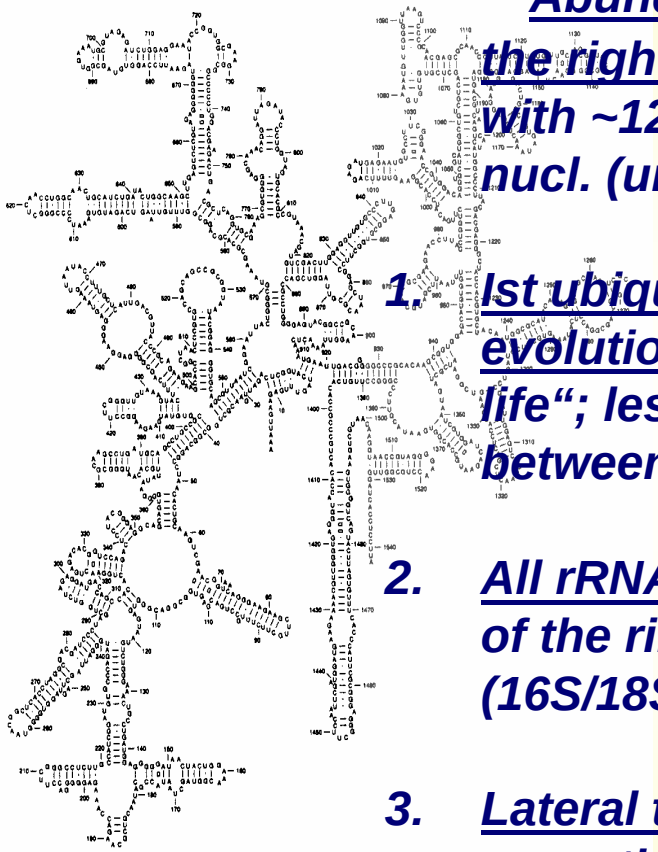
Most popular in molecular ecology: the 16rRNA gene



16S rRNA gene: part of the cell DNA carrying the information for the 16S rRNA



Why using 16S ribosomal RiboNucleic Acid (rRNA)?



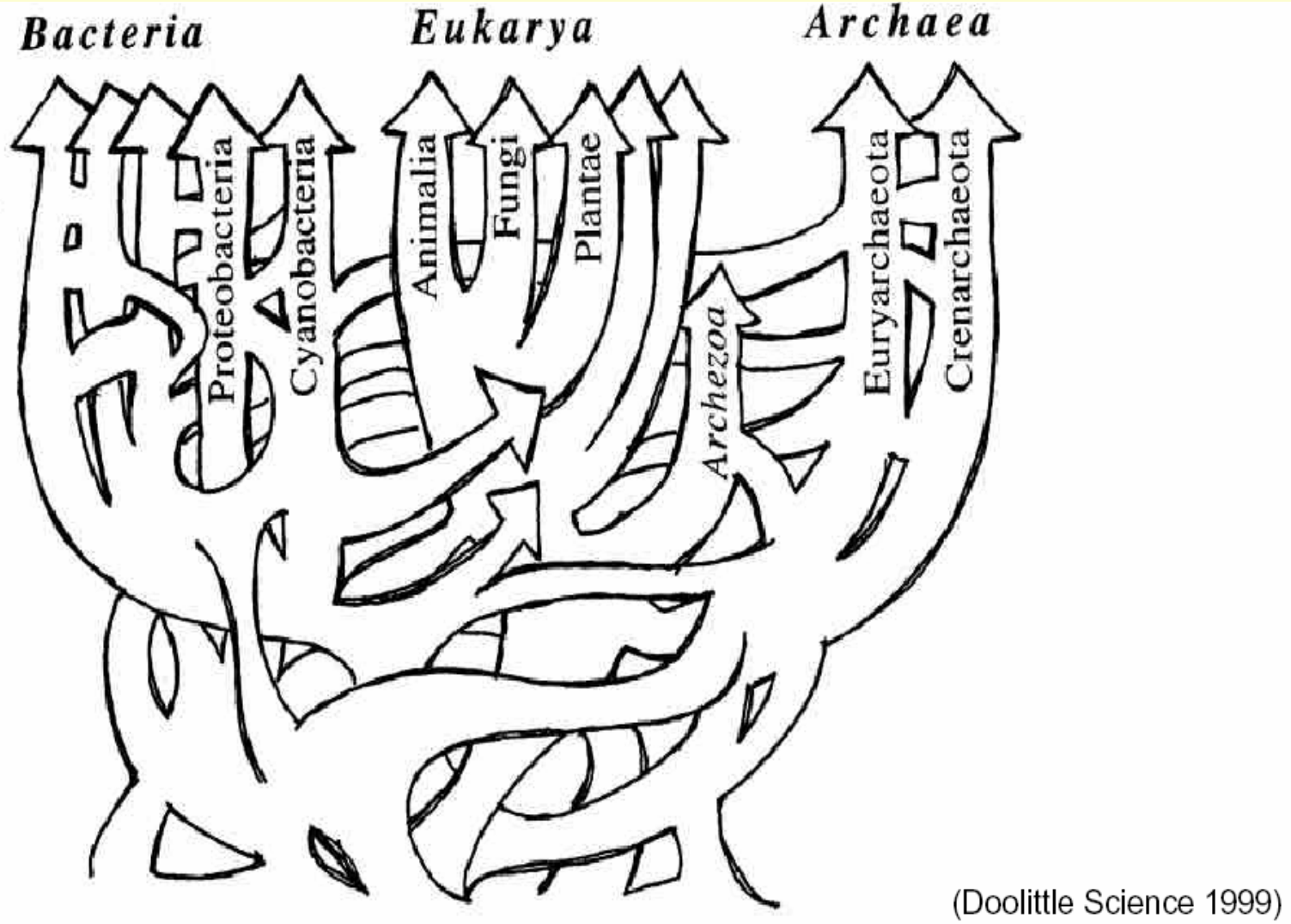
Abundant, easily isolated informative macromolecule of the right size, 1600 nucleotides as compared to the 5S rRNA with ~120 nucleotides too short) and 23S rRNA with ~3000 nucl. (unnecessarily long).

1. ***Ist ubiquitous presence in all organisms and relatively slow evolution enable the reconstruction of „universal trees of life“; less conserved RNA sequence regions often differ between microbial species.***

2. ***All rRNAs are functionally homologous! They make up 60% of the ribosomes, the protein synthesizers of all cells (16S/18S = SSU)***

3. ***Lateral transfer of rRNA genes is very unlikely, due to their essential function in the complex and fine-tuned translation machinery.***

Lateral gene transfer and the universal „bush of life“



(Doolittle Science 1999)

4.1. Exploring diversity: Who is out there?

A) The thorough way:

The full cycle rRNA approach

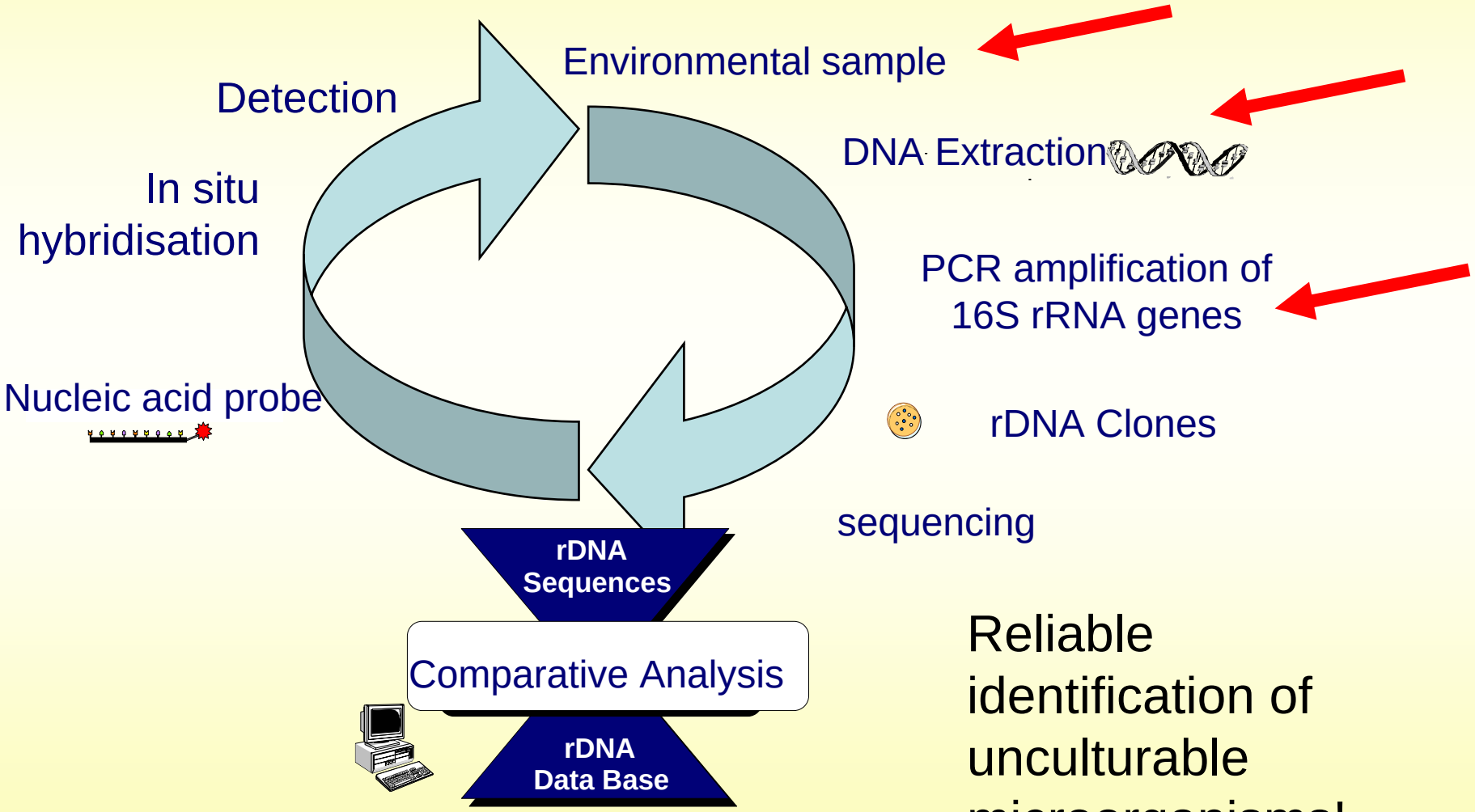
B) The faster and cheaper way:

Pattern Techniques
(Fingerprinting)

4.1. Exploring diversity: Who is out there?

A) The thorough way:

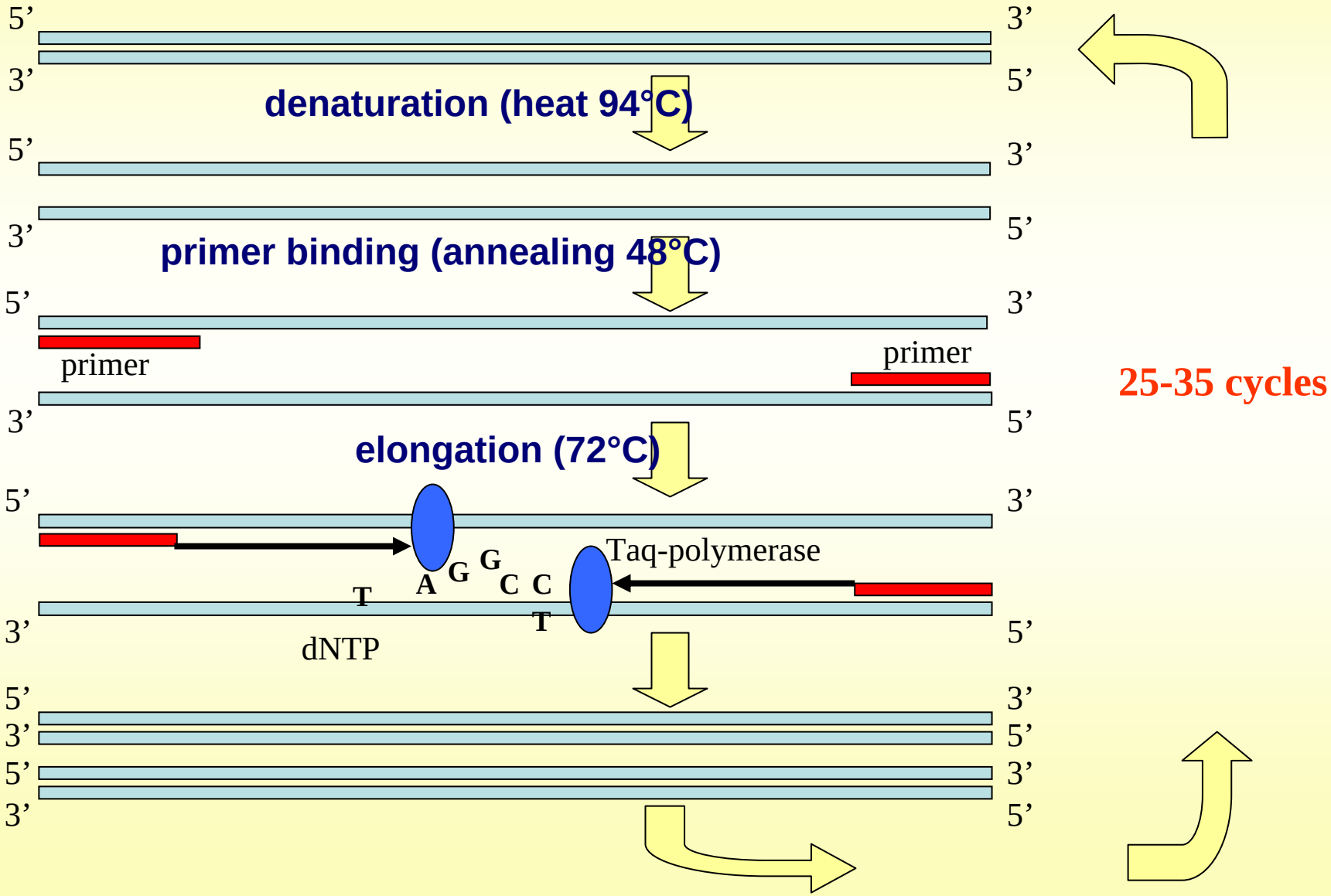
The full cycle rRNA approach



Reliable identification of unculturable microorganisms!

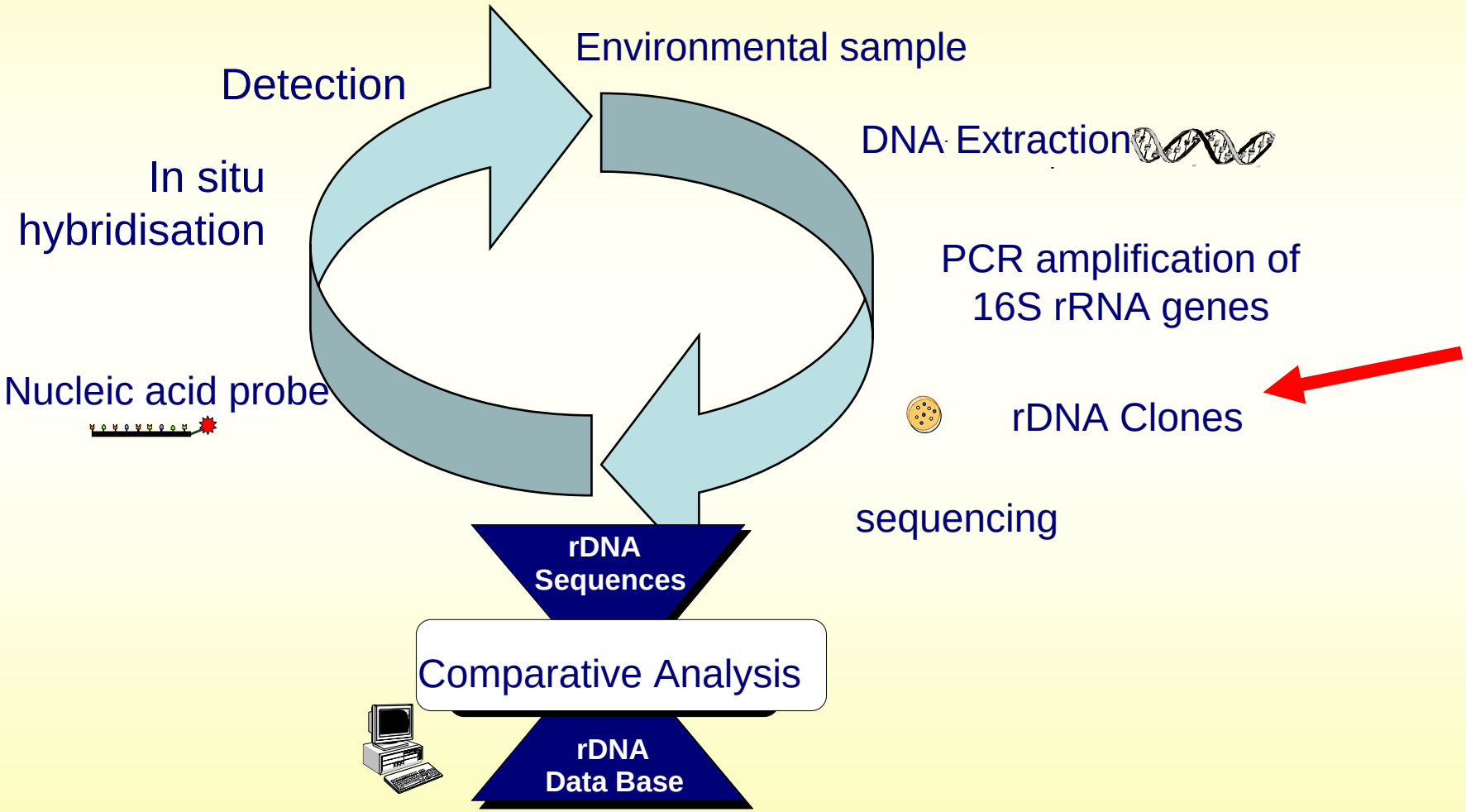
Sequencin

Polymerase chain reaction (PCR)



A) The thorough way:

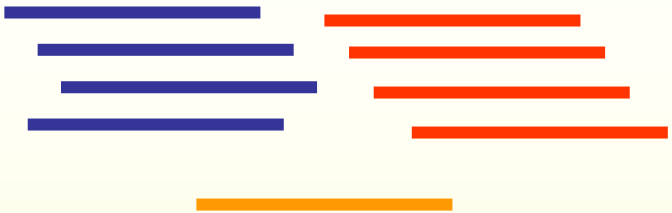
The full cycle rRNA approach



Cloning: separate and multi-copy your DNA mixture

Mixed environmental sample

„general“ PCR



<u>Biased</u> PCR product, can not be directly sequenced!

Ligation in vector
Transformation in *E. coli*

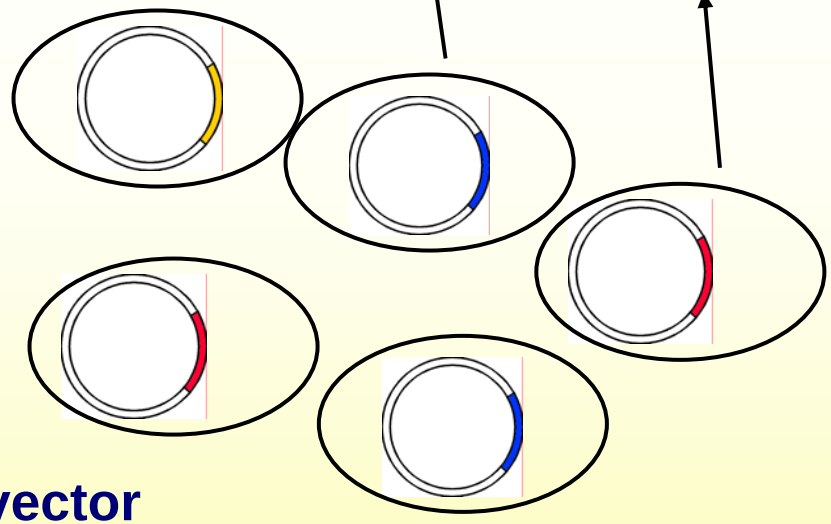


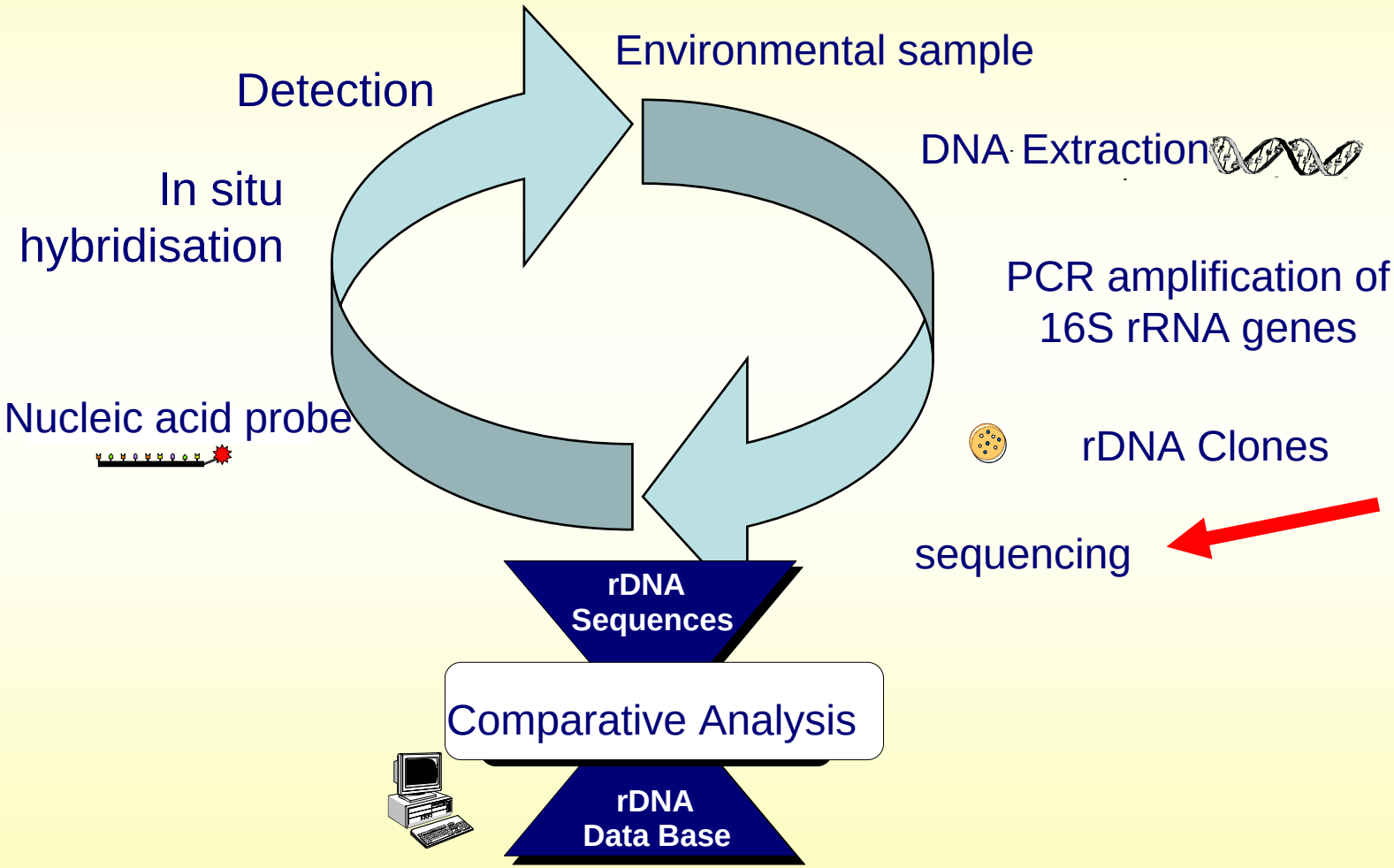
Plate and singularise clones

Screen and extract plasmids

sequence

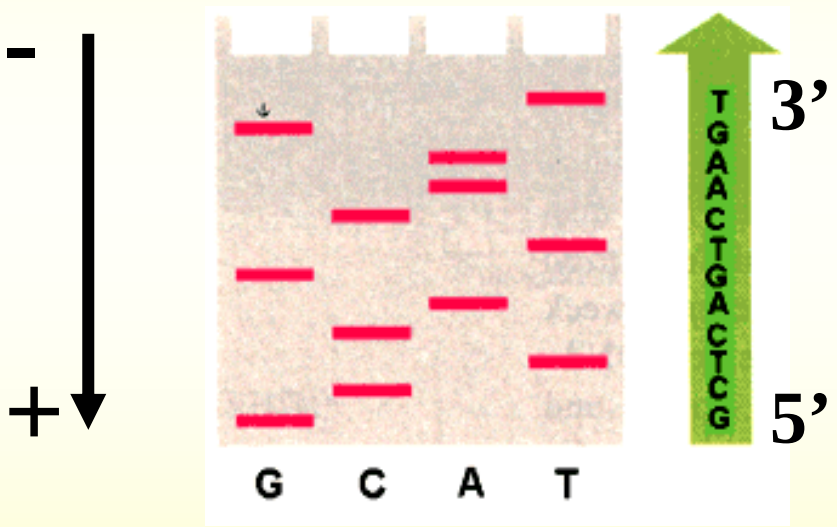
A) The thorough way:

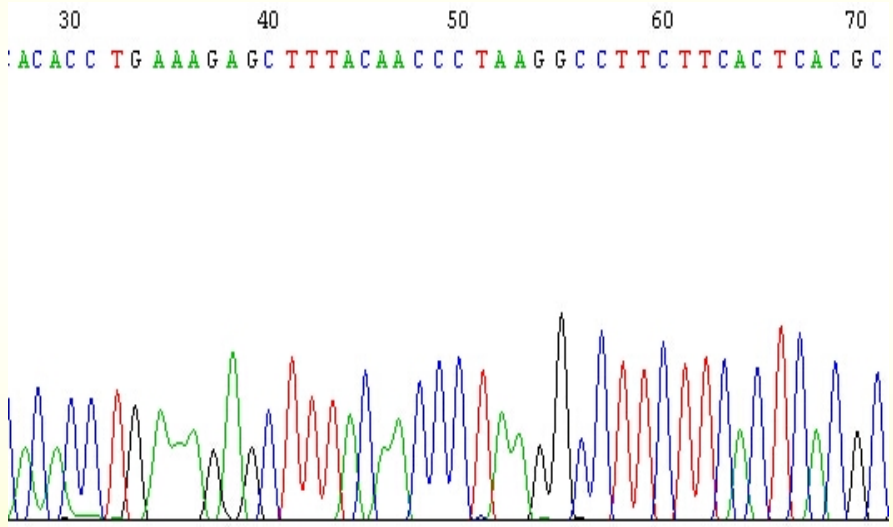
The full cycle rRNA approach



Sequencing

Different techniques available

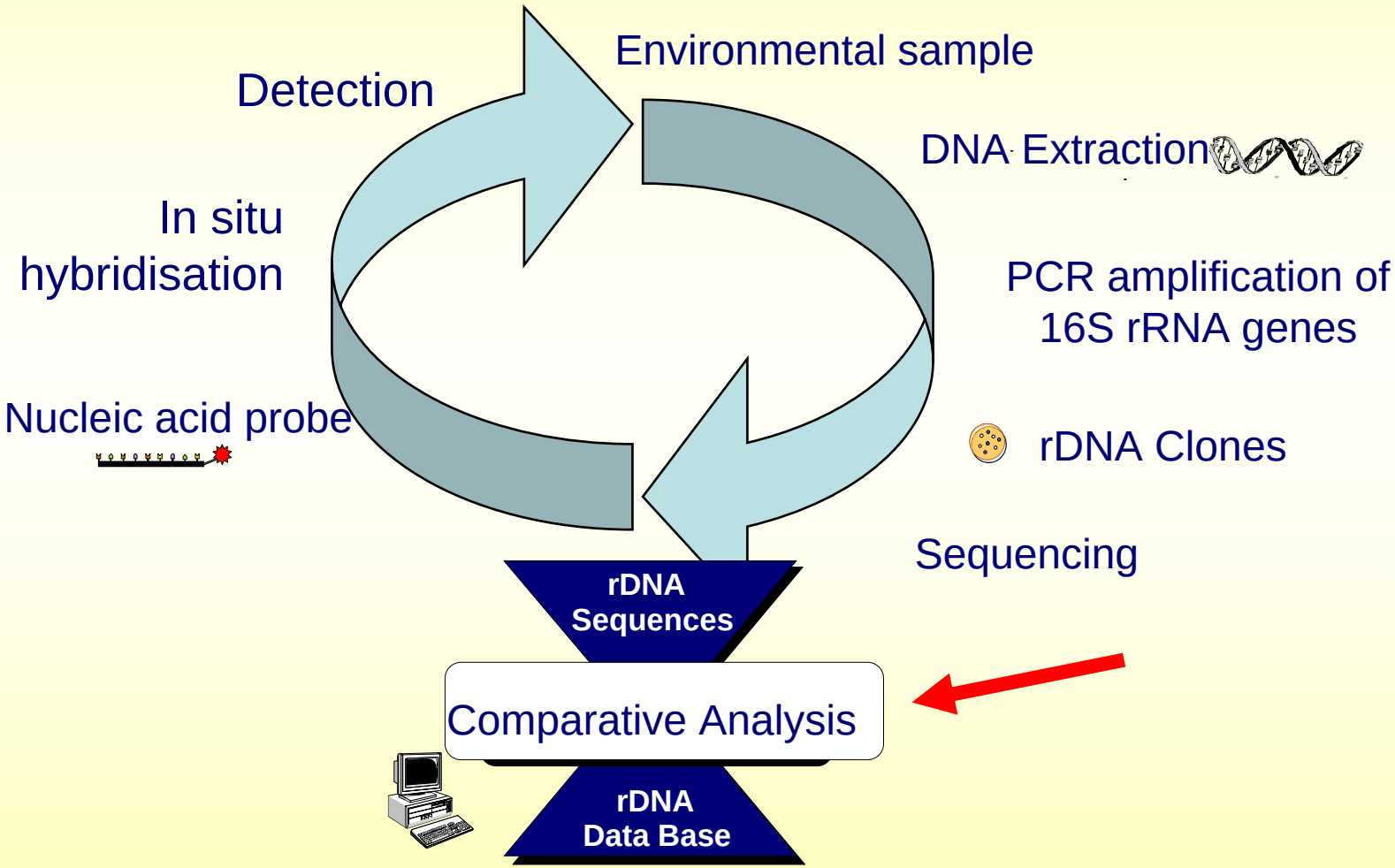




Result: DNA sequence

A) The thorough way:

The full cycle rRNA approach



Sequencin

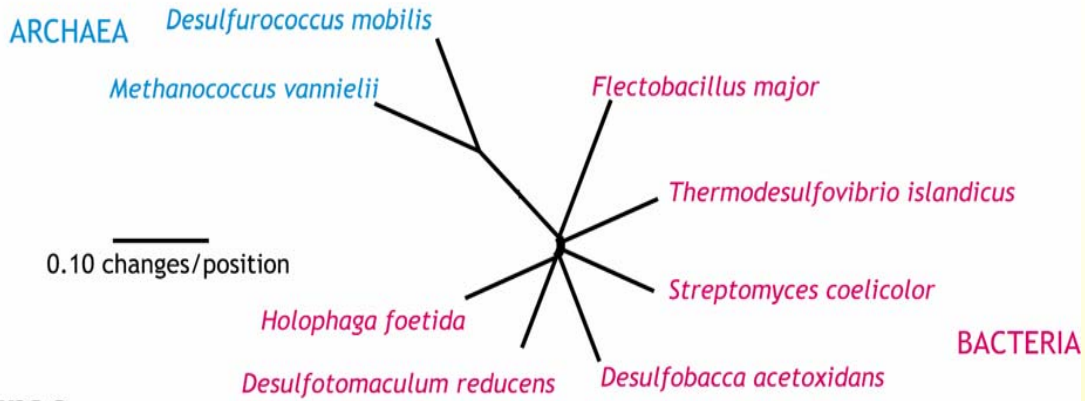
Sequence alignment

```
                          125       130       135       140       145       150       155
                          |         |         |         |         |         |         |
Holophaga foetida         ..TA--GG-AGA-CCT-AC-CTTT--TTGT-GG---GGAAT-AA-CGTTC-C..
Desulfotomaculum reducens ..TG--GA-TAA-CCT-GC-CTGA--TAGA-CC---GGGAT-AA-CAGCT-G..
Desulfobacca acetoxidans  ..TG--GG-TAA-TCT-AC-CTTC--GTTT-GG---GGGAT-AA-CCTAC-C..
Streptococcus coelicolor  ..TG--GG-CAA-TCT-GC-CCTT--CACT-CT---GGGAC-AA-GCCCT-G..
Thermodesulfovibrio islandicus ..TG--GG-TAA-CCT-GC-CCTT--AGGA-GG---AGGAT-AA-CTCGG-G..
Flectobacillus major      ..TA--TG-CAA-CCT-AC-CTAT--TATT-GG---GGGAT-AG-CCTTT-G..
Desulfurococcus mobilis   ..TG--GC-TAA-CCT-AC-CCTC--GGGA-GG---GGGAT-AA-CACCG-G..
Methanococcus vannielii   ..TG--GT-TAA-CTT-AA-CCTC--AGGT-GG---AGCAT-AA-CCTTG-G..
```

Distance matrix

```
Holophaga foetida
Desulfotomaculum reducens .195980
Desulfobacca acetoxidans  .214141 .209104
Streptococcus coelicolor  .222982 .197153 .224967
Thermodesulfovibrio islandicus .219530 .229107 .224000 .222707
Flectobacillus major      .264645 .265557 .255349 .254470 .262537
Desulfurococcus mobilis   .357243 .344189 .366644 .345017 .332329 .381429
Methanococcus vannielii   .358665 .342500 .377095 .347376 .349735 .367742 .233516
```

Radial tree



Linear tree

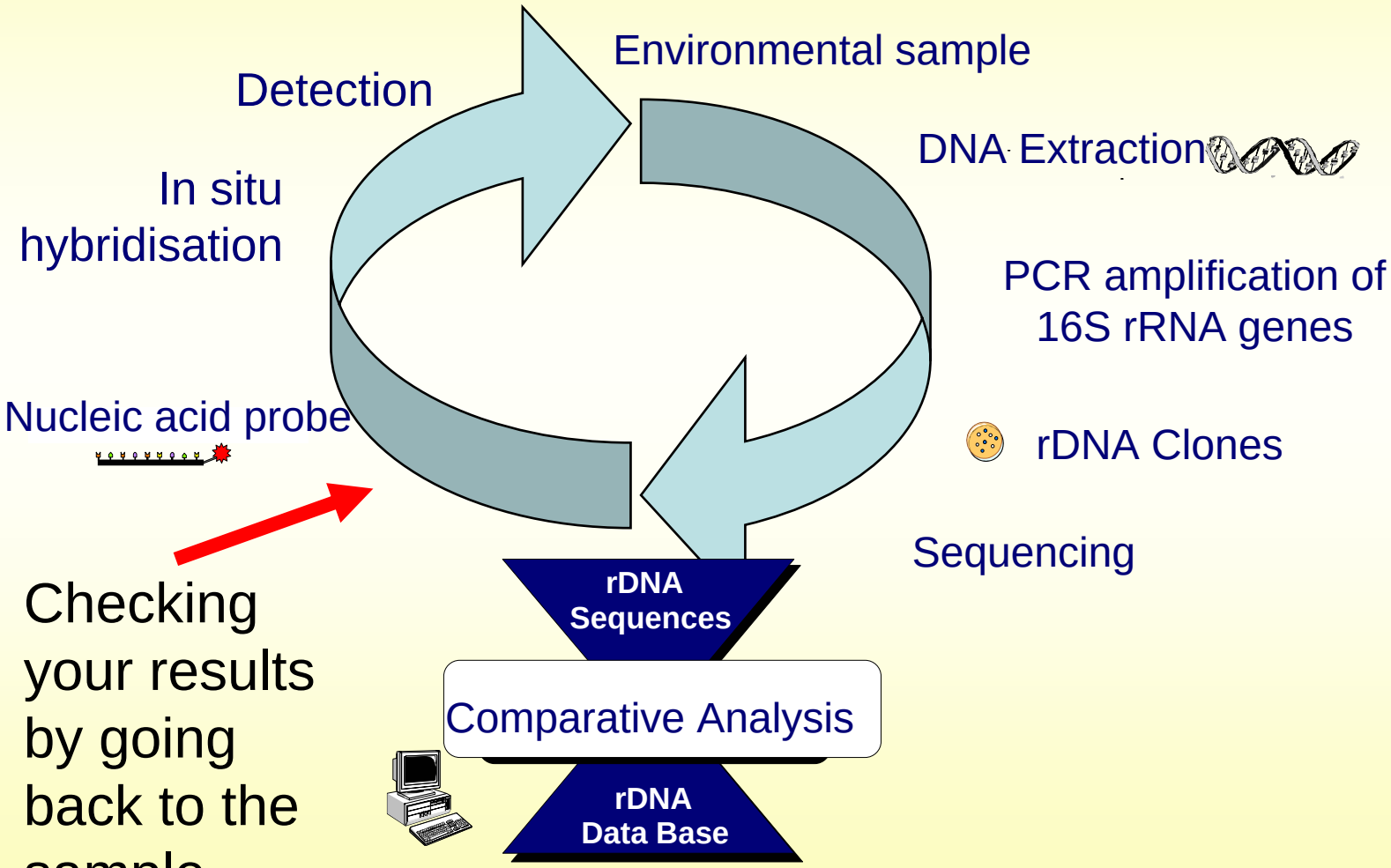
Example: phylogenetic tree of Archaea associated with the sponge *Tentorium semisuberites*

- Information about phylogenetic affiliation
- Restricted information about physiology

Master thesis A. Braunshausen

A) The thorough way:

The full cycle rRNA approach

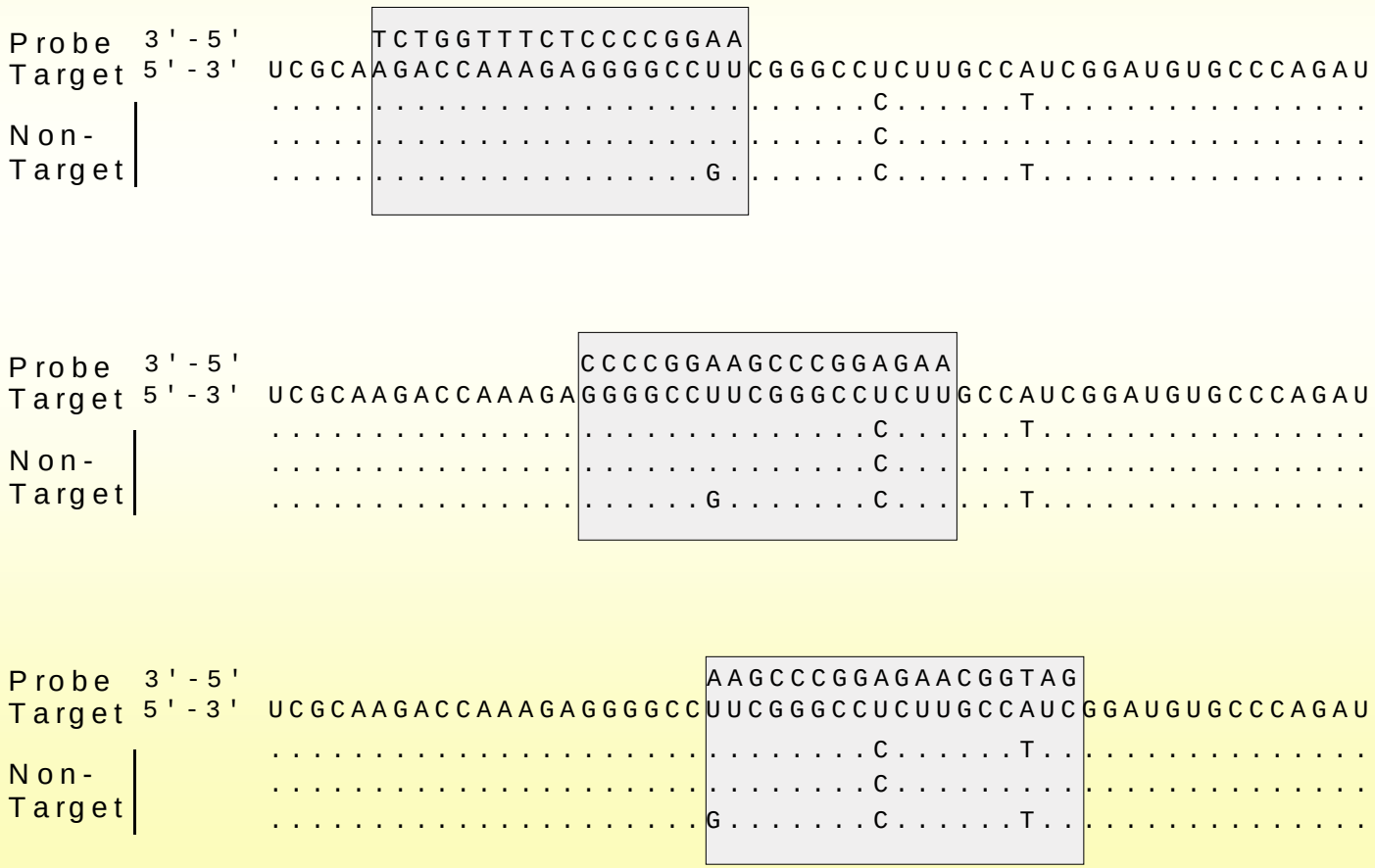


Checking your results by going back to the sample

Sequencin

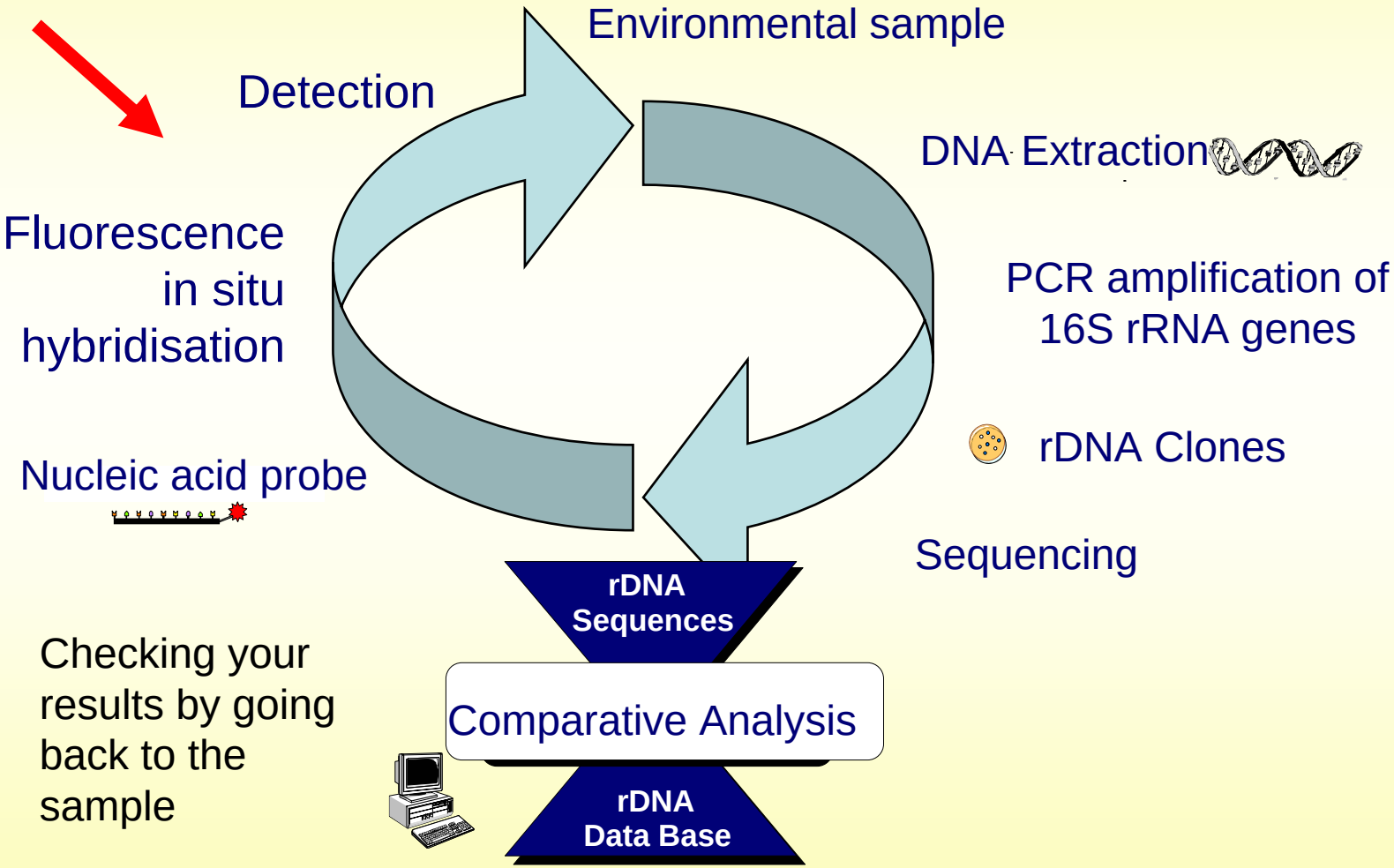
Designing nucleic acid probes

- Choose a small rRNA sequence of your target organsism(s) that differs from non-target organisms



A) The thorough way:

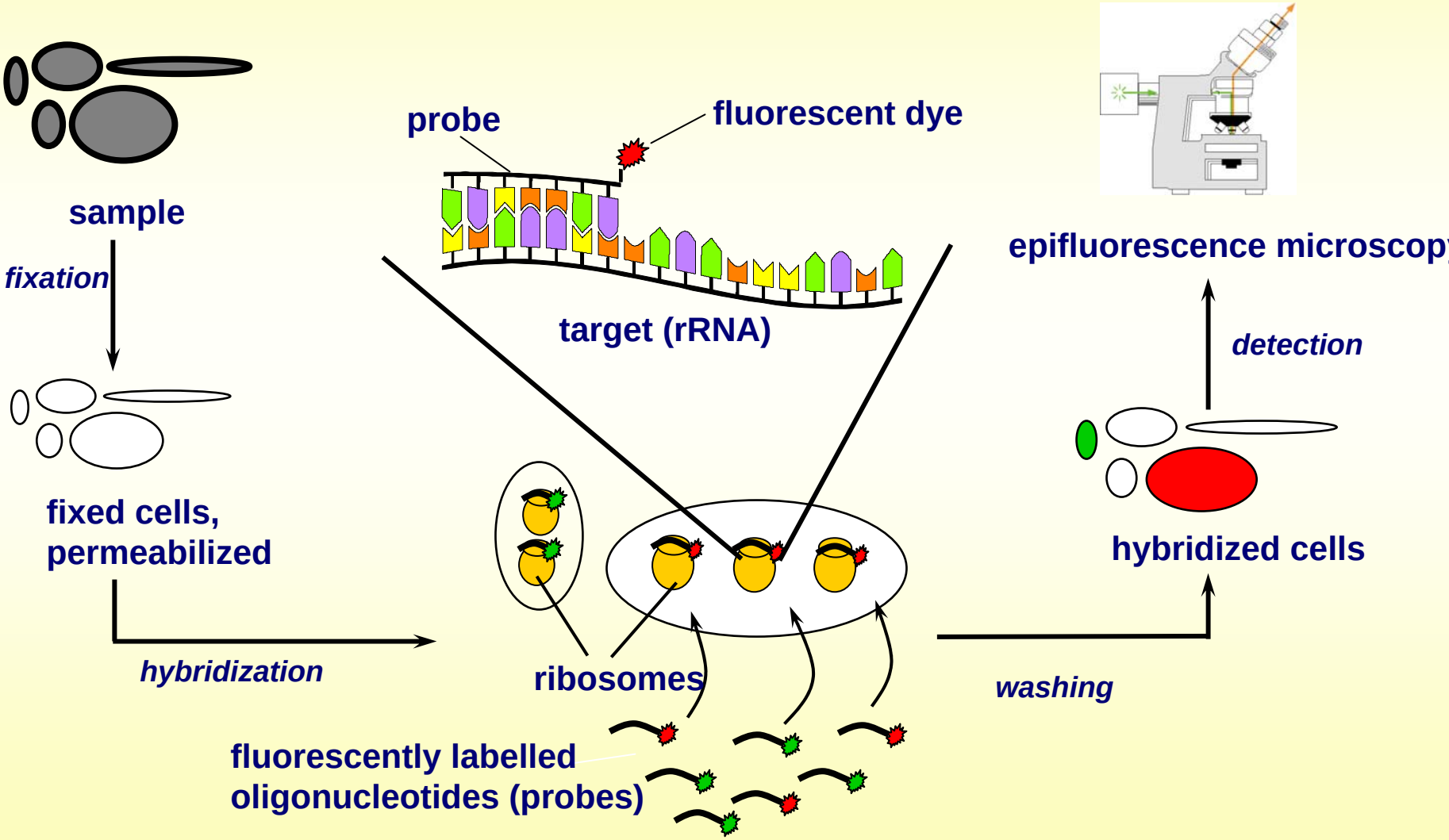
The full cycle rRNA approach



Checking your results by going back to the sample

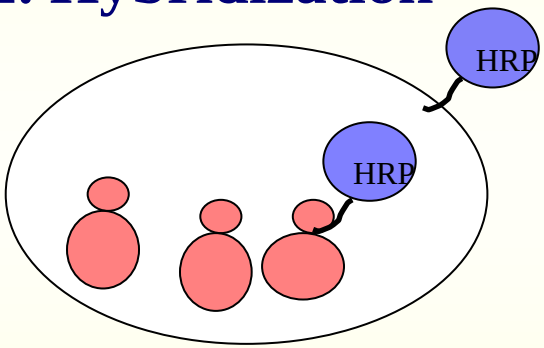
Sequencin

Fluorescence *In Situ* Hybridization (FISH)



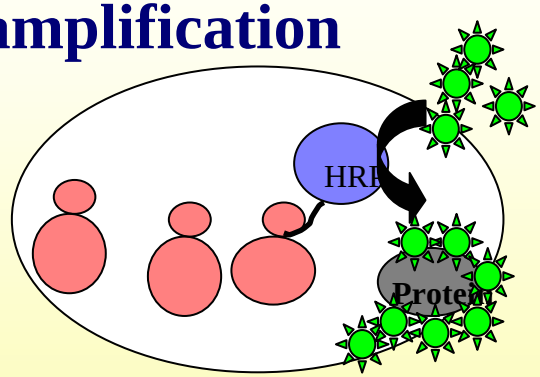
CARD-FISH: Enzymatic signal amplification in difficult samples

1. Hybridization



HorseRadish Peroxidase (HRP)
- labeled oligonucleotide

2. Signal amplification

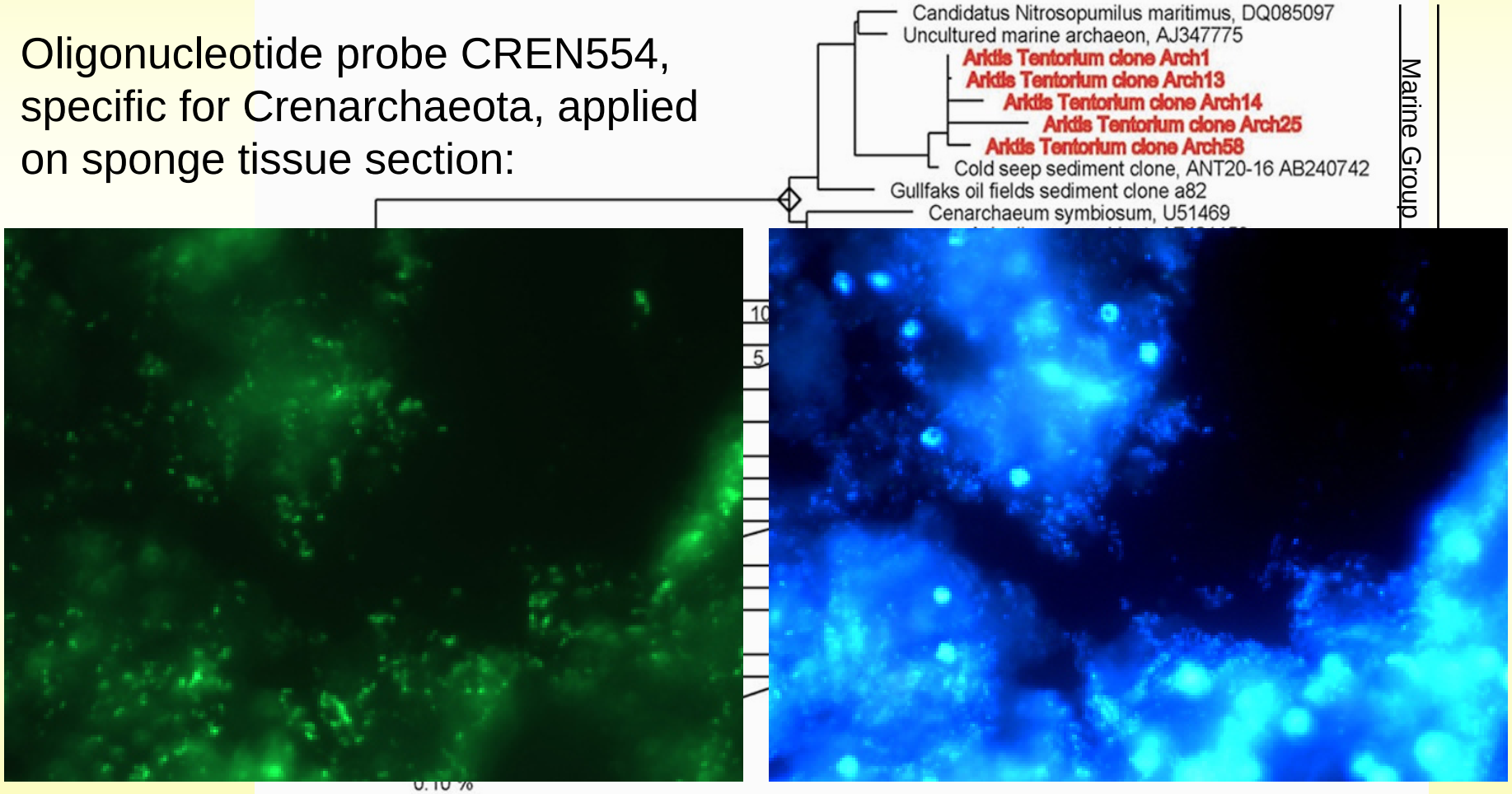


CAtalyzed Reporter Deposition (CARD) of fluorescently labeled tyramide

Example:

visualizing associated Crenarchaeota in the sponges *Tentorium semisuberites* with CARD-FISH

Oligonucleotide probe CREN554, specific for Crenarchaeota, applied on sponge tissue section:



Sponge-associated Crenarchaeota only!

DAPI staining: nuclei of sponge cells and all sponge-associated microbes!

The full cycle rRNA approach

Possible application in chemical ecology:

- Thorough information of microbial community structure in your sample
- Some ideas about function (if closely related to microorganisms with known function, e.g. producers of secondary metabolites)

Drawbacks:

- laborious
- expensive

4.1. Exploring diversity: Who is out there?

A) The thorough way:

The full cycle rRNA approach

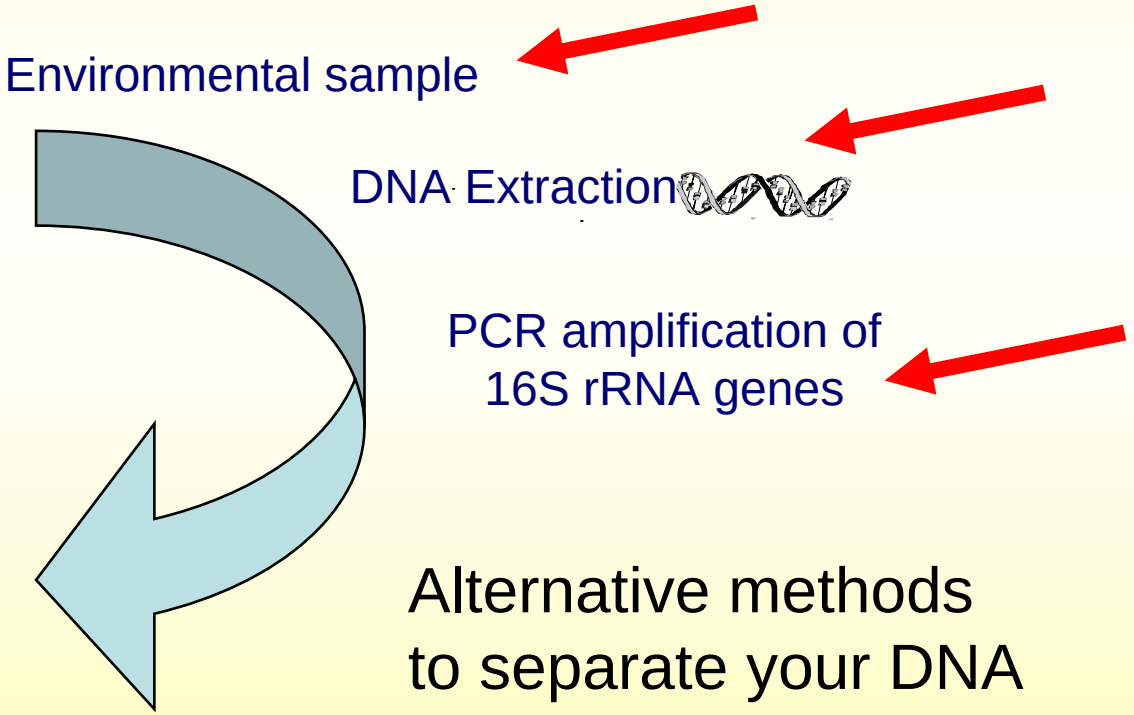
B) The faster and cheaper way:

Pattern Techniques
(Fingerprinting)

4.1. Exploring diversity: Who is out there?

B) The faster and cheaper way:

Pattern Techniques (Fingerprinting): doing not even the half cycle !



Alternative methods to separate your DNA mixture

Pattern Techniques (Fingerprinting)

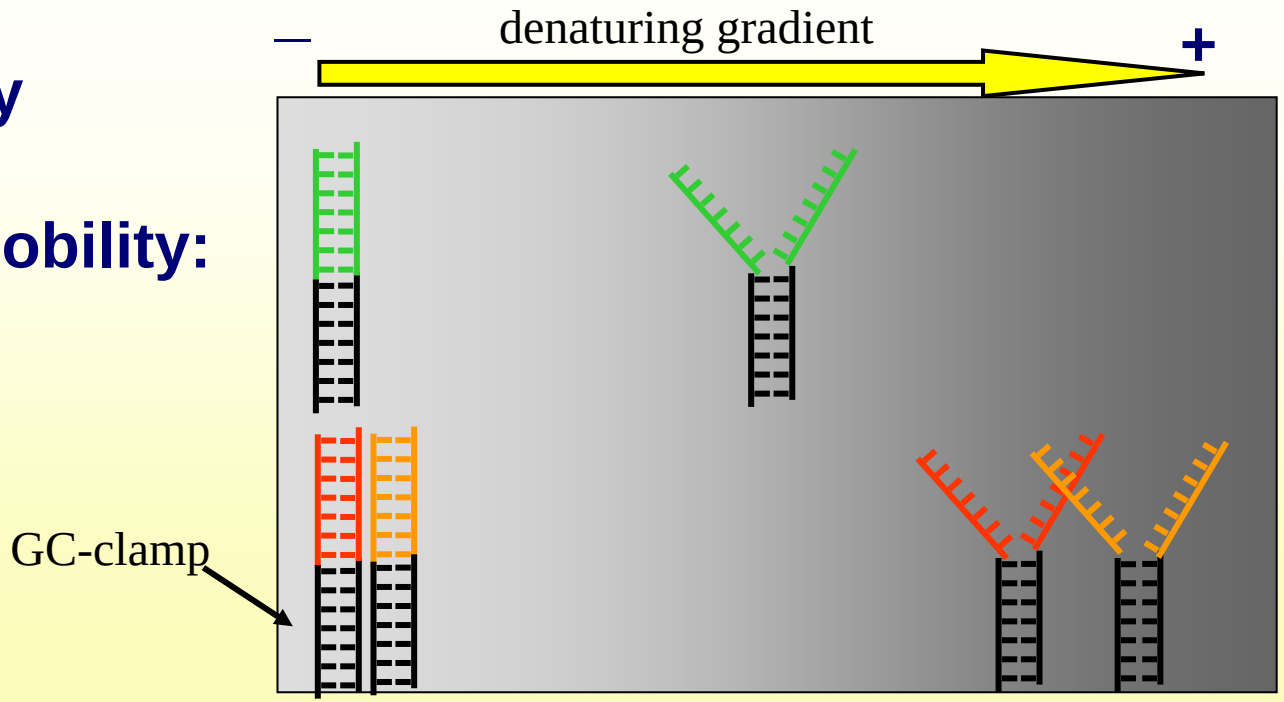
- **useful for**
  - **rapid screening of many samples**
  - **a first estimate of microbial diversity**
- **not recommended for in-depth diversity studies**

1) **RFLP/T-RFLP**
2) **DGGE/TGGE**
3) **LMW-RNA pattern**
4) **SSCP**
5) **SARST**
6) **ARISA**

DGGE/TGGE

Degenerating/Temperature Gradient Gel Electrophoresis

- **Two DNA fragments of almost identical length are hardly separated by standard gel electrophoresis.**
- **BUT, if they have a different GC-content, they have different melting characteristics!**
- **So: if the gel has a linearly increasing gradient of a denaturant (or when temperature is increasing during the run) two fragment might be separated due to differences in the melting behavior (i.e. denaturation occurs earlier or later).**
  - **Melting sharply decreases DNA fragment mobility:**
  - **Fragments are separated!**



Fingerprinting methods

Possible application for chemical ecology:

- First estimate of diversity
- Rapid screening of many samples
- Good for rapid comparison of different samples /sampling sites

Drawbacks:

- Only limited information about phylogeny
- No information about function

High density and high diversity of prokaryotes

Challenge and chance for chemical ecology

Focus on functional aspects

1. principles of cultivation and isolation

2. chemical communication between bacteria

3. considerations on a bioluminescent bioassay

4. cultivation-independent molecular studies
   - 4.1 Exploring diversity (who is out there?)
   - 4.2 Exploring function (what are they doing?)

5. porifera-microbe interactions

4.2. Exploring function: what are they doing?

- **Functional analysis:**

  DNA-based

  – **PCR for functional genes**

  – **Metagenomics**

  mRNA-based

  – **mRNA RT-PCR**

  – **mRNA FISH**

  Protein-based

  – **(Meta)Proteomics, 2D – gel electrophoresis**

  other

  – **tracer studies (radioisotopes)**

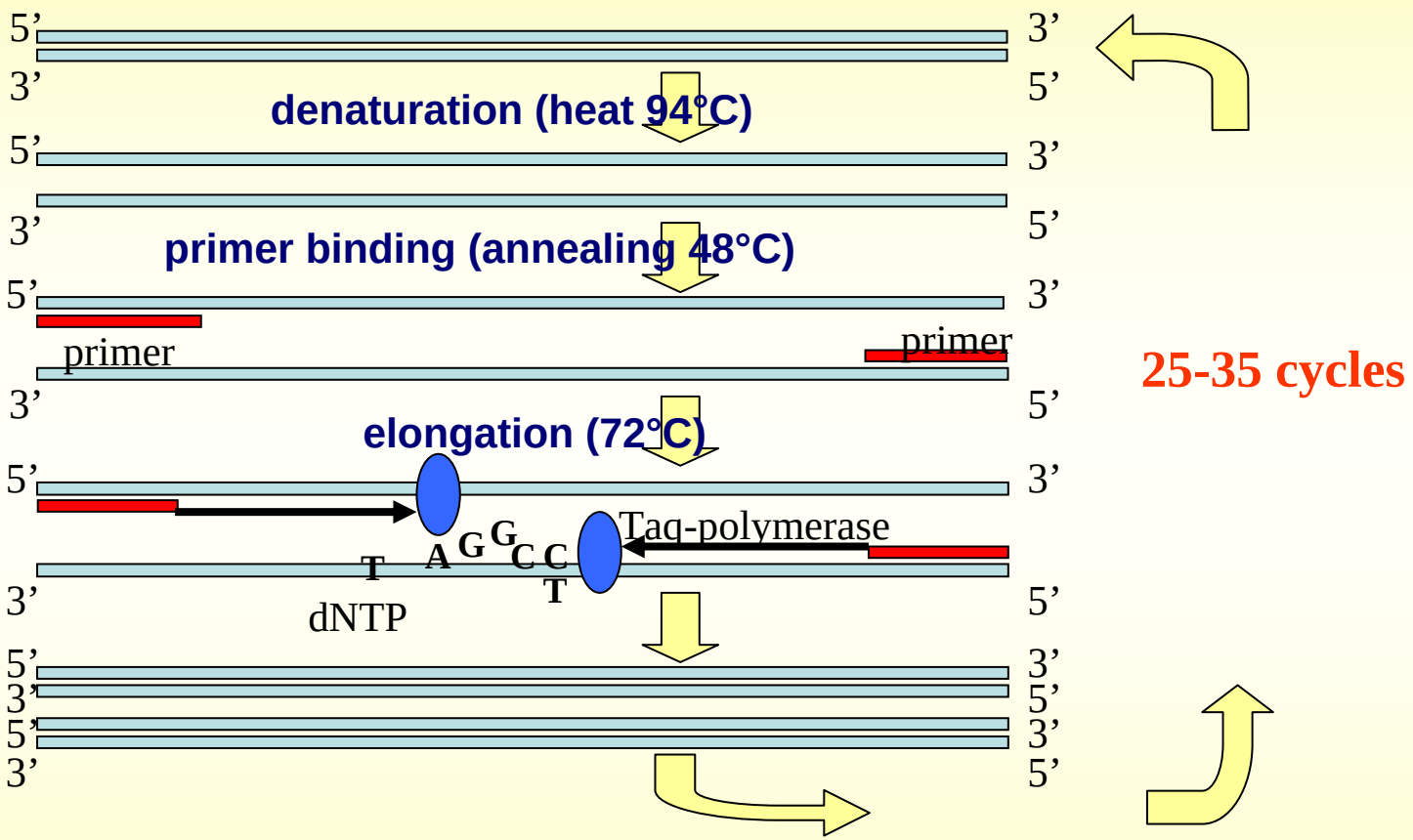
  – **Stable isotopes**

  – **enzyme tests**

  Combinations

  – **MAR-FISH**

Polymerase chain reaction (PCR) for functional genes



Same method as for 16S amplification, but using a different primer:

Specific for the “key” gene you are looking for

PCR for functional genes:

Gives you the information that there are organisms around which are capable of this specific biochemical process

Possible application in chemical ecology:

Looking for a gene which is essential for a biochemical function, e.g. the production of the target metabolite

4.2. Exploring function: what are they doing?

- **Functional analysis:**

  DNA-based

  – **PCR for functional genes**

  – **Metagenomics**

  mRNA-based

  – **mRNA RT-PCR**

  – **mRNA FISH**

  Protein-based

  – **(Meta)Proteomics, 2D – gel electrophoresis**

  other

  – **tracer studies (radioisotopes)**

  – **Stable isotopes**

  – **enzyme tests**

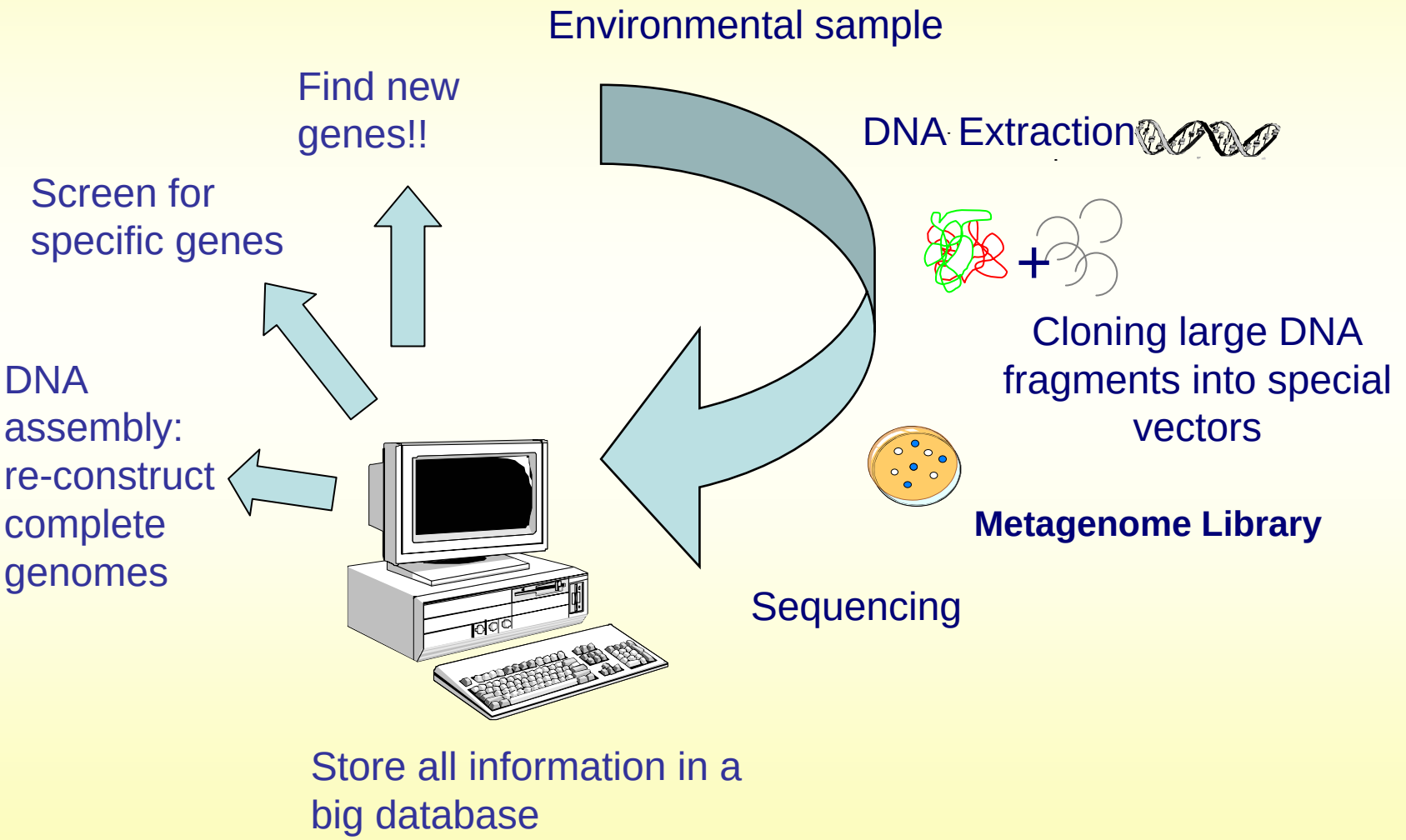
What is Metagenomics?

- Metagenomics is the genomic analysis of the collective genomes of an assemblage of organisms (Handelsman et al., 2002)

- culture-independent genomic analysis of microbial communities
- derived from the statistical concept of *meta*-analyses (the process of statistically combining separate analyses) and genomics (the comprehensive analysis of an organism‘s genetic material)

- **Synomyms:**
  - Environmental genomics (Stein et al., 1996)
  - Community genomics (Tyson et al., 2004)

Metagenome library: try to copy + store as much of the total genome as possible



Annotation Using GenDB

Meyer et al., Nucleic Acid Research 2003, 8:2187-2195

Metagenomics Libraries

- … contain DNA extracted directly from an environmental sample
- ... provide genomic sequences, and phylogenetic and functional information
- ... can be screened for functions, and genomic sequence surrounding genes required for those functions can provide insight into the organism from which the function was derived (e.g. 16S rRNA, *recA*)

⇨ Linking phylogeny, sequence and functional analysis provides a multifaceted approach to explore the uncultivated microbial community

Handelsman et al., Methods in Microbiology 2002, 33:241-255

4.2. Exploring function: what are they doing?

- **Functional analysis:**

  DNA-based

  - **PCR for functional genes**
  - **Metagenomics**

  mRNA-based

  - **mRNA RT-PCR**
  - **mRNA FISH**

  Protein-based

  - **(Meta)Proteomics, 2D – gel electrophoresis**

  other

  - **tracer studies (radioisotopes)**
  - **Stable isotopes**
  - **enzyme tests**

  Combinations

  - **MAR-FISH**

Tracer studies (radioisotopes)

Principle:

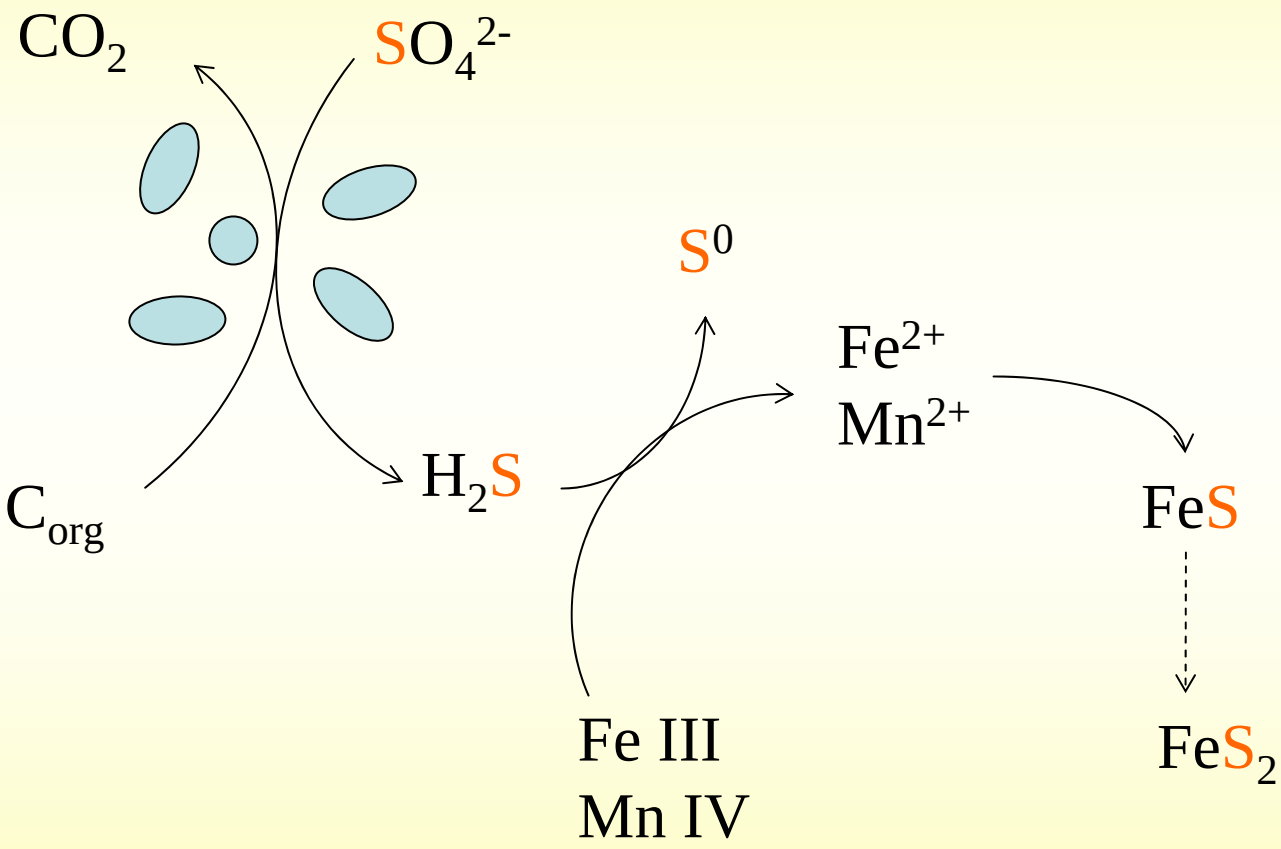
Add a substrate which is radioactively labelled (^{14}C, ^{35}S, ^{35}P)

Stable isotopes (^{13}C, ^{15}N) are also used

- Check for radioactive products or:
- Check for incorporation of radioactive substrate in microorganisms

Example:

Measuring microbial sulfate reduction with $^{35}SO_4^{2-}$



Hoffmann et al (2005): An anaerobic world in sponges. Geomicrobiology Journal 22: 1-10

Radioisotope/stable isotope studies:

Detect and quantify microbial activity!

Challenge:

Directly combining isotope technique and molecular methods

4.2. Exploring function: what are they doing?

- **Functional analysis:**

  DNA-based

  – **PCR for functional genes**

  – **Metagenomics**

  mRNA-based

  – **mRNA RT-PCR**

  – **mRNA FISH**

  Protein-based

  – **(Meta)Proteomics, 2D – gel electrophoresis**

  other

  – **tracer studies (radioisotopes)**

  – **Stable isotopes**

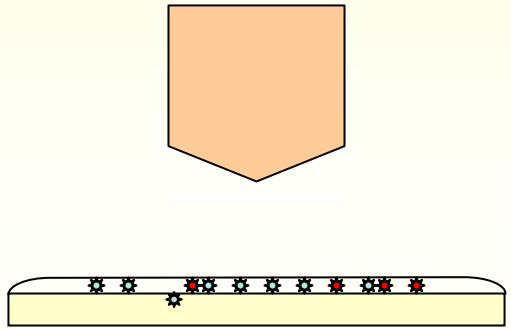
  – **enzyme tests**

  Combinations

  – **MAR-FISH**

Microautoradiography

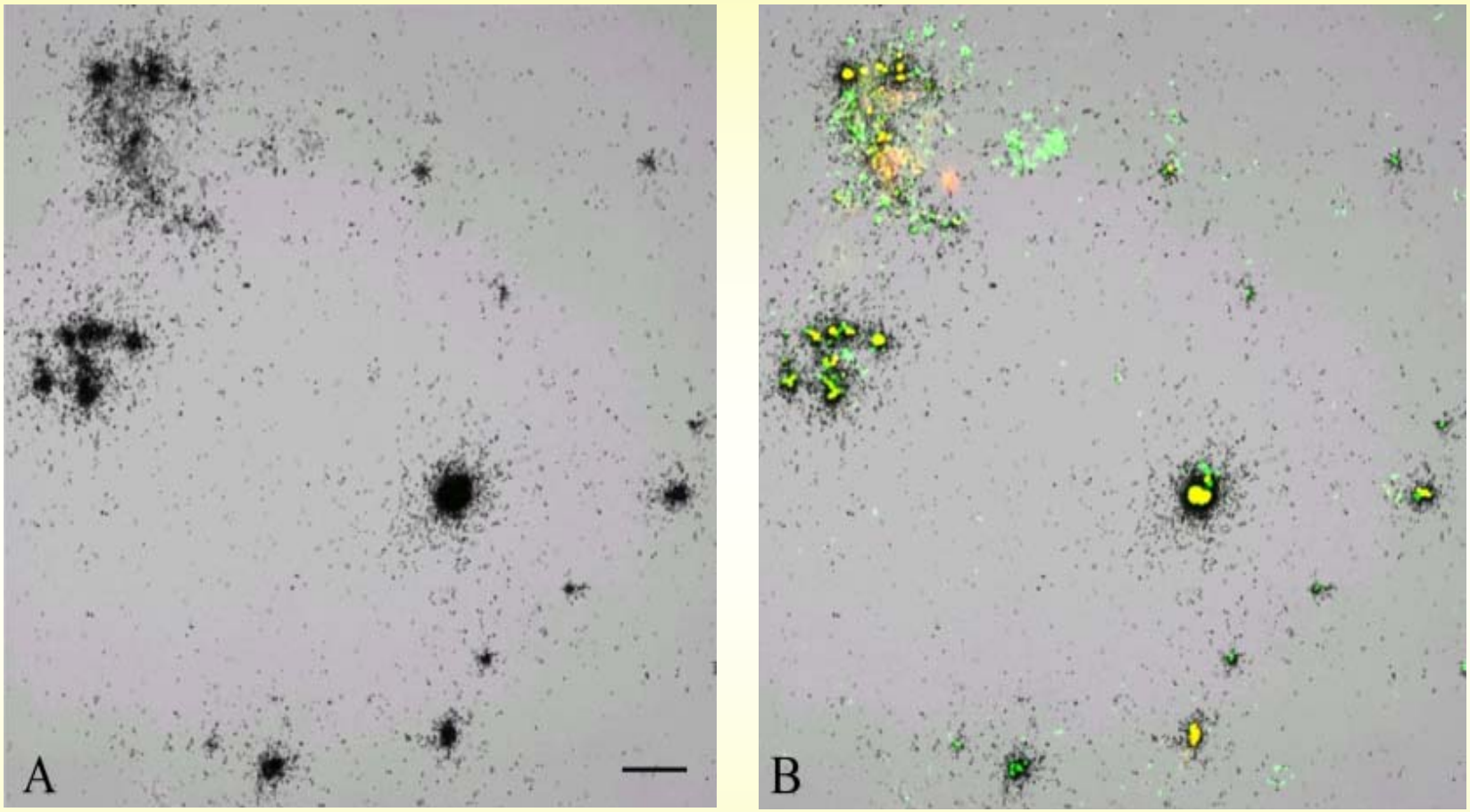
Based on the uptake of radioactively labled substrate



Upon incubation the radioactive material is covered with a photo-emulsion and the incorporated radioactive substrate is detected (semiquantitatively) within the photographic film as clusters of silver grains!

MAR-FISH:

Combining microautoradiography and fluorescence in situ hybridisation



Only method available directly linking substrate incorporation to bacterial identification! (“who is doing what?”)

MAR-FISH

Possible application for chemical ecology:

Offers the possibility to check which microbe is processing the target metabolite, e.g. by adding a radiolabeled precursor

High density and high diversity of prokaryotes

Challenge and chance for chemical ecology

Focus on functional aspects

1. principles of cultivation and isolation

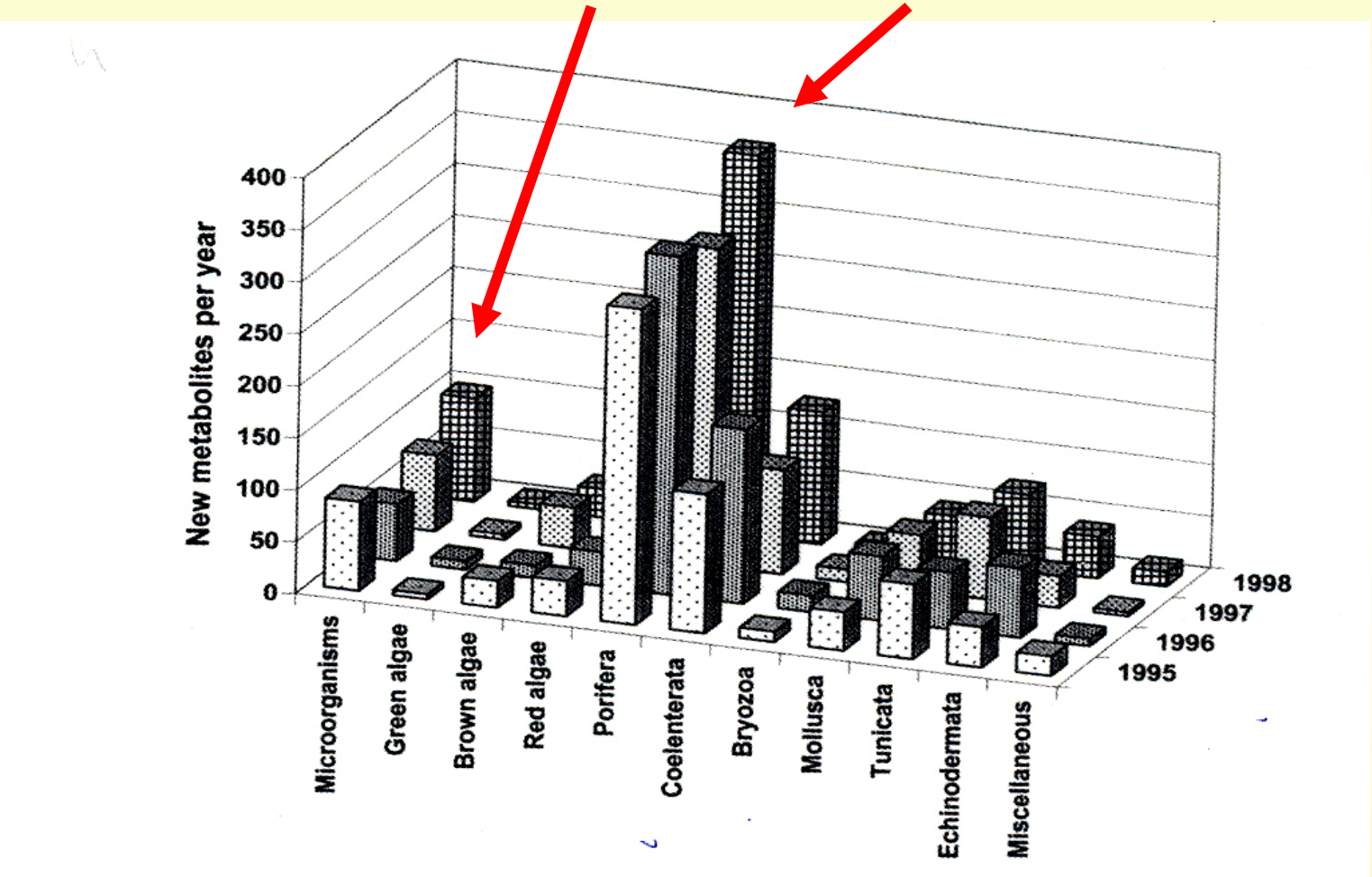
2. chemical communication between bacteria

3. considerations on a bioluminescent bioassay

4. cultivation-independent molecular studies
    - 4.1 Exploring diversity (who is out there?)
    - 4.2 Exploring function (what are they doing?)

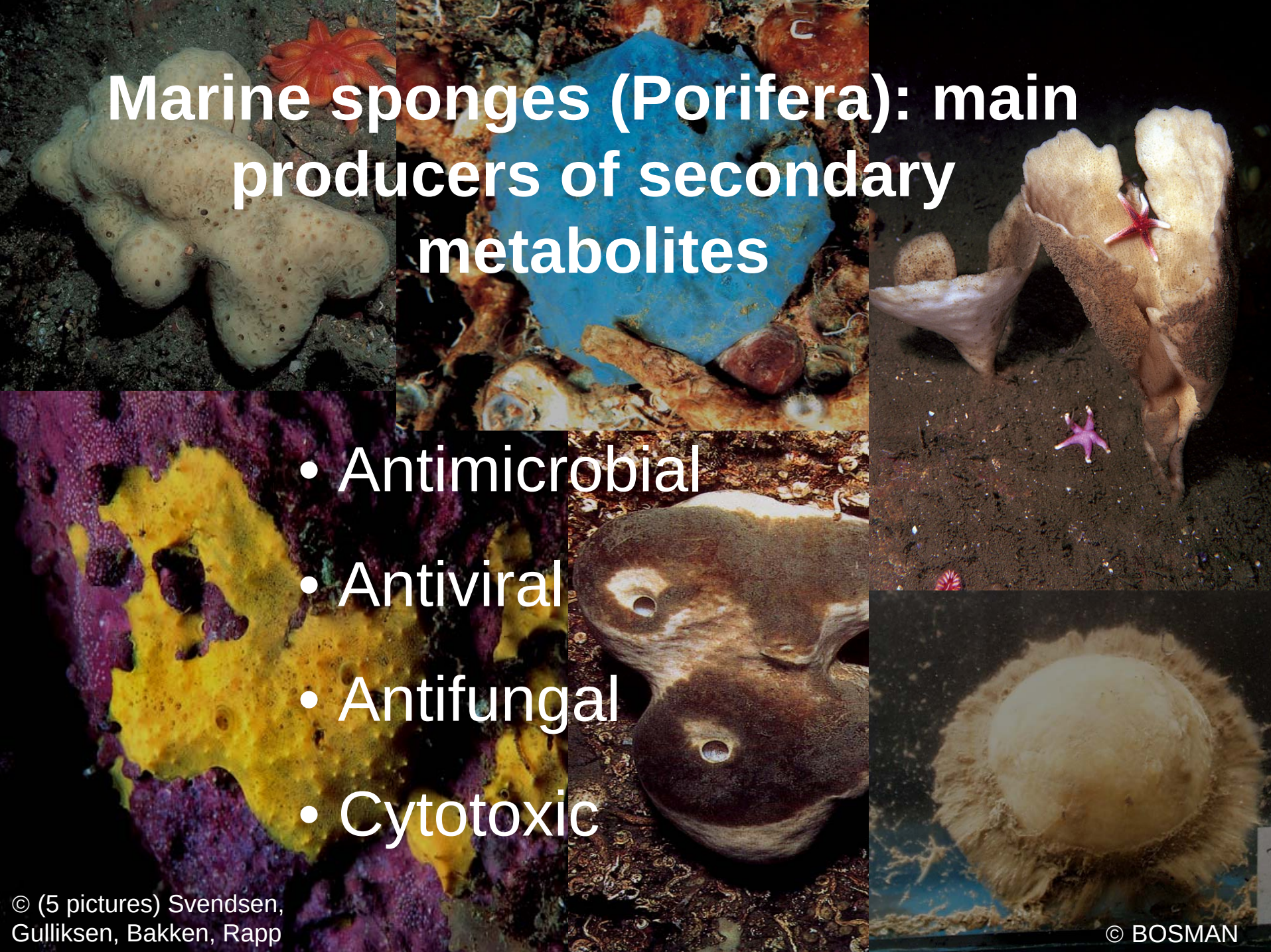
5. porifera-microbe interactions

Main producers of marine natural products



Nickel et al (2001) J Biotechnol 92: 169-178

Marine sponges (Porifera): main producers of secondary metabolites



- Antimicrobial
- Antiviral
- Antifungal
- Cytotoxic

Commercial potential: *Geodia barretti*, Barettin

Geodia barretti

Total extract

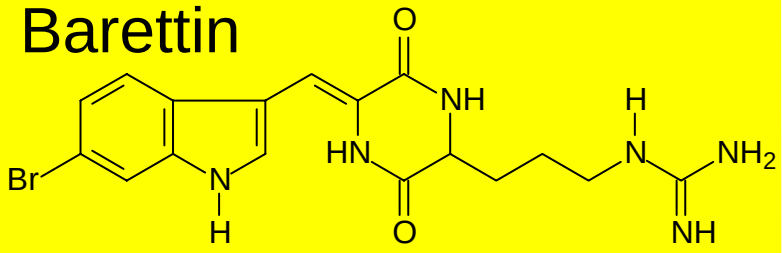
Bioassays, toxicity tests

Purification, structural analysis of secondary metabolites

Antifouling activity

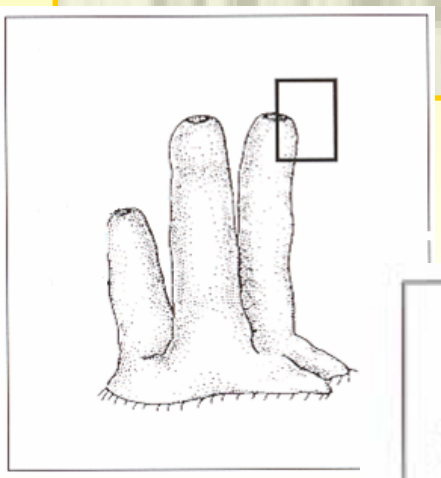
Sjögren et al 2004, J Nat Prod 67,368-372

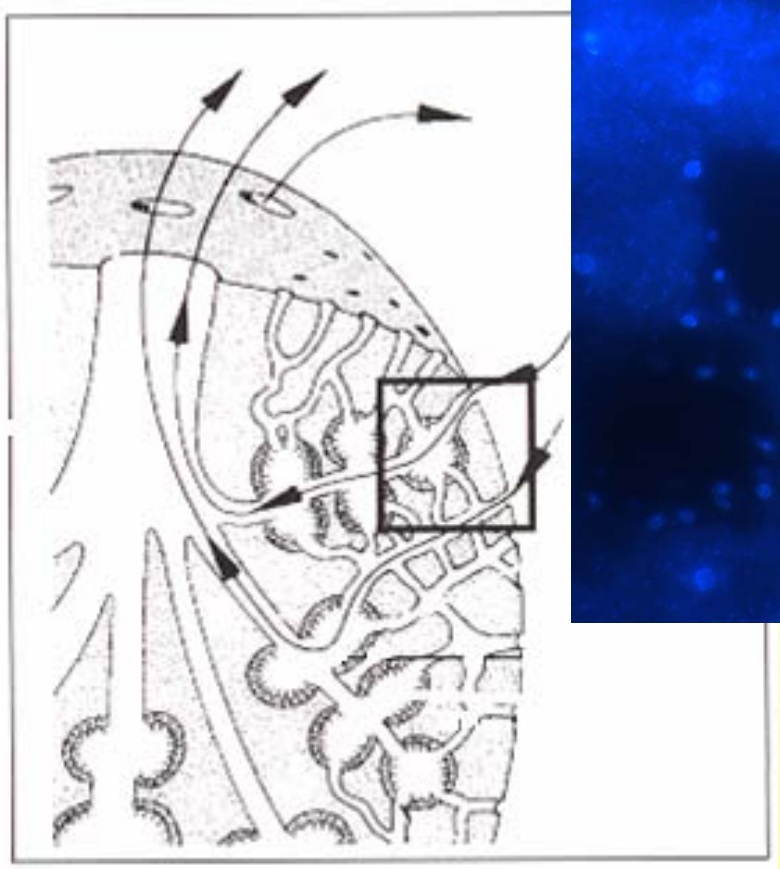
Barettin

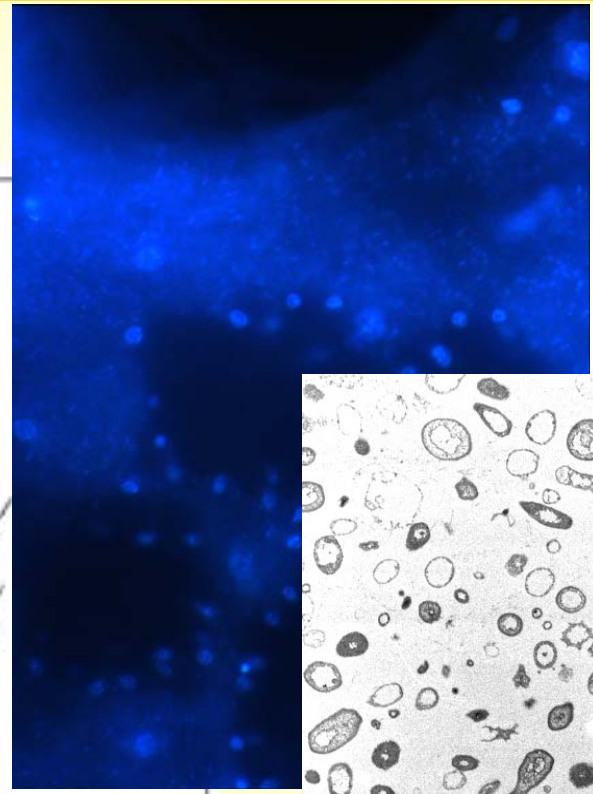


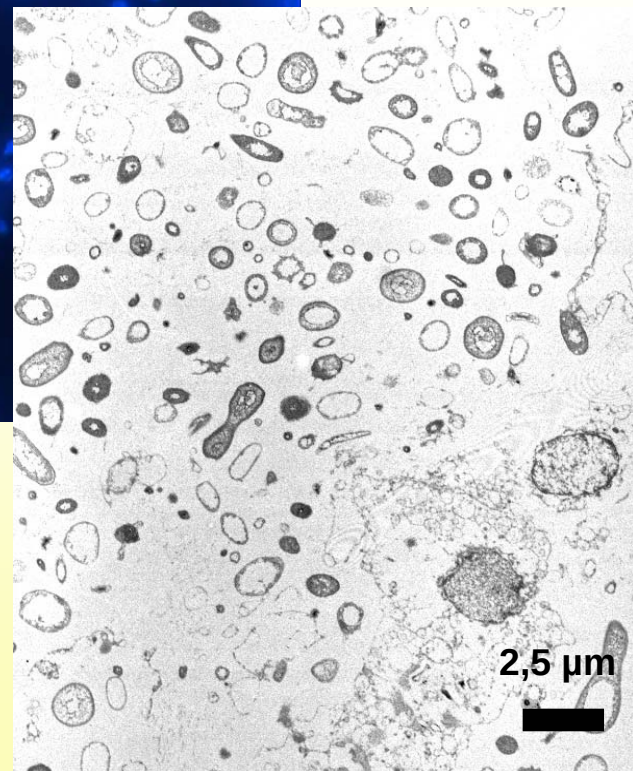
Sölter et al 2002, Tetrahedron L 43 (18), 3385-3386

Sponge Anatomy









all pictures: Hentschel et al. (2003)

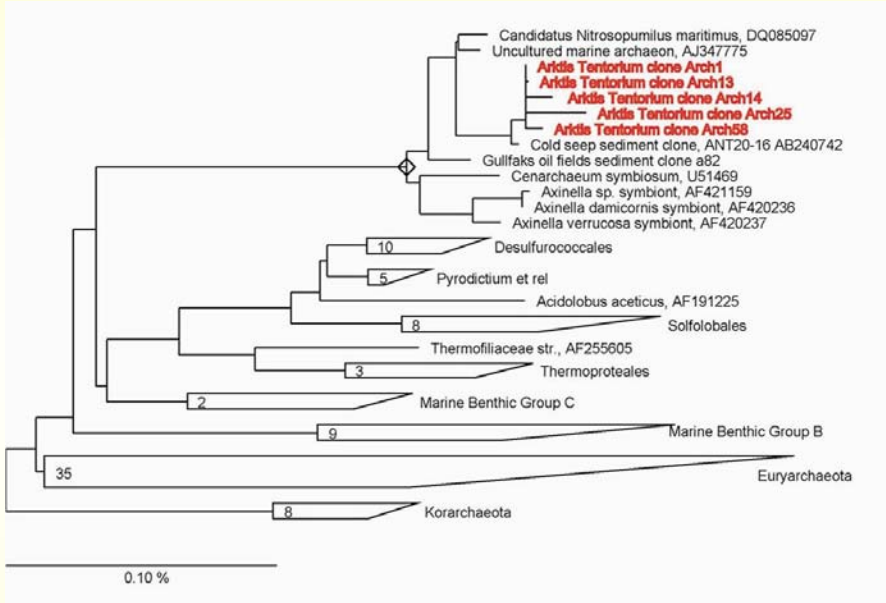
DAPI staining on sponge sections

Sponge with few microbes

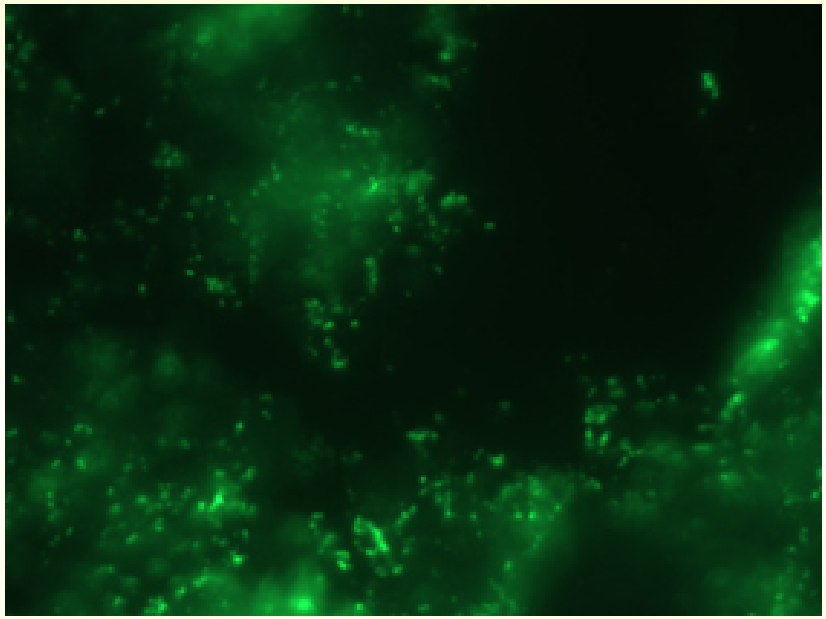
Sponge with many microbes

Exploring microbial diversity in sponges:

Who is out there?



16S rRNA approach: Clone libraries + phylogenetic trees

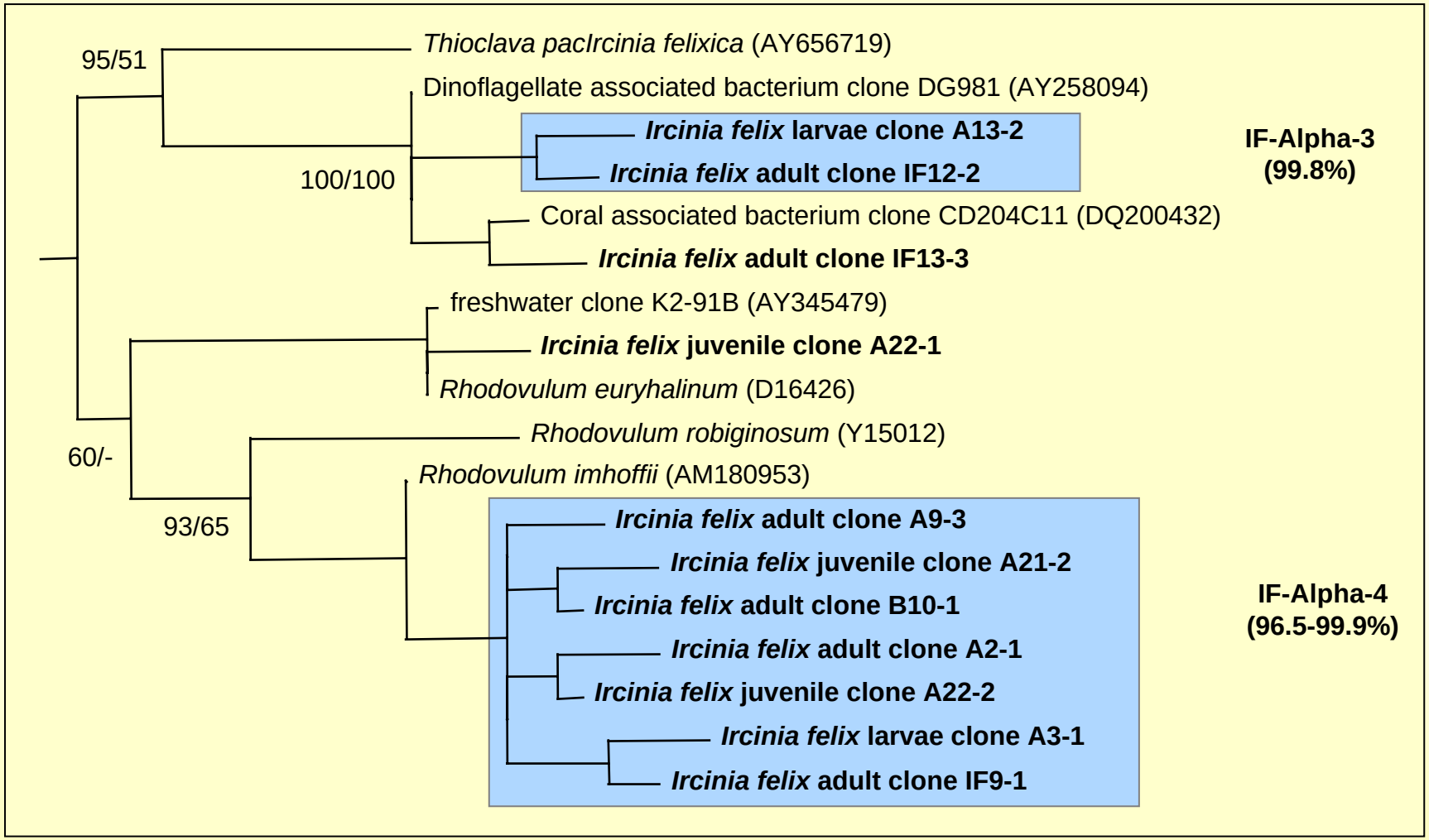


Fluorescence in situ hybridisation (FISH)

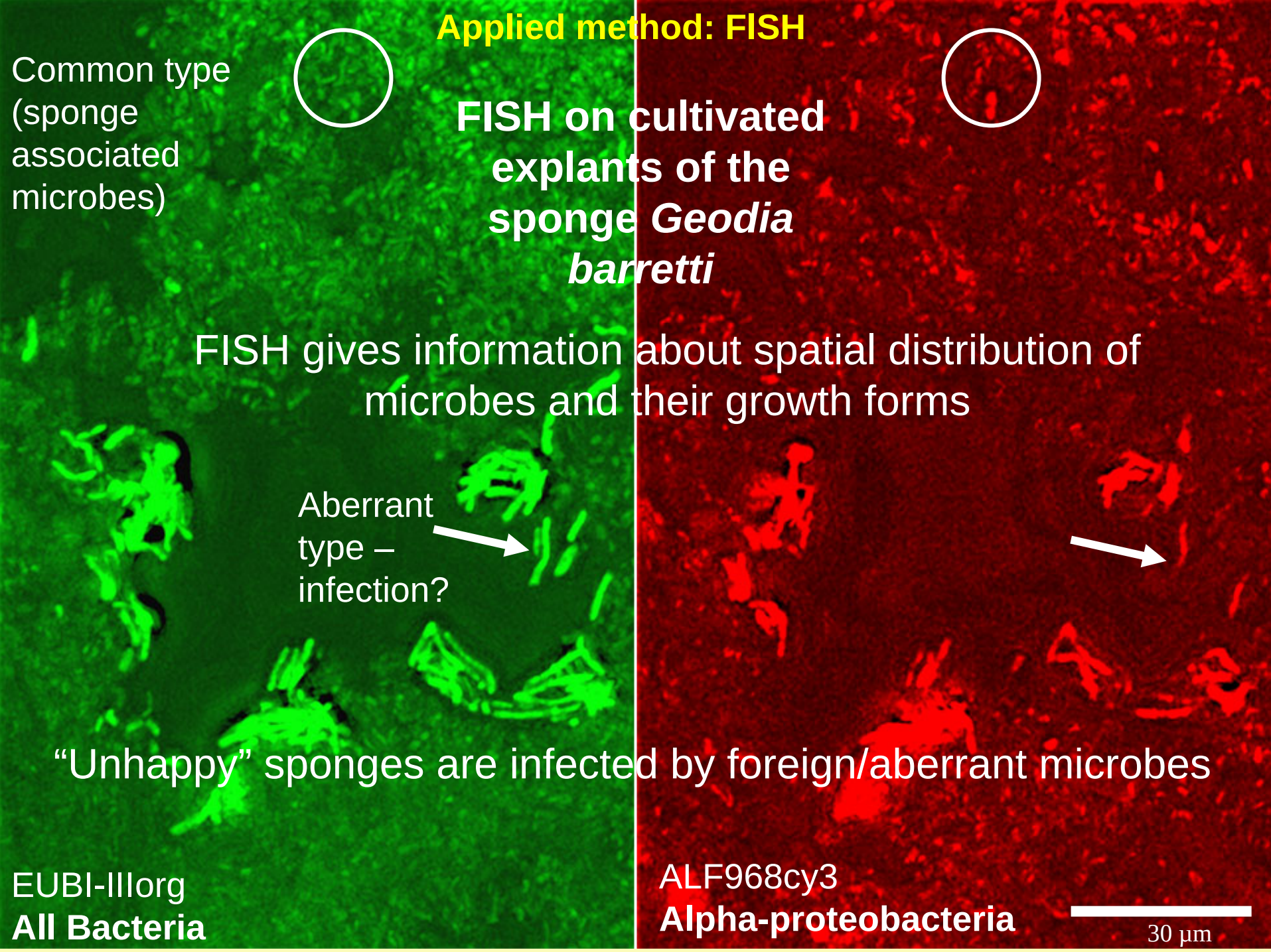
Cultivation-independent studies most popular !

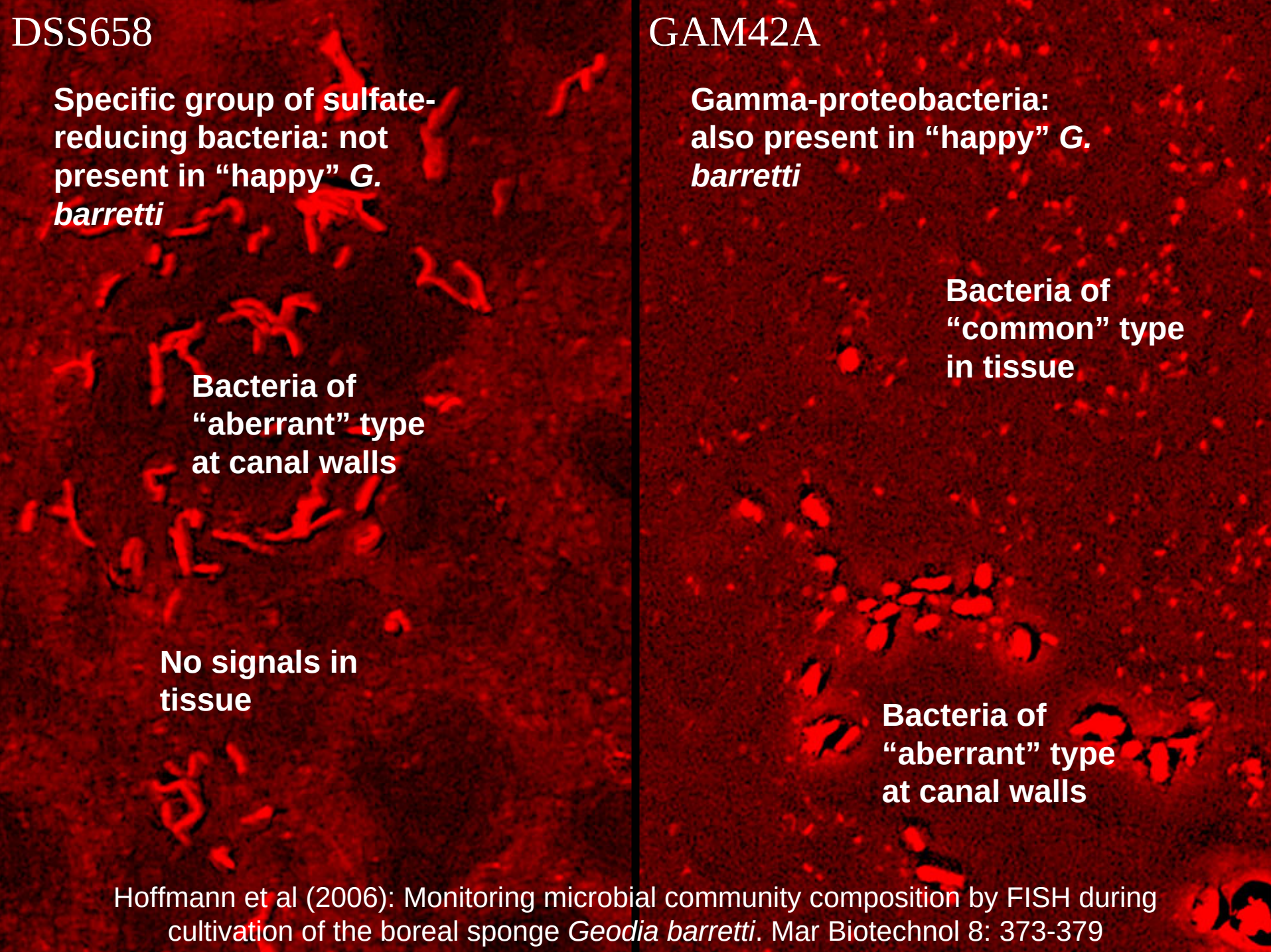
Master thesis of A. Braunshausen

16S rRNA approach can give information about vertical symbiont transmission



Schmitt, Weisz, Lindquist, Hentschel. AEM submitted


Applied method: FISH
Common type (sponge associated microbes)
FISH on cultivated explants of the sponge *Geodia barretti*
FISH gives information about spatial distribution of microbes and their growth forms
Aberrant type – infection?
“Unhappy” sponges are infected by foreign/aberrant microbes
EUBI-IIIorg
All Bacteria
ALF968cy3
Alpha-proteobacteria
30 µm



Hoffmann et al (2006): Monitoring microbial community composition by FISH during cultivation of the boreal sponge *Geodia barretti*. Mar Biotechnol 8: 373-379

Sponge cells:
Coordination/communication without a neuronal network

The language of the sponge-microbe system: chemical communication

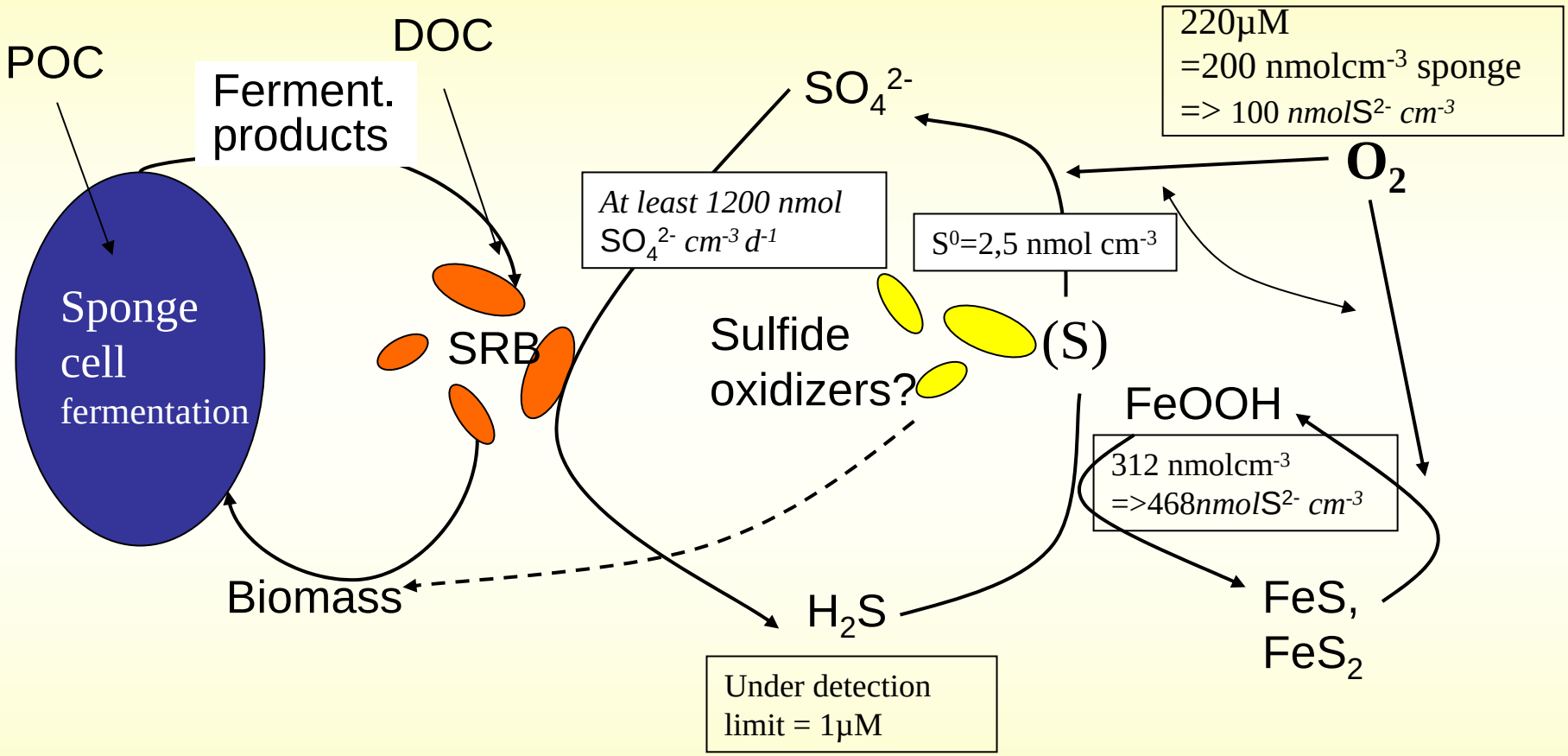
Exploring function of sponge microbes:

What are they doing?

Sponge-microbe interactions??

Applied method: tracer studies with radioisotopes

<u>Proving</u> activity of sponge associated sulfate reducing microbes in the sponge *Geodia barretti* by tracer studies (radioisotope $^{35}SO_4^{2-}$)

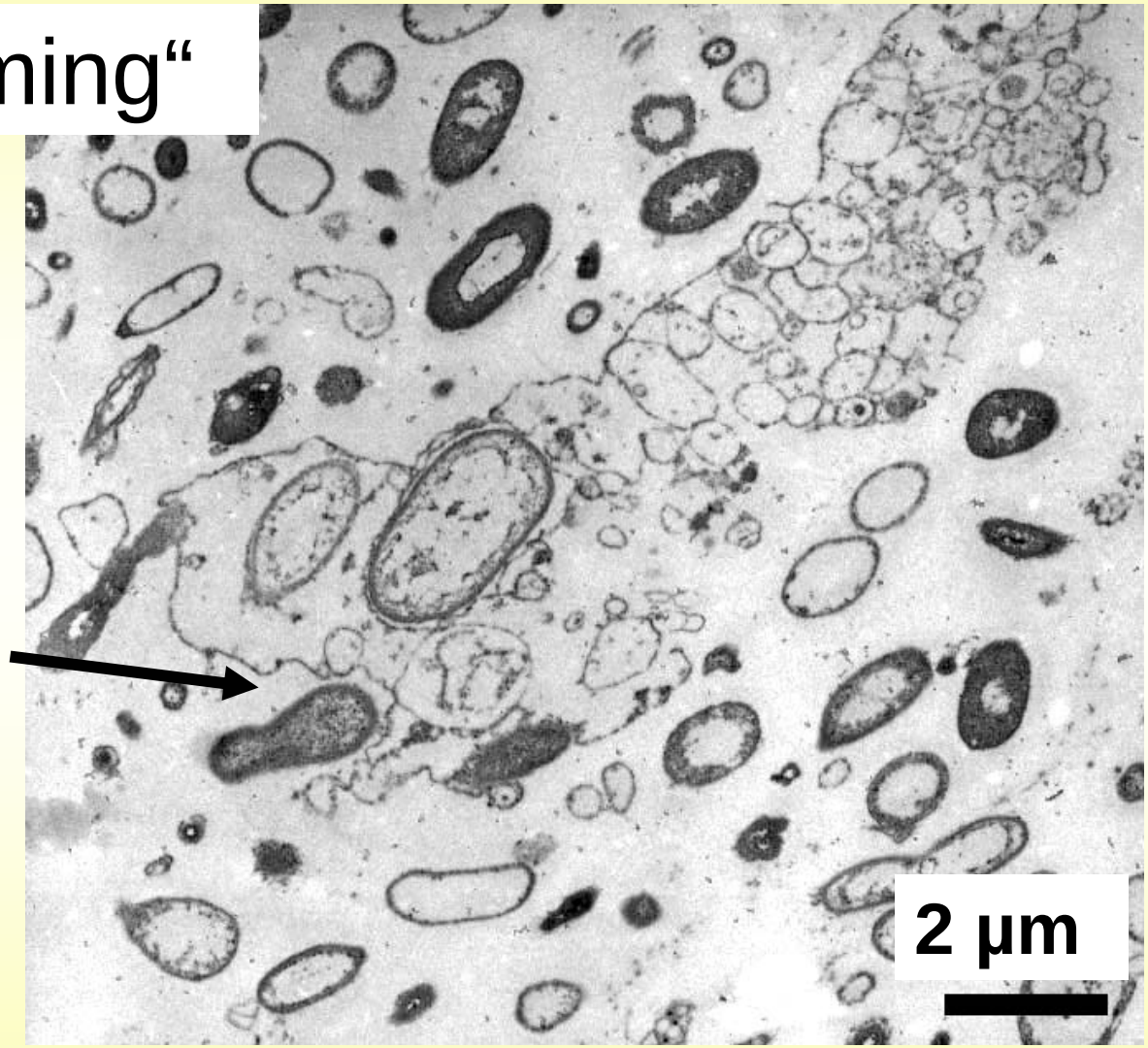


<u>Proposing</u> mutualistic interaction by "microbial farming"

Hoffmann et al (2005): An anaerobic world in sponges. Geomicrobiology Journal 22: 1-10

Mutualistic interaction 1:

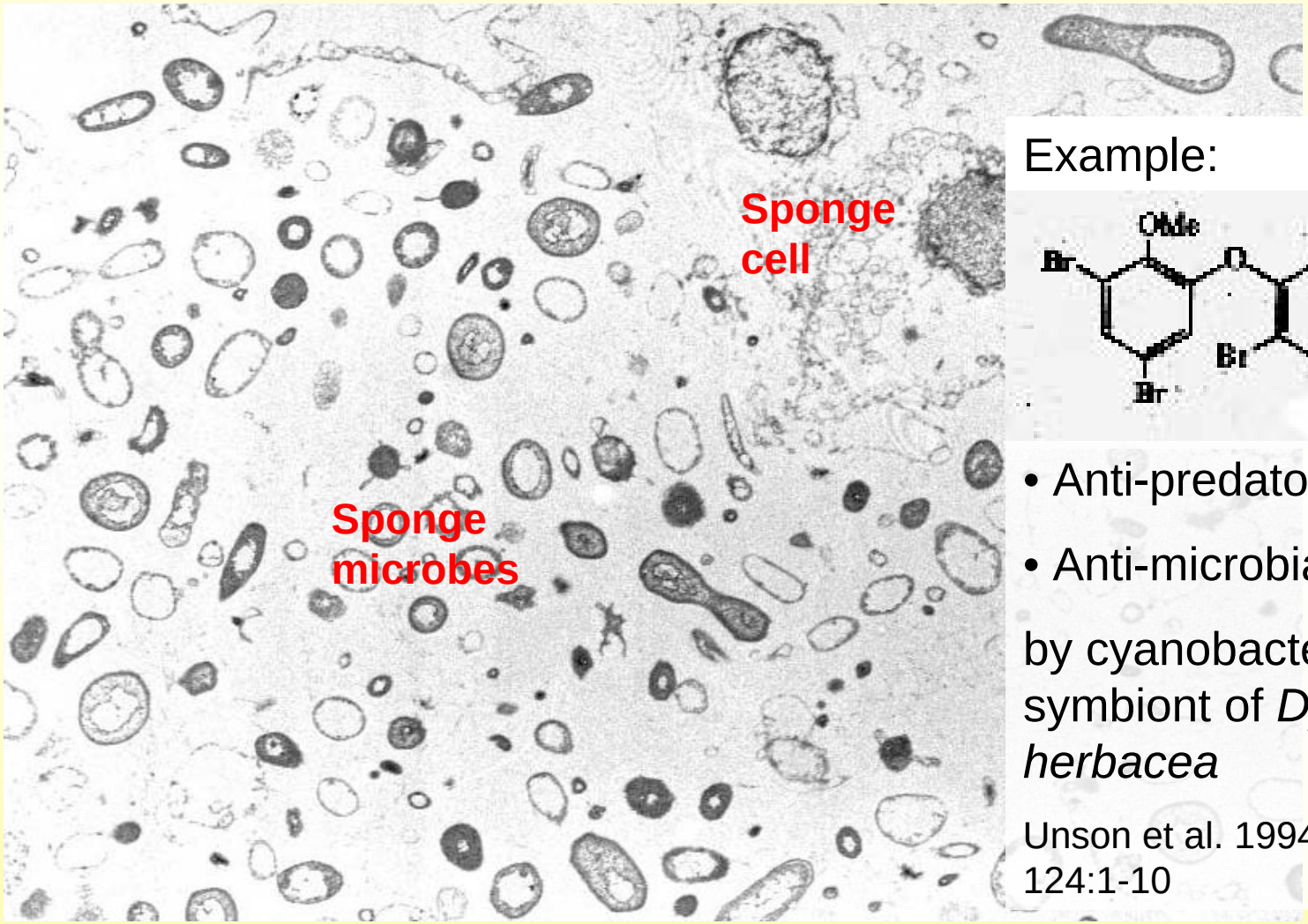
„Microbial farming“



“Microbial farming” as observed in *G. barretti* (TEM)

Mutualistic interaction 2:

Synthesis of natural products



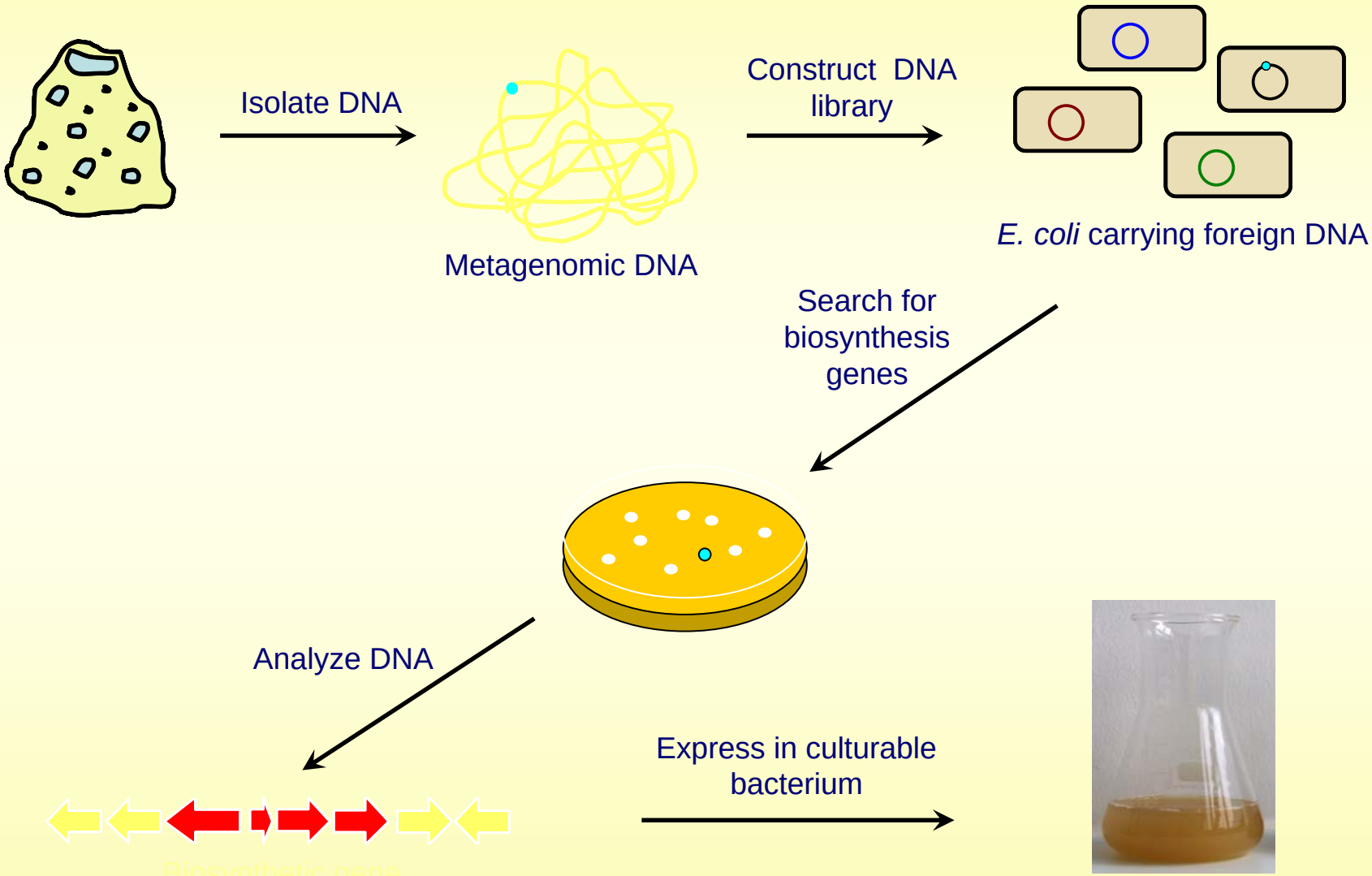
Example:

- Anti-predator
- Anti-microbial

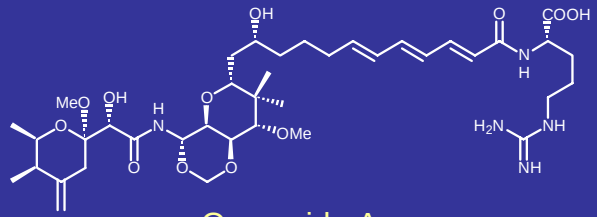
by cyanobacterial symbiont of *Dysidea herbacea*

Unson et al. 1994: Mar.Biol 124:1-10

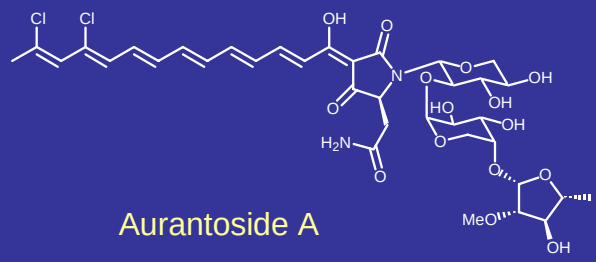
A Metagenomic Strategy to Study Symbiotic Producers



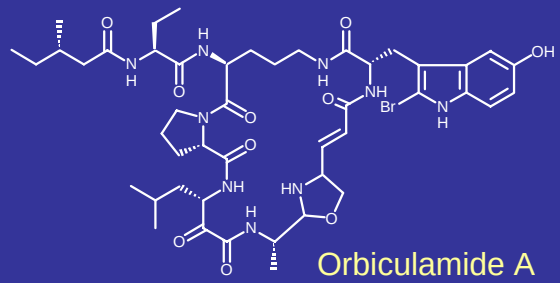
Model Sponge *Theonella swinhoei*



Onnamide A

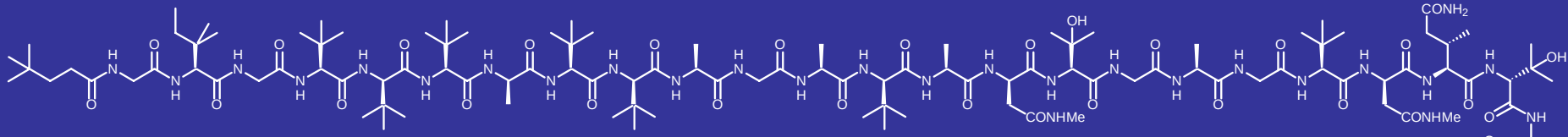


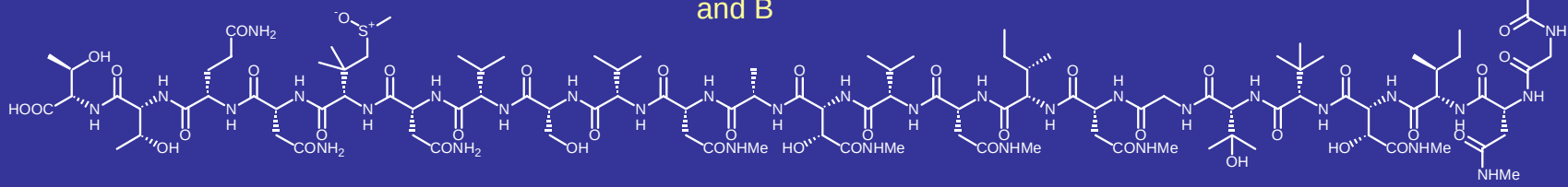
Aurantoside A



Orbiculamide A

Pseudotheonamide A_2



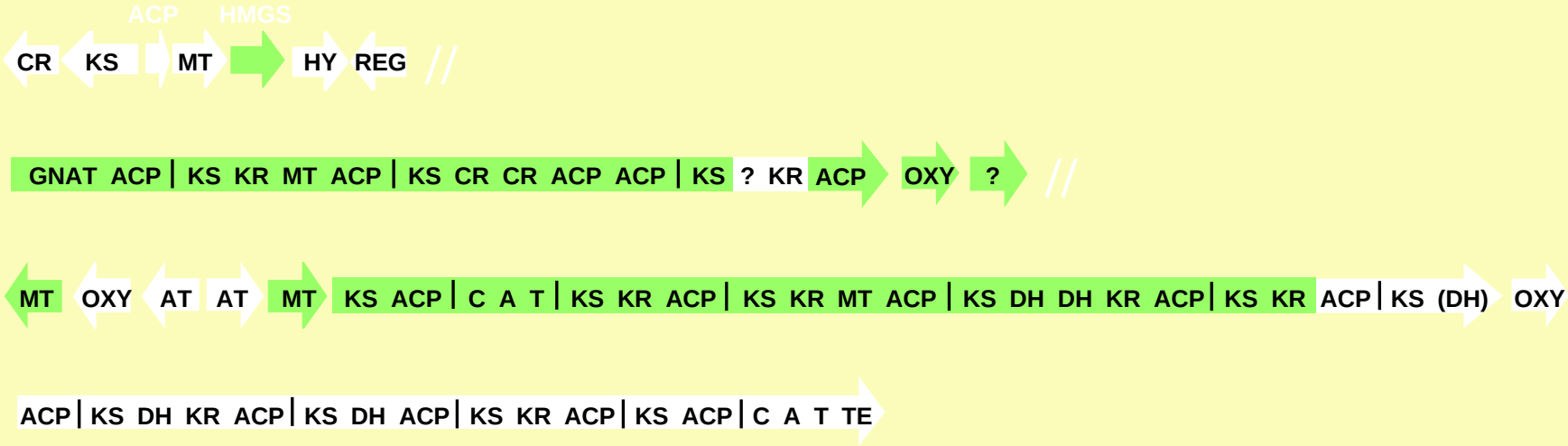


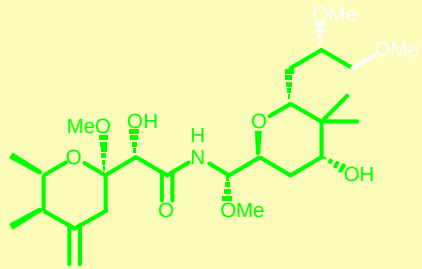
Polytheonamides A and B

S. Matsunaga, N. Fusetani, *Curr. Org. Chem.* **7**, 945-966 (2003)

The First Natural Product Genes from a Sponge

Pederin genes **Onnamide genes**





Pederin

Genes are of bacterial origin!

J. Piel, G. Wen, D. Hui, D. Butzke, M. Platzer, S. Matsunaga, N. Fusetani, *Proc. Natl. Acad. Sci. USA* **101**, 16222-16227(2004)

- Microbes are important players in the world of chemical communication

(though often overlooked at first glance)

- Culture-independent methods provide powerful tools to identify target microbes (or genes)

- Challenge: directly link phylogeny (who is out there?) to function (what are they doing?)

- Application: biotechnological production of secondary metabolites!

Thanks to:

Rudi Amann, Nicole Dubilier, Anke Meyerdierks, Michael Richter, Jörn Piel, and various internet sources who provided figures, slides and knowledge for this talk!