Technical Note TN9

Test Plan and Procedure Document for long-term axeny study

July 2002

Number of pages including front page: 67

ESA/ESTEC

ESTEC contract number 15680/01/NL/ND

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DOCUMENT CHANGE LOG

Version	Issue	Date	Observation
0	1	27/03/2002	Draft 1
1	1	30/08/02	Draft 2
1	1	14/01/03	Final version

TABLE OF CONTENTS

1. PCR-based genomic analysis of microbial samples 11

1.1. Aims and outline 11

1.2. Development of highly specific primers 11

1.3. Materials and methods 16

2. Horizontal gene transfer in the MELISSA loop 18

2.1. Presence of plasmids, free DNA and Viruse in the MELISSA strains 18

2.1.1. Presence of plasmids 18

2.1.1.1. Extraction of plasmid DNA 18

2.1.1.2. Plasmid DNA extraction of E. Coli strains Based on the method of birnboim and Doly 18

2.1.2. Presence of free DNA 19

2.1.2.1. Determination of total extracellular DNA 19

2.1.2.2. Determination of transformable DNA 19

2.1.3. Presence of Viruses 19

2.1.3.1. Extraction and concentration of bacteriophages in the sample 20

2.1.3.2. Extraction of phage-encapsulated DNA 20

2.1.3.3. PCR amplification 20

2.2. Conjugation and retrotransfert 20

2.2.1. Isolation of plasmids containing selectable markers 20

2.2.1.1. Heterologous conjugation experiment 21

2.2.1.2. Heterologous retrotransfer experiment 21

2.2.1.3. Homologous conjugation experiment 21

2.2.1.4. Homologous retrotransfer experiment 21

2.2.2. Isolation of mobilizing plasmids using triparental exogenous isolation 22

2.3. Analysis of plasmids, free DNA and viruses 22

2.3.1. Analysis of plasmids 22

2.3.1.1. Characterization of incompatibility group by hybridisation 22

2.3.1.2. Incompatibility testing using a mating protocol 23

2.3.2. Analysis of viruses 23

2.3.2.1. Phage identification 23

2.4. investigating general dissemination 23

2.4.1. Testing Melissa strains as recipients 23

2.4.2. Testing Melissa strains as donors 23

3. Proteomic research.. 25

3.1. Membrane or surface accessible proteins detection 25

3.1.1. Determination of protein concentration 25

3.1.2. Specific labelling of bacterial surface proteins 25

3.1.3. Two-dimensional gel electrophoresis procedure 25

3.1.3.1. Sample preparations 26

3.1.3.2. Two-dimensional gel electrophoresis 26

3.1.3.3. Evaluation of 2-DE patterns 27

3.1.4. Identification of proteins 27

3.1.4.1. Electroblotting 28

3.1.4.2. N-terminal aminoacid microsequencing analysis 28

3.1.4.3. Mass spectrometry 28

A. Enzymatic digestion 28

B. Electrospray ionisation mass spectrometry 29

C. Matrix-assisted laser desorption ionization/time-of-light mass spectrometry 29

3.2. Intact cell MALDI-TOF-MS 29

3.2.1. Sample preparation. 30

3.2.2. Mass spectrometry measurements 30

3.2.3. Data analysis 31

3.3. Supernatant cell MALDI-TOF-MS 31

3.3.1. Sample preparation. 32

3.3.2. Mass spectrometry measurements 32

3.3.3. Data analysis 32

4. Analysis by flow cytometry 33

4.1. Basic thechnical flow cytometric requirements 33

4.1.1. Sheath fluid 33

4.1.2. Calibration of the cytometer flow rate 33

4.1.3. Accuracy of measurements 34

4.1.4. Detection and threshold 34

4.1.5. Data acquisition 34

4.2. Fresh or Fixed Material? Preservation and storage of EPAS and UAB samples 35

4.2.1 Preservation and storages of the samples 35

4.3. Optimisation of cell individualisation for Arthrospira Platensis filaments 35

4.4. Estimation of the abundance of the MELISSA strains and any bacterial contaminants 36

4.4.1. Materials 36

4.4.2. Protocol 36

4.5. Determination of size variations of MELISSA strains and any bacterial contaminant 37

4.5.1. Flow cytometry protocol 37

4.6. Determination of genome size of MELISSA strains and any bacterial contaminants 37

4.6.1. Materials 38

4.6.2. Protocol 38

4.7. Assessment of viability, survival, membrane permeability and membrane potential and cell cycle of MELISSA strains and any bacterial contaminant 40

4.7.1. Determination of membrane permeability of MELISSA strains and any bacterial contaminant 40

4.7.1.1. Membrane permeability indicators 40

4.7.1.2. Membrane permeability assessment 40

4.7.1.3. Flow cytometry measurements 41

4.7.2. Determination of membrane potential of MELISSA strains and any bacterial contaminant 41

4.7.2.1. Membrane potential-sensitive dyes 41

4.7.2.2. Determination of membrane potential 41

4.7.2.3. Flow cytometry measurements 41

4.7.3. Determination of metabolic activity (esterase activity) of MELISSA strains and any bacterial contaminant 41

4.7.3.1. Materials 42

4.7.3.2. Equipments 42

4.7.4. Determination of intracellular pH variations in MELISSA strains and any bacterial contaminant 42

4.7.4.1. CFdase staining protocol 43

4.7.5. Determination of respiratory activity in MELISSA strains and any bacterial contaminant 43

4.7.5.1. Protocol 43

4.7.6. Cell cycle analysis of MELISSA strains any bacterial contaminants 44

4.7.6.1. Coefficient of variations 44

4.7.6.2. Protocol 45

4.8. Flow cytometric detection of virus in MELISSA strains by DNA staining and fluorescent detection 46

4.8.1. Materials 47

4.8.2. Protocol 47

4.9. Flow cytometric of viruses 48

4.9.1. Viruses used 48

4.9.2. Viruses preparation and staining procedure to optimise 50

4.10. Flow cytometric evaluation analysis of MELISSA strains viability following viral infection 51

4.10.1. Protocol to be optimised 51

4.10.1.1. Live/dead cell staining 51

4.10.1.2. Experimental set-up 52

4.10.1.3. Bacterial and virus count 52

4.10.1.4. Flow cytometry 53

5. References 55

5.1. References of PCR-based genomic analysis of microbial samples 55

5.2. References of horizontal gene transfer in the Melissa loop 55

5.3. References of proteomic research 56

5.4. References of cytometry analysis 56

6 Addendum 60

LIST OF FIGURES

Figure. 1: The ARB database of 16S rRNA sequences (ssjun02.arb).

Figure. 2: Search the target organism in ARB.

Figure. 3: Probe design window of ARB.

Figure. 4: Search results window.

Figure. 5: Results sheet of the NCBI listing matches of the oligonucleotide probe sequence with entries of the database.

Figure. 6: Layout of used PCR primers for detection /identification purposes

Figure 7. 2.DE of total proteins of *Arthrospira* and *R. Rubrum*

Figure 8 : Estimation of the genome size of the photosynthetic prokaryote *Prochlorococcus* (Proc), using a culture of *E. coli* pretreated with rifampicin as internal reference.

Figure 9. Example of FDA staining: Daily frequency distributions of FDA derived fluorescence (relative units) in *Chrysochromulina hirta* kept in darkness with (Chr+) and without (Chr-) addition of bacteria

Figure 10. Cell cycle analysis on a natural sample of Prochlorococcus collected in the Pacific Ocean (Cast 94, 65 m). The coefficient of variation of the G,-like peak is an indicator of the quality of the DNA staining.

Figure 11. DNA frequency distributions (relative units) in noninfected and virus-infected cultures of *Micromonas pusilla* obtained by flow cytometry using the nucleic acid–specific green fluorescent stain SYBR-I.

Figure 12. Histograms of green viral nucleic acid fluorescence of different viruses (see Table 1 for details concerning nomenclature and characteristics of the viruses) after staining with 1 x 10^{-4} of the commercial stock solution of SYBR Green I.

Figure 13. Representative biparametric plot of green fluorescence versus chlorophyll red fluorescence representing axenic algal populations after staining with SYTOX-Green for 10 min in the dark at room temperature.

Figure 14. Representative biparametric plot of green fluorescence versus chlorophyll red fluorescence representing axenic algal populations after staining with Calcein-AM for 1 h under culture conditions.

ABBREVIATIONS

ACNPV	Autographa California Polyhedrosis Virus
BHR	Broad Host Range (plasmids)
BSA	Bovine serum Albumin
bp	Base pair
CNBr	Cyanogene Bromide
CTC	5-Cyano-2,3-ditolyl Tetrazolium Chloride
CV	Coefficient of Variation
dCTP	Deoxycytidine TriPhosphate
DiBAC4(3)	bis-(1,3-dibutylbarbituric acid) trimethine oxonol {$DiBAC_4(3)$}
DiOC2(3)	Diethyloxacarbocyanine iodide
DMSO	DiMethylSulfoxide
DNA	DesoxyriboNulceic Acid
dNTP	DeoxyNucleotide TriPhosphate
DTE	Dithioerythreitol
ESI-MS/MS	Electrospray ionisation tandem mass spectrometry
FDA	Fluorescein DiAcetate
FISH	Fluorescent In Situ Hybridization
G+C	Guanine and Cytosine content
HIV-1	Human Immunodeficiency Virus 1
HPLC	High Pressure liquid Chromatography
HSV-1	Human Herpes Simplex Virus 1
ICM-MS	Intact Cell MALDI-TOF mass spectrometry
Immobiles NL	Immobiles Non Linear
pI	Isoelectric Point
ISAV	Infectious Salmon Anemia fish Virus
ISS	International Space Station
IPG	Immobilines polyacrylamide gels
ITS	Internally Transcribed Spacer
Kbp	Kilo base pair
MELISSA	Microbial Ecological LIfe Support System Alternative
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization/Time-of-Flight mass spectrometry
MALDI LR	Matrix-Assisted Laser Desorption Ionization/Time-of-Flight linear and reflectron
nanoESI	Nano ElectroSpray Ionization
nt	nucleotide
PAR	Photosynthetic Active Radiation
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PMSF	Phenymethylsulfoxide
PV-1	Human Poliovirus 1
PTH	Phenythiohydantoine
RNA	RiboNucleic Acid
RMS	Root Mean Square
RP-HPLC	Reverse Phase –High pressure Liquid Chromatography

RPM	Revolution Per Minute
RT	Room Temperature
SAPD	Surface Accessible Proteins Detection
SCM-MS	Supernatant Cell MALDI-TOF-MS
SDS	Sodium DodecylSulfate
SDS-PAGE	Sodium DodecylSulfate PolyAcrylamide Gel Electrophoresis
TCA	TriChloroacetic Acid
TE	Tris Ethylenediamine buffer
Tm	Melting Temperature
2-DE	Two-Dimensional Electrophoresis
5(6)-CFDase	CarboxyFluorescein Diacetate

In an initial approach we will use three different approches (PCR, proteomic research and flow cytometry analysis) to detect artificial contamination in a spiking experiment in which a pure culture of Rhodospirillum rubrum is contaminated with Arthrospira platensis and vice versa. In a parallel experiment, pure cultures of R. rubrum and A. platensis will be contaminated with Ralstonia metallidurans, the model organism that was choosen in order to study possible gene transfer.

1. PCR-BASED GENOMIC ANALYSIS OF MICROBIAL SAMPLES

1.1. AIMS AND OUTLINE

In order to detect and unambiguously identify bacterial strains we decided to develop PCR primers that are directed towards the 16S ribosomal RNA encoding DNA region (16S rDNA). The small subunit ribosomal RNA gene is highly conserved throughout the three domains of life (Eukarya, Bacteria, and Archaea), but also contains variable regions that may be used as signature sequences. The presence of highly conserved and more variable regions allows amplification (and hitherto detection) by PCR. The principle of PCR and the general technical aspects of PCR-based amplification (i.e. primer design, procedures and methods) have been discussed extensively in TN70.8.

In an initial approach we will use PCR to detect artificial contamination in a spiking experiment in which a pure culture of *Rhodospirillum rubrum* is contaminated with *Arthrospira platensis* and vice versa. In a parallel experiment, pure cultures of *R. rubrum* and *A. platensis* will be contaminated with *Ralstonia metallidurans*, the model organism that was choosen in order to study possible gene transfer. The four spiking experiments will be undertaken using 16S rDNA based primers that are highly specific for the respective bacterial species. Note that only cross-contamination of these three bacterial species is being investigated, and that external contamination by other organisms can not be monitored with this approach. This would entail the design of a PCR primer pair that detects any prokaryotic and /or eukaryotic species except for the underlying bacterial species, e.g. *A. platensis*. In our opinion the development of such primer pairs is quasi-impossible using 16S rDNA as a target. However, it is possible that strains or even species lack a particular gene or pathway (operon) that is otherwise present in all other lineages of the bacterial domain (and preferably also in the other 2 domains). For instance, it is well known that Buchnera species lack the – otherwise omnipresent - *recA* gene, presumably owing to its endosymbiont status and that chlamydia lack FtsZ, otherwise essential to bacterial cell division. Unfortunately, the genome sequences of *A. platensis* and *R. rubrum* are ‘terra incognito’ and the genome sequence of *R. metallidurans* is not yet completed, preventing genome-wide comparative analysis and the search for a suitable target. In the future, given that sufficient genome data are available, such a search may be undertaken or alternatively, Melissa strains may be genetically altered as to contain or lack a particular marker gene.

1.2. DEVELOPMENT OF HIGHLY SPECIFIC PRIMERS.

As a first measure, Probase[1] (Very recently, a new website, **probeBase**[2], was established by Prof. Michael Wagner and collaborators of the Microbial Ecology group at the TU Munchen. This database contains published rRNA-targeted oligonucleotide probe sequences, DNA microarray layouts, and associated information. An interesting feature of proBase is the possibility to search for target organisms or a particular probe name. See TN70.8) was checked for the presence of suitable 16S rRNA derived oligonucleotide probes. No probes were available for *Arthrospira*, *Rhodospirillum*, and *Ralstonia*. As outlined in TN70.8, suitable PCR primers for 16S rDNA based detection / identification can be searched for *ab initio* by using the Probe Design facilities of the ARB software[3]. The procedure is as follows:

- Load the database. The most recent database of small subunit rRNA genes was used (11 June 2002). This database contains 17,825 prokaryotic (of which 17,034 bacterial and 791 archaeal) and 6,793 eukaryotic sequences **(Fig. 1)**.

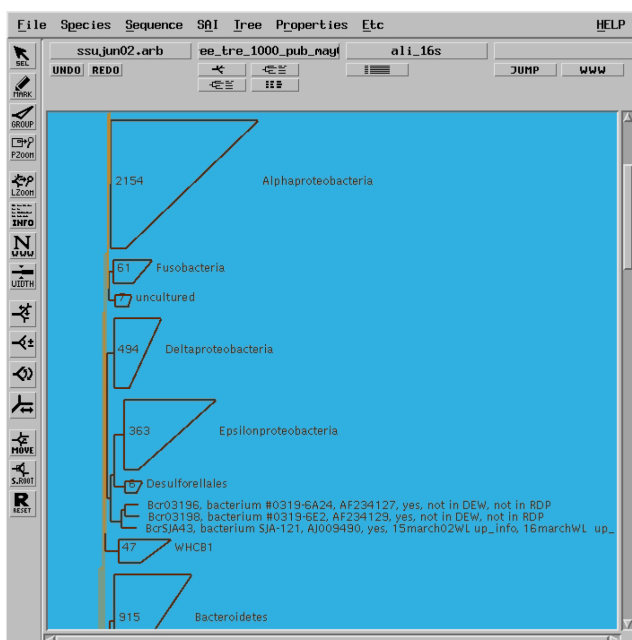


Fig. 1: The ARB database of 16S rRNA sequences (ssjun02.arb)

- Search for the target organism(s) (e.g. *Ralstonia metallidurans*) for which a probe needs to be designed by filling in the search field(s). Click on “SEARCH” **(Fig. 2)**. Mark the target sequence(s) (*) by clicking on “MARK/UNMARK”, thereby unmarking all other species in the database.

[1] http://www.microbial-ecology.de/probebase/
[2] http://www.microbial-ecology.de/probebase/
[3] http://www2.mikro.biologie.tu-muenchen.de/arb/

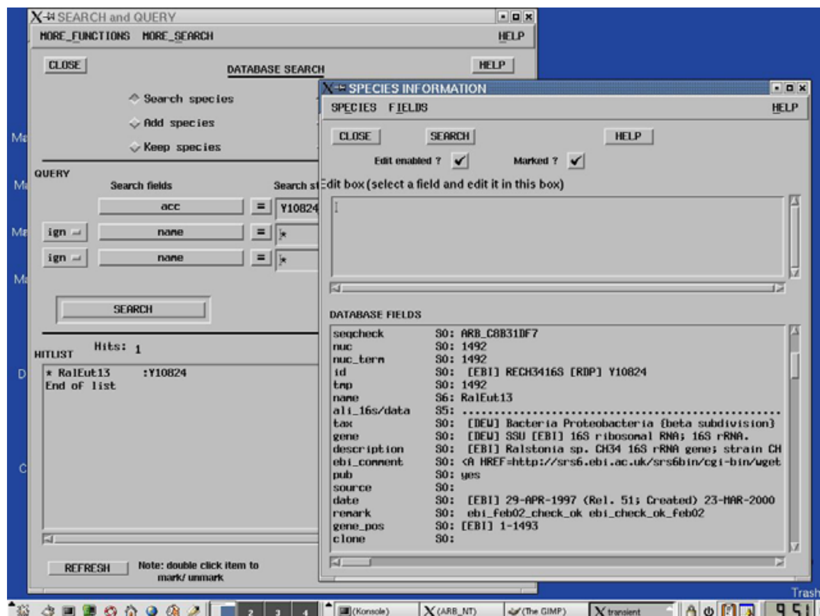


Fig. 2: search the target organism in ARB

- Open probe design window (Etc/Probe/Functions/Probe Design…). Select a PT_SERVER (in this case SSU_rRNA.arb) and define the parameters **(Fig. 3)**. Length of output (set at 0) is the number of sequences reported in the results list, Max. non group hits means highest specificity, Max. hairpin bonds (set at 3) avoids cruciform formations, Min. main group hits (set at 50%; default) means that at least half of the target organisms contain a perfect match. Physical parameter follow common sense, e.g. temperature range set at 45-65 °C and GC%-content at 40-60.

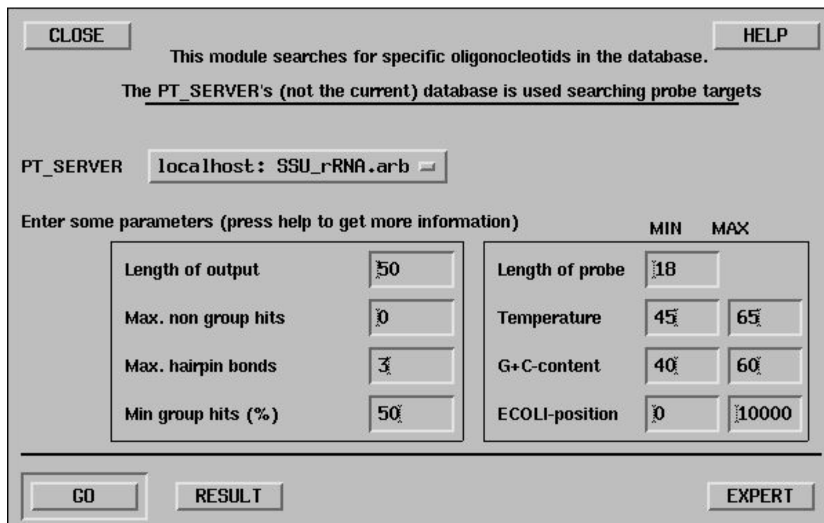


Fig. 3: Probe design window of ARB

- Click on "GO". Results appear in PD RESULT window **(Fig. 4)**. Potential target sequences and associated parameters such as length, location (absolute position; *E. coli* number), G+C% content, and melting temperature are displayed. Close variations of a potential probe site (e.g. A) are given, shifted one or more nucleotides upstream

(A+, A++, etc.) or downstream (A-, A--, etc.). A set of 20 colums indicate predicted aspecific probe characteristics when lowering the annealing temperature (n non-group species for 0.3°C decrease per column).

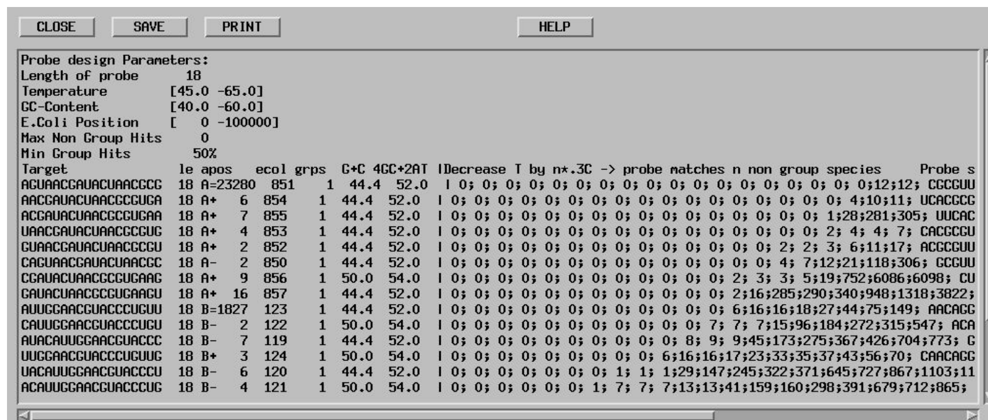


Fig. 4: Search results window

- Confirm probe specificity against all publicly available DNA sequences using the BLASTN program at the National center for Biotechnology Information (NCBI) website[4]. An exact match will have a score (bits) in the BLAST Search Results descriptions output **(Fig. 5)** twice the value of the number of nucleotides of the submitted probe sequence (e.g. a 18-mer will have an exact match score of 36). Sequences with mismatches to the probe sequence will have scores less than 36. The database (GenBank+EMBL+DDBJ+PDB) contains 1,332,196 sequences with a total of 6,002,469,361 total nucleotides.

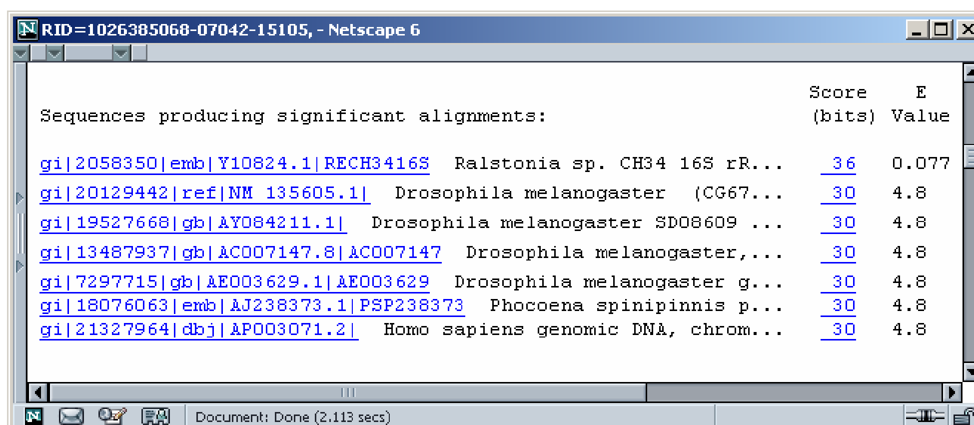


Fig. 5: Results sheet of the NCBI listing matches of the oligonucleotide probe sequence with entries of the database.

- *RESULTS*

Using the probe search facility of the ARB software package a list of suitable candidate probe sequences was prodiced for each of the three target organisms (*A. platensis*, *R. rubrum*, *R. metallidurans*) - (**Addendum A**). However, for *A. platensis*, no 16S rRNA based probe

[4] http://www.ncbi.nlm.nih.gov/

could be found, i.e. the proposed probes all fell within the 16S-23S intergenic region (known as the Internally Transcribed Spacer or ITS; Baurain *et al.*, 2002). A choice was made taking into account possible palindromic sequences, sequence bias (avoiding long stretches of nucleotides such as AAAAA), G+C% content, and predicted Tm. Also, the actual position of the probe sequence was taken as a criterium as this determines the amplicon size.

The following primer sequences are proposed:

code	target	5'-3' sequence	length	GC%	Tm
EUB338F	ACTCCTACGGGAGGCAGC	ACTCCTACgggAggCAgC	18	66,7	55
EUB338R	ACTCCTACGGGAGGCAGC	gCTgCCTCCCgTAggAgT	18	66,7	55
UNIV1390F	TTGTACACACCGCCCGTC	TTgTACACACCgCCCgTC	18	61,1	53
UNIV1390R	TTGTACACACCGCCCGTC	gACgggCggTgTgTACAA	18	61,1	53
RRUB999R	CGTGACACTTCCAGAGAT	ATCTCTggAAgTgTCACg	18	50	54
RMET110F	CATTGGAACGTACCCTGT	CATTggAACgTACCCTgT	18	50	54
APLA1759R	AGTTGGGGTGAGGTAGTC	gACTACCTCACCCCAACT	18	55,6	54

Verification of sequences. **The 16S rDNA and ITS probe sequences were verified using Probe Match of the Ribosomal Database Project website[5]. Attention was given to the uniqueness of the 3'- end region of the PCR primers to avoid aspecific DNA chain elongation. All three primers (RRUB999R, RMET110F, APLA1759R) passed this check.**

This results in the following amplicon sizes:

species	primer pair	amplicon size
Rmet	RMET110F / EUB338R	233
Apla	UNIV1390F / APLA1759R	469
Rrub	EUB338F / RRUB999R	654

The primer binding sites and expected amplification products are schematically represented in **Fig. 6**. The *E.coli* 16S rRNA gene (1542 nt) is used as reference. Note that for *A. platensis* no suitable probe could be found within the boundaries of the 16S rRNA. Suitable *A. platensis* ITS probe sequences proposed by ARB were however rejected because they map partly within the $tRNA^{Ile}$ or $tRNA^{Ala}$ genes, which are highly conserved (but these gene sequences are not necessarily part of ARB entries). Consequently, a suitable primerbinding site was choosen manually 20 nt downstream of $tRNA^{Ala}$. Sequence details can be found in **Addendum B**.

[5] http://rdp.cme.msu.edu/html/analyses.html

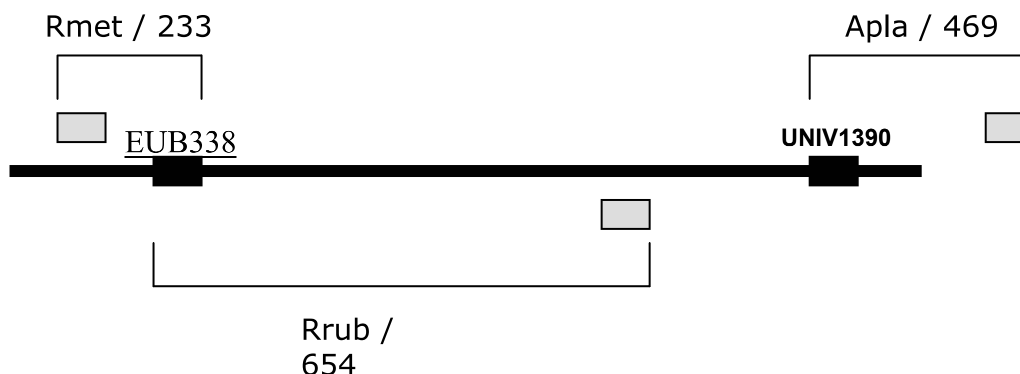


Fig. 6: Layout of used PCR primers for detection /identification purposes

The size of the amplicons was choosen to differ approximately 200 bp from each other. This has the advantage that PCR products can be loaded simultaneously in the same well of the gel. Multiplex PCR will be tried out, but in the first instance, separate PCRs will be performed.

1.3.MATERIAL AND METHODS.

- **DNA preparation:** several DNA extraction methods have been used successfully in the laboratory of ULg to extract DNA from *Arthrospira* strains, of quality enabling subsequent amplifications by PCR. They include a modification of the Pitcher method (Pitcher *et al.*, 1989) used with *Arthrospira* strains by Scheldeman *et al.* (1999) and Baurain *et al.* (2002). This method uses lyzozyme, proteinase K, guanidium thiocyanate and the classical extraction with phenol/chloroform. In addition, we have tested with success the commercial kit 'DNeasy Plant mini kit' of Quiagen. This kit uses a silica-gel membrane technology to isolate genomic DNA. The same procedure or similar will be used for the preparation of total DNA from *R. rubrum* and *R. metallidurans*.
- **PCR reaction mix:** PCR will be generally performed in voluma of 25-50µl (total volume), containing DNA, 1x PCR buffer (10mM Tris.HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$), 0.2 mM dNTPs, 50 ng of each primer, 1 mg ml^{-1} BSA (Sigma, USA), 1U Super Taq Plus polymerase with a proof reading activity (HT Biotechnology, UK. The composition of the reaction mix will be finetuned and may change in due course.
- **Reaction conditions:** 1 cycle of 5 min at 94°C, 10 cycles of 45 s at 94°C, 45 s at 57°C, 2 min at 68°C; 25 cycles of 45 s at 92°C, 45 s at 54°C, 2 min at 68°C and a final elongation step of 7 min at 68°C. Optimal annealing temperatures will be emperically determined using the iCycler (Bio-Rad, Hercules, CA) allowing gradient PCRs.
- **Experimental setup:** spiking will be performed by adding increasing amounts of contaminating cells to an axenic culture of *A. platensis* or *R. rubrum*. Total chromosomal DNA will be prepared from the axenic and spiked cultures and species specific PCR will be performed, at first in separate reactions, later in a multiplex

fashion. The proposed ratios of the number of original/contaminant cells for PCR detection is as follows:

1/0; E+5/1; 50,000; 10,000/1; 5,000/1; 1,000/1; 500/1; 100/1; 50/1; 10/1; 5/1; 0/1

PCR products will be loaded on a 1.2 % agarose gel and the detection level will be measured (e.g. at which level of contamination can a contaminant-specific amplicon be seen). All three primer pairs will also be tested on *E. coli* chromosomal DNA and “no DNA” as negative controls.

2. HORIZONTAL GENE TRANSFER IN THE MELISSA LOOP.

A study will be required to define the plasmid status of the three Melissa strains (C2 to C4): presence of plasmids, their number, their stability, and their genetic content. The presence of functional restriction endonucleases in the four Melissa strains should be tested as well: it may be important to check in which extent the four strains can “restrict” foreign DNA. The presence of conjugative plasmids in C1 bacteria will surely deserve a study even if we know that C1 will never go into contact with the rest of the Melissa loop and if the (moderate) thermophilic character of C1 may counterselect some undesired genetic determinants.

2.1. PRESENCE OF PLASMIDS, FREE DNA AND VIRUSES IN THE MELISSA STRAINS

2.1.1. *PRESENCE OF PLASMIDS*

2.1.1.1. *EXTRACTION OF PLASMID DNA (KADO AND LIU, 1981)*

1. grow a 3ml culture overnight in selective medium at 30°C
2. centrifuge 1.5ml during 10 min at 12000 rpm and remove the supernatant
3. resuspend the pellet in TE-buffer (0.04M Tris-acetate, 0.002M EDTA) (pH 7.9) with a sterile toothpick
4. add 0.2ml alkaline lysis buffer, gently shake for 15 min till the solution looks clean, homogeneous and viscous
5. incubate the mixture for 60 min at 68°C
6. add 0.6 ml fenol-chloroform solution (saturated with H_2O; 1:1 v/v) and agitate the mixture for 10 min until it appears milky white
7. centrifuge at 4°C for 15 min at 12000 rpm and rescue the supernatant in a sterile 1.5 ml tube (ready for electrophoretical analysis)
8. add 1 volume of diethylether and shake gently
9. centrifuge for 15 min at 12000 rpm
10. rescue the lower phase and incubate at 37°C with open cap
11. add 2.5 volumes of 100% ethanol (-20°C) and 0.1 volumes of 3M Na-acetat solution (pH4.8) (60 ml 5 M Na-acetat; 11.5 ml ijsazijn) and incubate for 1 hour at -70°C
12. centrifuge for 15 min at 12000 rpm and wash the pellet twice with 200 ml 70% ethanol
13. centrifugate for 6 min at 12000 rpm, dry the pellet and suspend it in 10 µl H_2O

2.1.1.2. *PLASMID DNA EXTRACTION OF E. COLI STRAINS BASED ON THE METHOD OF BIRNBOIM EN DOLY (BIRNBOIM AND DOLY, 1979)*

1. Grow a culture of 5 or 50 ml in LB medium overnight at 37°C, 100 rpm.
2. Centrifuge the 5 ml culture during 5 min at 11000 rpm at room temperature. A 50-ml culture is centrifuged at 5000 rpm during 5 min at 4°C, whereafter the pellet is resuspended in 10 ml 0.01 M $MgSO_4$ and centrifuged again, for another 5 min, at 4°C and 5000 rpm.

3. Add 100 µl or 2 ml SOL1 (50mM glucose; 10 mM EDTA; 25 mM Tris-Hcl ; pH8)to the pellet and resuspend by vortexing. Incubate during 5 min at room temperature.
4. Add 200 µl or 4 ml of freshly prepared SOL2 (0.2M NaOH ; 1% SDS) and mix by converting the tube a few times. Incubate for 5 min on ice.
5. Add 150µl or 3 ml SOL3 (3M Na-acetate, pH4.8) and mix by converting the tube a few times. Incubate for 15 min on ice.
6. Centrifuge the mixture for 10 min at 12000 rpm in an 1.5 ml tube or 30 ml glass corex tube that can resist centrifugation at high speed. Save the supernatant.
7. Extract the supernatant with 1 ml (9 ml) phenol-chloroform solution and separate the phases by centrifugation (20 min, 10000 rpm, 4°C).
8. Extract the water phase with 1 ml (9 ml) chloroform-isoamylalkohol solution and separate the phases by centrifugation (20 min, 10000 rpm, 4°C).
9. Rescue the water phase again and add 2.5 volumes of 100% ethanol (-20°C). Incubate the mixture for 30 min at –20°C.
10. Centrifuge the mixture during 10 min, at 10000 rpm and 4°C. Wash the pellet with 70% EtOH, and centrifuge again 10 min, at 10000 rpm and 4°C.
11. Dry the pellet completely. Then add respectively 50 µl or 200 µl sterile H_2O_{bidest}. DNA extracts can be stored at –20°C.

2.1.2 *PRESENCE OF FREE DNA (LORENZ ET AL., 1991)*

2.1.2.1. *DETERMINATION OF TOTAL EXTRACELLULAR DNA (LORENZ ET AL., 1991)*

1. filtrate the sample with a membrane filter (0.45 µm)
2. concentrate the filtrate twentyfold by ethanol precipitation and dissolution in water
3. add $CaCl_2$ and $MgCl_2$ at the final concentrations 1 and 10 mM respectively
4. measure fluorescence after addition of 0.1ml H33258 solution (10 µg/ml in 1 M NaCl, 0.5 M Na_2HPO_4, pH 7.4) to portions (0.9 ml) either untreated or incubated with DNaseI for 45 min at 37°C
5. make a standard using known concentrations of phage lambda DNA treated the same way as the samples and tested on fluorescence
6. fluorescence measurements: 354 nm excitation and 458 nm emission
7. the difference in fluorescence between untreated and DNaseI-treated (mixtures with known concentrations of DNA and the unknown samples) have to be taken as a result of DNA degradation
   F=F1-F2; F is the fluorescence of DNA; F1 is the fluorescence of the untreated sample; F2 is the fluorescence of DNaseI treated sample
8. calculate the concentration of free DNA in your sample

2.1.2.2. *DETERMINATION OF TRANSFORMABLE DNA*

In order to determine the presence of transformable DNA a bioassay will be developed using a specially genetically manipulated derivative of the highly competent *Acinetobacter* sp. BD413 (sui generic).

2.1.3. *PRESENCE OF VIRUSES (SANDER AND SCHMIEGER, 2001)*

2.1.3.1 *EXTRACTION AND CONCENTRATION OF BACTERIOPHAGES IN THE SAMPLE*

1. take the supernatant from a sample
2. remove coarse particles by centrifugation (5,000 rpm, 30 min)
3. filtration with standard folded filters (Schleicher & Schuell)
4. filter sterilize with a membrane filter (0.45 µm)
5. concentrate the sample by ultracentrifugation (3.85 ml tubes, 2h, 22.000 rpm, 5°C, Kontron TGA-50, rotor TST 28.38)
6. resuspend the sediment in 500 µl of DNase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$) and spiked with 1 µg of the control plasmid
7. to degrade nucleic acids, 100 µl of DNase I, 30 µl or RNase, and 30 µl of lysozyme have to be added, shaken overnight at 6°C
8. extract sample with an equal volume of chloroform and centrifuged for 30 min at 3,000 rpm at room temperature
9. incubate the supernatant with 100 µl of lysozyme for 1h at 37°C
10. repeat step 8
11. resuspend in 100µl of DNase, 30 µl of RNase, and 30 µl of lysozyme and incubate overnight at 6°C.

2.1.3.2. *EXTRACTION OF PHAGE-ENCAPSULATED DNA*

1. extract DNA using the phenol-chloroform method of Maniatis et al. (1982)
2 precipitate DNA at -80°C overnight with addition of 0.4 volumes of 5 M ammonium acetate and 2 volumes of 99% (vol/vol) ethanol
3 after centrifugation, wash DNA with 70% (vol/vol) ethanol
4 dry DNA under vacuum
5 resolve DNA overnight in double-distilled H_2O and shaking at 4°C
6 determine the DNA concentration spectrophotometrically at 260 nm
7 check quality and size of the DNA electrophoretically

2.1.3.3. *PCR AMPLIFICATION*

1. PCR amplification mixture: 10 ng target DNA, 5 µl 10×PCR buffer, 1.5 mM $MgCl_2$ (for 16S ribosomal DNA [rDNA]) or 2.5 mM $MgCl_2$ (for plasmid control amplification), 2 U of native Taq polymerase, 10 mM deoxynucleotide triphosphates (each) and 0.3 mM primers (each), with final reaction/mixture volume of 50 µl
2. cycling program: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 1 min, primer annealing at 50°C for 1 min, and DNA synthesis at 72°C for 2 min; final extension step at 72°C for 2 min

2.2.CONJUGATION AND RETROTRANSFER

2.2.1. *ISOLATION OF PLASMIDS CONTAINING SELECTABLE MARKERS*

Donor sample = *R. rubrum*; *A. platensis*; unknown consortium of compartment I
Recipient = *R. rubrum*; *A. platensis*; reference strain *R. metallidurans*
(Alternative for recipient strain: *gfp* labeled derivative of phe^- mutant of AE104)
Helper = *E. coli* CM120(pRK2023) (in some cases) (for the transfer of Tra^- Mob^+ Rep^+)

2.2.1.1. *HETEROLOGOUS CONJUGATION EXPERIMENT*

1. test growth of sample bacteria on rich medium with a range of antibiotics
2. select the antibiotic resistances that are not present in the sample for tagging recipient strains and the helper plasmid (in the following it is assumed that the sample bacteria are sensitive to rifampicin and kanamycin)
2. grow recipient culture rifampicin resistant *R. metallidurans* AE104 derivative AE815 overnight in 2 ml LB, shaking at 30°C
3. grow helper strain *E. coli* CM120(pRK2023) overnight in 2 ml LB Km (50 µg/ml), shaking at 37°C
4. Centrifuge a mixture of 500 µl of the AE815 culture and 500 µl of the sample, with and without 500 µl of the CM120(pRK2023) culture at 3,000 rpm
5. resuspend the pellet in 50 µl of LB broth
6. spot the suspension on a filter on an LB plate and incubate overnight at 30°C
7. resuspend the mating patch in 1 ml of 0.01 M $MgSO_4$ and dilute up to 10^{-8} in 0.01 $MgSO_4$
8. plate 100 µl of each dilution on selective plates (recipients on Schatz, 0.2% gluconate, Rif; helpers (when present) on LB, Km; donors on media containing a range of antibiotics; transconjugants on Schatz, 0.2% gluconate, Rif, + the same range of antibiotics used to select for donors)
9. for negative control, donor, recipients, (helpers), and samples were incubated separately on filters under the same conditions

2.2.1.2. *HETEROLOGOUS RETROTRANSFER EXPERIMENT*

1. follow the same instructions for the 'heterologous conjugation experiment' but use a recipient strain containing a transferable plasmid with an appropriate selectable marker
2. select for retrotransconjugants by a medium that selects for the donor organism containing the plasmid of the recipient strain (i.e. inheritance of characters in the conjugative plasmid bearing strain)

2.2.1.3. *HOMOLOGOUS CONJUGATION EXPERIMENT*

1. follow the same instructions for the 'heterologous conjugation experiment' but use a homologous plasmid free derivative of the recipient strain.

2.2.1.4. *HOMOLOGOUS RETROTRANSFER EXPERIMENT*

1. follow the same instructions for the 'heterologous conjugation experiment' but use a homologous recipient strain containing a transferable plasmid with an appropriate selectable marker

2. select for retrotransconjugants by a medium that selects for the donor organism containing the plasmid of the recipient strain

2.2.2. ISOLATION OF MOBILIZING PLASMIDS USING TRIPARENTAL EXOGENOUS ISOLATION (Top et al., 1994)

Strains containing possible mobilizing plasmids = sample = *R. rubrum*; *A. platensis*; unknown consortium of compartment I
Recipient = *R. rubrum*; *A. platensis*; reference strain *R. metallidurans*
(Alternative for recipient strain: *gfp* labeled derivative of phe^- mutant of AE104)
Donor = *E. coli* CM120(pRK2023)

1. test growth of sample bacteria on rich medium with a range of antibiotics
2. select the antibiotic resistances that are not present in the sample for tagging recipient strains and the helper plasmid (in the following it is assumed that the sample bacteria are sensitive to rifampicin and chloramphenicol)
2. grow recipient culture rifampicin resistant *R. metallidurans* AE104 derivative AE815 overnight in 2 ml LB, shaking at 30°C
3. grow donor strain *E. coli* CM330(pMOL155) overnight in 2 ml LB, shaking at 37°C
4. Centrifuge a mixture of 500 µl of the AE815 culture and 500 µl of the sample, and 500 µl of the CM330(pMOL155) culture at 3,000 rpm
5. resuspend the pellet in 50 µl of LB broth
6. spot the suspension on a filter on an LB plate and incubate overnight at 30°C
7. resuspend the mating patch in 1 ml of 0.01 M $MgSO_4$ and dilute up to 10^{-8} in 0.01 $MgSO_4$
8. plate 100 µl of each dilution on selective plates (recipients on Schatz, 0.2% gluconate, Rif; donors on LB media with Cm; transconjugants on Schatz, 0.2% gluconate, Rif., 0.8 mM Zn^{2+})
9. for negative control, donor, recipients, and samples were incubated separately on filters under the same conditions

Isolation of mobilizing plasmids will likewise be performed by homologous or heterologous triparental matings, selecting for transconjugants as well as retrotransconjugants (see previous paragraph).

2.3. ANALYSIS OF PLASMIDS, FREE DNA AND VIRUSES

2.3.1. ANALYSIS OF PLASMIDS (TOP ET AL., 1994)

2.3.1.1. *CHARACTERIZATION OF INCOMPATIBILITY GROUP BY HYBRIDISATION*

1. perform colony and blot hybridization with nylon membranes (Hybond N, Amersham Corp.) according to the supplier's instructions, using specific DNA probes (Couturier et al., 1988)
2. label DNA using ^{32}P-labeled dCTP with a Multiprime labeling reagent kit (Amersham)
3. rinse hybridized membranes twice with 2x SSC-0.1% sodium dodecyl sulfate (SDS) (1xSSC is 0.15 M NaCl plus 0.015 M sodium citrate)

4. rinse once with 1xSSC-0.1%SDS, each time for 1h at 65°C
5. perform southern blot analysis with (DIG)-dUTP-labeled probe DNA labeling and detection kit (non-radioactive) (Boehringer Mannheim) according to the manufacturer's instructions (high-stringency washes: twice with 2xSSC-0.1% SDS for 5 min at room temperature and then twice in 0.1xSSC-0.1% SDS for 15 min at 68°C; low stringency washes: twice with 2xSSC-0.1% SDS for 5 min and then twice for 15 min at 40°C

2.3.1.2. *INCOMPATIBILITY TESTING BY USING A MATING PROTOCOL*
(only applicable for plasmids that are replicable in the same host)

1. transconjugants (AE815+pMOL155+additional plasmid) are used as recipients in the plate mating with E. coli CM120 (RP4) as the donor strain
2. after 24h incubation of the mating patch new transconjugants containing RP4 can be selected on LB medium rif (100 µg/ml), tet (20 µg/ml).
3. pick up four transconjugant from every mating experiment and purify on selective medium plates
4. transfer the transconjugant strain to LB broth and subculture twice after 24h of growth
5. extract plasmid and analyse extraction electrophoretically

2.3.2. ANALYSIS OF VIRUSES (SANDER AND SCHMIEGER, 2001)

2.3.2.1. *PHAGE IDENTIFICATION*

1. analyse amplification products electrophoretically
2. clone fragments into a TOPO vector (Invitrogen, Groningen, The Netherlands) and transform into *E. coli* strain TOP10F' (Invitrogen, Groningen, The Netherlands).
3. detect transformants on lB plates containing 100 µg ampicillin/ml, 50 µg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid/ml, and 200 mM isopropyl-β-D-thiogalactopyranoside
4. isolate putative positive recombinant plasmids
5. verify positive clones by EcoRI digestion
6. plasmid can thereafter be extracted and used for sequencing using M13 reverse, M13 forward and 525F primers

2.4. INVESTIGATING GENERAL DISSEMINATION

2.4.1. *TESTING MELISSA STRAINS AS RECIPIENTS*

1. perform filter mating experiment with AE815 containing test plasmids (of every Inc group) (see above) as donor organism with CM102(pRK2023) as helper strain and MELISSA strains as recipients on rich medium described for the MELISSA strains (TN70.1)
2. select for transconjugants as described above

2.4.2 *TESTING MELISSA STRAINS AS DONORS*

1. use MELISSA transconjugant strains from the previous paragraph as donor strains

2. if no recipients were retrieved electroporate the MELIiSSA strain with the used plasmid DNA
3. perform filter mating experiment with the plasmid free AE815 as recipient organism with CM102(pRK2023) as helper strain and MELISSA strains containing various plasmids as donors on rich medium described for the MELISSA strains (TN70.1)
4. select for transconjugants as described above

3. PROTEOMIC RESEARCH

3.1. MEMBRANE OR SURFACE ACCESSIBLE PROTEINS DETECTION (SAPD)

Flow cytometry can detect and separate specific populations of bacteria by the application of cell sorting. Using an antibody against specific surface bacterial protein would improve the detection of contaminants in a specific culture, for instance in a Melissa compartment. The proteomic approach is one of the best approaches to identify a specific surface protein for each Melissa bacterial culture. A proteomic approach will be used to identify specificsurface proteins for *Arthrospira* and *R. Rubrum* .

3.1.1. DETERMINATION OF THE PROTEIN CONCENTRATION

The protein concentration will be estimated by the classical Bradford technique using BSA as standard. This procedure will be used to determine the required volume to the SCM-MS technique.

Disolve 100mg Coomassie brillant blue G250 in 50ml 95% ethanol.
Add 100 ml concentrated phosphoric acid.
Add Waterto a final volume of 200 ml.
Prepare samples of 100,50, 25 and 12.5ug/100ul of BSA in the same solution as your protein sample
Dilute the Bradford dye concentrate 5 X with water, filter if precipitations occurs.
Add 5 ml of the diluted dye to each sample. The red dye will turn blue when binding to the protein, allow color to develop for at least 5 min, not longer than 30 min.
Read the absorbance at 595nm, prepare a linear standard curve and calculate concentration.

3.1.2. *SPECIFIC LABELLING OF BACTERIAL SURFACE PROTEINS*.

In the first step, bacterial surface proteins are labelled by a specific surface probe (Sabarth, 2002) . Bacteria were suspended in ice-cold PBS (PBS : Phosphate buffer saline), 1mM $CaCl_2$, 0.5mM $MgCl_2$ at an optical density at 600nm of 2.5-3.5. The bacteria were surface-labeled by incubation with 200uM (final concentration) sulfosuccinimidyl-6-(biotinamido)-hexanoate [s-NHS-LC-biotin (Pierce)] for 30 min on ice. The reaction was stopped by adding two volumes of TNKCM (50mM Tris , pH 7.4, 100mM NaCl, 27 mM KCl, 1mM $CaCl_2$, 0.5 mM $MgCl_2$). After 10 min incubation at room temperature, the bacteria were sedimented by centrifugation at 3500 g for 10 min and washed three times with TNKCM. The biotin surface proteins are purified by affinity chromatography on reversibly binding avidin-agarose (Boehringer). After extraction, these proteins are separated by two-dimensional gel electrophoresis and biotinylated proteins were detected on the blots using NeutrAvidin-peroxydase staining (Pierce) and chemiluminescent visualization (ECL, Amersham). Proteins will be identified by mass spectrometry. The specificity of these surface proteins can be further analysed by comparison with the genes included in the different genomic and proteomic databases.

3.1.3. *TWO-DIMENSIONAL GEL ELECTROPHORESIS PROCEDURE*

3.1.3.1. *SAMPLE PREPARATION*

The samples of arthrospira and Rhodospirillum are filtrated through a glass filter system (Millipore Inc) at 4°C. The pellet are resuspended immediately in the sample solution (7M urea, 2M thiourea, 0.5% (V/V) triton X-100, 2% (V/V) Pharmalyte 3-10, 65 mM DTE, and 8mM PMSF). The samples are treated by sonication (2 min. at 4°C) and, finally, centrifuged at 30000 X g for 30 min at 4°C in order to remove any undissolved material. The supernatants are used for 2-DE analysis. The samples were frozen in liquid N, and stored at -80°C.

3.1.3.2. *TWO-DIMENSIONAL GEL ELECTROPHORESIS*

2-DE was performed in a horizontal Multiphor 2-DE set-up (Pharmacia, Uppsala, Sweden) according to Wattiez et al., 1999. IPG strips (0.5 X 3 X 180 mm), containing Immobilines NL 3-10 and 4-7, were rehydrated overnight in rehydration solution (8M Urea, 0.5% (V/V) triton X-100, 2% (V/V) Pharmalyte 3-10 and 9.7 mM DTE). The first dimension was then performed by applying 250 µg protein/sample in a volume of 150 µl at the anodic side. The gels were run on a Pharmacia Multiphor II system equipped with a Multidrive XL power supply (Pharmacia Biotech., Uppsala, Sweden) using a 3-phase program. The first phase was set at 500V for 5h, the second phase was a linear gradient spanning from 500 V to 3500 V in 5 h, and the final phase was set at 3500 V for 14 h. After electrophoresis the strips were kept at –80°C or prepared directly for the second-dimension electrophoresis, as follows: the gels were equilibrated twice for 15 min., under gentle shaking, first in equilibration buffer (50mM Tris-HCl, pH 6.8, 6M urea, 30% glycerol, 1% (W/V) SDS) containing 20mM DTE and, next, in equilibration buffer containing 244 mM iodoacetamide. Second-dimension separations (SDS-PAGE) were carried out with ExcelGel XL SDS 12-14 (Pharmacia) (0.5 X 180 X 245 mm) running at 20-40 mA for about 4h. ExcelGel SDS buffer strips from Pharmacia were used. Staining with Silver nitrate of the 2-DE gels wasperformed as described by Rabilloud et al. (24). Protein pI and Mr were respectively assigned by calibration of 2D-PAGE gels with carbamylyte pI calibration markers and molecular weight standard proteins (Pharmacia Biotech, Uppsala, Sweden).These different conditions have been tested in preliminary experimentations (figure 7)

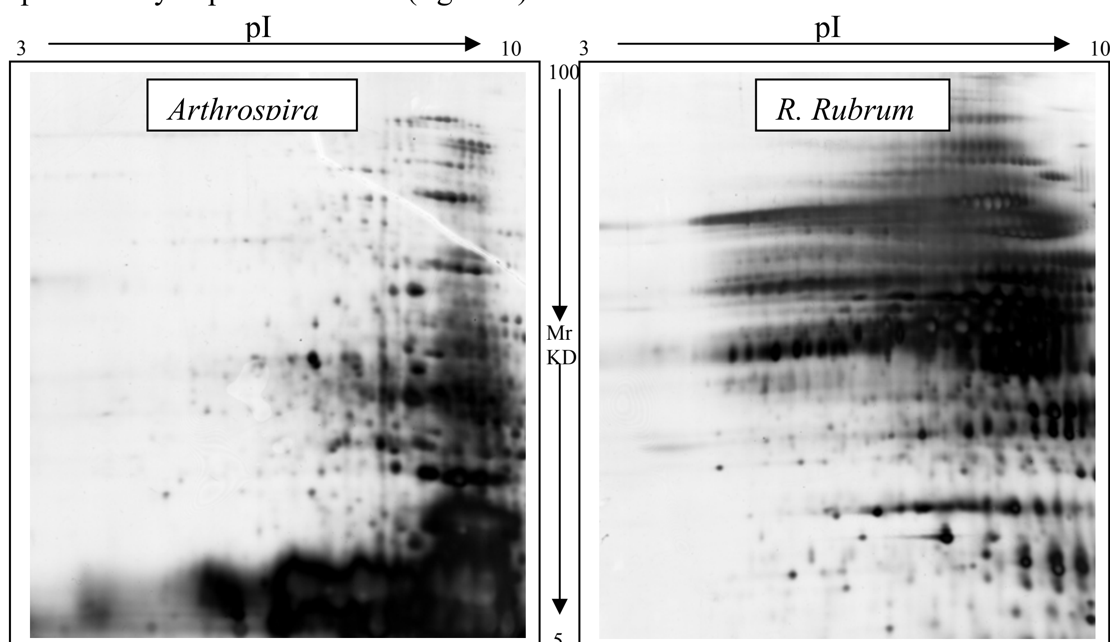
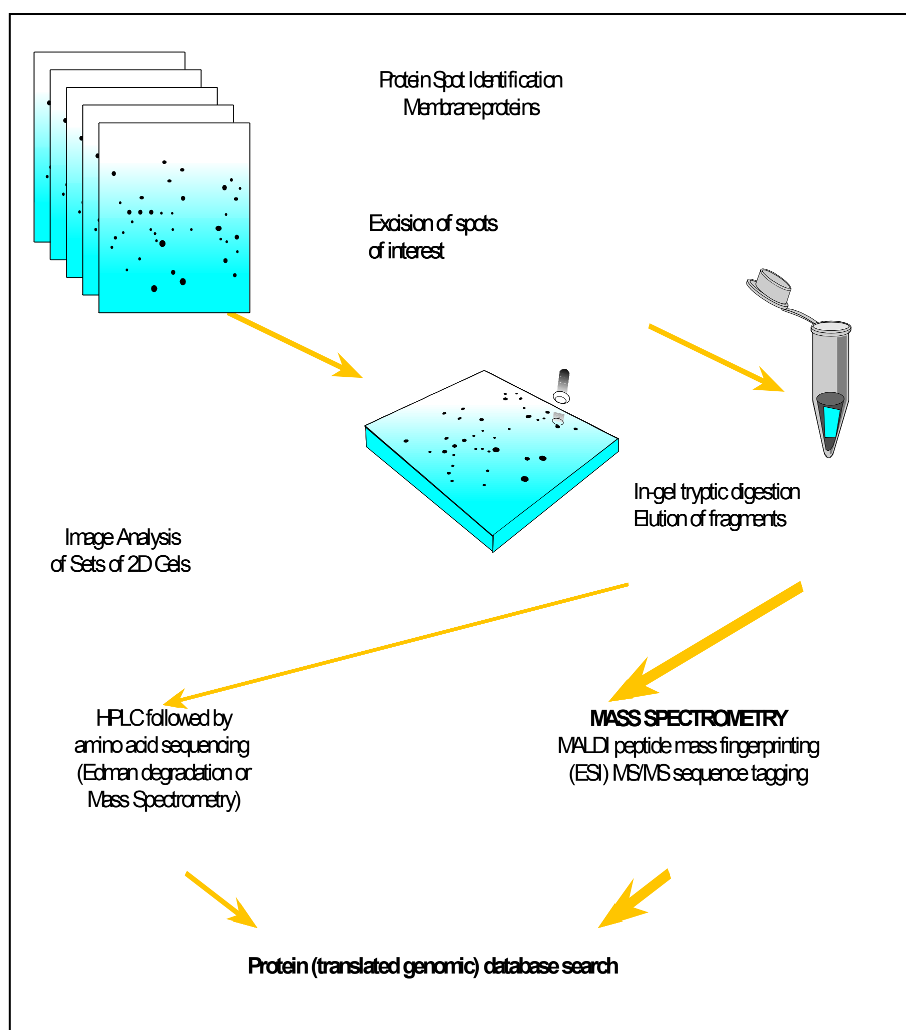


Figure 7. 2.DE of total proteins of *Arthrospira* and *R. Rubrum*

3.1.3.3. *EVALUATION OF 2-DE PATTERNS*

The protein patterns in the gels were analysed as digitalized images using a high resolution scanner (8 bits/pixel) in combination with PDQuest software (Bio-Rad, Richmond, CA, USA). The amount of protein in a spot was assessed as background-corrected optical density, integrated over all pixels in the spot and expressed as integrated optical density (IOD). In order to correct for differences in total staining intensity between different 2-DE images, the amounts of different spots were expressed as the percentage of the individual spot IOD per total IOD of all the spots (% IOD).

3.1.4. *IDENTIFICATION OF PROTEINS*



3.1.4.1. *PROTEIN TRANSFER BY ELECTROBLOTTING*

Proteins were electroblotted onto PVDF membranes (Sequi-Blot PVDF membrane; Bio-Rad, Richmond, CA, USA) using 25mM Tris, 192 mM Glycine, 0.1 % SDS as cathode buffer and 25 mM Tris, 192 mM Glycine, 2% methanol as anode buffer. Before the electrotransfer, the membranes were soaked in methanol for 30s and at least 10 min in anode buffer. Protein transfer was carried out using a semi-dry blotting apparatus (Biolyon, France) for 1h at 24V. Just before the staining step, the membranes were washed four times with MilliQ water. The PVDF membrane-bound proteins were visualised by staining with Coomassie Brillant Blue R-250. The membranes were incubated overnight at RT with 250ml of 1.1% (W/V) of Coomassie Brillant Blue R-250 in methanol/ acetic acid (50%/10% (V/V)). The membranes were destained with 250 ml of a methanol/ acetic acid solution(50%/10% (V/V)). Just after, the membranes were dried at RT and stocked at **-4°C.**

3.1.4.2. *N-TERMINAL AMINO ACID MICROSEQUENCE ANALYSIS*

The spots on the PVDF membrane were excised and the protein N-terminal amino acid sequences were currently determined at the picomole level by automated Edman Degradation of 1 pmol of protein using a Beckman LF3400D protein-peptide microsequencer equipped with an on-line model 126 Gold system microgradient HPLC and a model 168 diode array detector (Beckman Instruments). Carboxylmethylcysteine eluted just after glutamic acid in the PTH chromatograms. All samples were sequenced using the standard Beckman sequencer procedure 4. All sequencing reagents were from Beckman. Amino acid sequence comparisons were carried out using the FASTA or the TFASTA computer program of the Genetics Computer Group (GCG) for screening protein or nucleic acid databases. The International databases were SWISS-PROT (release 34), PIR (release 50), Genpept, Genpeptnew, GenEMBL (release 53), Genbank (release 105), GenEMBLnew (release 53.+) and IMGT (release 97.06).

3.1.4.3. *MASS SPECTROMETRY*

A ENZYMATIC DIGESTION

Spots on the gel were excised using a sample corer (Fine Science Tools Inc.). Excised gel pieces were placed in 1.5ml polypropylene Eppendorf tubes and washed twice in 50 ul of 50 mM NH_4HCO_3. The gel pieces were destained and dehydrated with 50 ul of 50 mM NH_4HCO_3/50% acetonitrile (V/V) and then were dried in a centrifugal evaporator. Enzymatic digestion was performed with the addition of 10 ul of 0.02ug/ul trypsin (Promega Madison, WI, USA) in 25mM NH_4HCO_3, to each gel piece, and incubated at 37°C overnight. The peptide solutions were recovered and the gel piece were extracted twice with 10 ul of 50% acetonitrile in 5% formic acid. The combined solution were concentrated in a vacuum concentrator and stored frozen until use.

B ELECTROSPRAY IONISATION TANDEM MASS SPECTROMETRY (ESI-MS-MS)

Mass spectrometry (MS) and collision-induced dissociation (MS/MS) were accomplished with a Q-TOF 2 mass spectrometer (Micromass, Manchester) equipped with a Z-spray nano flow electrospray ion (nanoESI) source and a high-pressure collision cell. Samples were dissolved in 50% acetonitrile in 5% formic acid, and loaded into borosilicate nanoflow tips (Protana, Denmark). For MS/MS studies, the quadrupole was used to select the parent ions, which were subsequently fragmented in a hexapole collision cell using argon as collision gas and a appropriate collision energy (typical 20-35 ev). Data acquisition was performed on a MassLynx system based on windows NT. MS/MS data were processed by a maximum entropy data enhancement program, MaxEnt 3TM (Micromass, Manchester). Amino acid sequences were manually deduced with the assistance of Micromass's peptide sequencing program PepSeq. The resulting sequences were search against SWISS-PROT (release 34), PIR (release 50), Genpept, Genpeptnew, GenEMBL (release 53), Genbank (release 105), GenEMBLnew (release 53.+) and IMGT (release 97.06) and JGI *Rhodospirullum* databases using BLAST.

C. MATRIX-ASSISTED LASER DESORPTION IONIZATION/TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF)

The peptides (after enzymatic digestion) were characterized using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (**MALDI-TOF** : M@LDI LR, Micromass). Samples were mixed (1/1, v/v) with cyano-4-hydroxycinnamic acid on the target and dried in room temperature. The energy of the nitrogen laser (337 nm) was between 1.7 to 5.5 ∝J. Ions were submitted to a 28 kV accelerating voltage towards the detector (4.75 kV). Analysis was carried out in positive polarity, without a filter, after a calibration with standard peptides in the molecular mass range 1 0086 968.7 (oxytocin, vasopressin, angiotensin I, somatostatin, insulin, hirudin). Resulting fingerprint from the MALDI spectrum (usually using the top 50-100 peaks at most) can then be used to search for corresponding proteins using either the Proteinlynx Global Server of Micromass, the MS-FIT programs at the UCSF Protein Prospector site, MASCOT at Matrix Sciences,Peptide Search at Heidelberg, Profound at the Prowl site at Rockefeller, PeptIdent or MultiIdent at the Expasy site, MassSearch at the CBRG in Switzerland (Computational Biology Research Group), or MOWSE at the UK Human Genome Mapping Project Resource Centre. The resulting sequences were search against SWISS-PROT (release 34), PIR (release 50), Genpept, Genpeptnew, GenEMBL (release 53), Genbank (release 105), GenEMBLnew (release 53.+) and IMGT (release 97.06) and JGI *Rhodospirullum* databases.

3.2. INTACT CELL MALDI-TOF-MS (ICM-MS)

Mass spectrometry combined with novel bio-informatic approaches provides a powerful new strategy for the rapid speciation and typing of microorganisms. This revolutionary concept of Bacterial "Mass-Fingerprinting" offers greater sensitivity, selectivity and speed of analysis as compared to classical identification methods that are currently used in clinical microbiology, food science, biotechnology, water quality and pharmaceutical analysis. Mass spectrometry combined with novel bio-informatic approaches provides a powerful new strategy for the

rapid speciation and typing of microorganisms. This revolutionary concept of Bacterial "Mass-Fingerprinting" offers greater sensitivity, selectivity and speed of analysis as compared to classical identification methods that are currently used in clinical microbiology, food science, biotechnology, water quality and pharmaceutical analysis.

Intact cell MALDI-TOF-MS (ICM-MS) applies established biopolymer Mass Spectrometry technology to the analysis of intact bacteria. This method allows the rapid sampling of a population of macromolecules expressed on the surface of bacteria and characterisation of these molecules by molecular weight. The resulting mass spectrum provides a unique physico-chemical fingerprint for the species tested. Mass-Fingerprints of unknown species can be reliably matched against databases of quality controlled reference mass spectra leveraging this simple analytical method into a powerful new tool for real-time detection and sub-typing of bacteria.

Mass-Fingerprinting is very rapid - the entire process from sample preparation to result takes only a few minutes for each test microorganism. Sample preparation is quick and easy - intact cells from primary culture are smeared across a stainless steel target plate and allowed to co-crystallise with a UV-absorbing matrix. After drying, the target is placed into the MALDI-TOF mass spectrometer. The microorganisms in the matrix are illuminated with a pulse from a nitrogen laser (337 nm). The matrix absorbs energy from the laser and macromolecules from the surface of the microorganisms are desorbed and ionised. The resulting ionised macromolecules are mass analysed and the results reported as a mass spectrum, i.e. a plot of mass (X axis) versus abundance (Y axis).

The Mass-Fingerprint of the test microorganism is then submitted to the MicrobeLynx™ search algorithm, which challenges an appropriately selected database from a range of quality controlled bacterial reference mass spectra.

In an initial approach, we will use ICM-MS to build a fingerprint database of *Rhodospirillum rubrum* and *Arthrospira platensis*. In a second approach we will use ICM-MS to detect artificial contamination in a spiking experiment in which a pure culture of is contaminated with and vice versa. In a parallel experiment, pure cultures of *R. rubrum* and *A. platensis* will be contaminated with *Ralstonia metallidurans*, the model organism.

3.2.1 *SAMPLE PREPARATION*

Differents sample preparation conditions will be used and tested. First, National Collection of Type Culture (NCTC) strains were stored at -85 °C, sub-cultured three times on Columbia blood agar (CBA) containing 5% (v/v) horse blood (TCS Microbiology, Botolph Claydon, Bucks., UK). The CBA plates were incubated for 24 h at 30°C. Second, bacterial strains were sub-cultured on liquid medium as in the MELISSA compartments for 24 h at 30°C and filtrated just before the analysis.

3.2.2. *MASS SPECTROMETRY MEASUREMENTS*

Mass spectrometry measurements were performed on a M@LDI LR (Micromass, Manchester, UK) laser desorption time-of-flight instrument equipped with a nitrogen UV laser (λ=337 nm). The laser fluence was set just above the threshold for ion production. The mass spectrometer was used in the positive ion detection mode using an acceleration voltage

of +15kV. On loading each target plate, automatic, accurate indexing of sample/reference wells was performed, followed by calibration of the m/z range of the instrument, using the average molecular weights from a standard peptide mixture (Bradykinin, angiotensin, Glu-fibrinopeptide B, renin substrate tetraddecapeptide, ACTH (18-39) all at 1 pmol/ul, bovine insulin, 2pmol/ul and ubiquitin, 10pmol/ul). The acquisition mass range was from m/z 500 to 10000 Da. For maximum throughtput of samples the bacterial mass fingerprints were acquired automatically. Sample supernatant were mixed (1/1, v/v) with cyano-4-hydroxycinnamic acid on the target and dried in room temperature. Twelve target wells were used for each organism to be entered into the database. A 1ul aliquot of matrix was applied to each target spot allowed to air-dry prior to mass spectrometry analysis. The matrix for Gram-positive bacteria was a saturated solution of 5-chloro-2-mercaptobenzothiazole (Aldrich Chemical) and, for Gram-negative bacteria, it was a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) both dissolved in 1:1:1 water, acetonitrile and methanol with 0.1% (v/v) formic acid and 0.01 M 18-crown-6. The formic acid promotes positive ion production and 18-crown-6 removes metal ion adducts (Evason et al., 2000).

3.2.3. *DATA ANALYSIS*

Spectral data were exported from M@LDI LR as .TXT files of mass, intensity pairs and to the MicrobeLynx™ search algorithm, which challenges an appropriately selected database from a range of quality controlled bacterial reference mass spectra. A bacterial fingerprinting database (>1500 different bacterial species) is now accessible from the Manchester Metropolitan University in collaboration with the National Collection of Type Cultures (NCTC), Central Public Health (London). The quality of the replicates was determined by comparing each of the twelve replicate spectra to each other using the root mean square (RMS) value. An RMS rejection value of 3 used to identify significant outliers and an average mass spectral fingerprint obtained from a minimum of ten accepted replicate spectra of an individual bacterium was used to populate the database. The pattern recognition algorithm uses all the mass and intensity data in the mass spectrum to give the best database match with a probability score. A comparative display of the test spectrum and the differences from the best database match is produced in a browser format.

3.3. SUPERNATANT CELL MALDI-TOF-MS (SCM-MS)

As in the intact cell mass spectrometry technique, the supernatant, mainly its protein content, is a characteristic of one bacterial species . Until now, the protein content of the different bacterial supernatants has been characterized by two-dimensional gel electrophoresis. Mass spectrometry is also able to characterise the protein supernatant of bacterial cultures by a similar way as the ICM-MS technique. Each bacterial Melissa compartment, especially of the compartment III, will be characterized by a specific supernantant fingerprint. Evolution of a specific fingerprint will be also analysed. A characterisation of bacterial protein supernatant fingerprints will be done in different culture time points.

3.3.1 *SAMPLE PREPARATION*

Differents sample preparation conditions will be used and tested. Supernatants will be prepared by filtration through an 2um filter system (Nalgene disposable filter ware : 150mL filter unit with sterile receiver) at 4°C and immediately stored at -80°C. In the first, supernatant will be tested after filtration without other treatment. In a second experiment, peptides and proteins will be precipitated by TCA treatment before analysis. After filtration at 4 °C, the protein in the supernatant will be precipitated by TCA (trichlorooacetic acid) precipitation (10% V/V TCA, overnight at 4°C). After centrifugation (15000 RPM, 1h, 4°C), the pellet will be laid directly with a Maldi-tof matrix on the sample plate.

3.3.2. *MASS SPECTROMETRY MEASUREMENTS*

Mass spectrometry measurements were performed on a M@LDI LR (Micromass, Manchester, UK) laser desorption time-of-flight instrument equipped with a nitrogen UV laser (λ=337 nm). The laser fluence was set just above the threshold for ion production. The mass spectrometer was used in the positive ion detection mode using an acceleration voltage of +15kV. On loading each target plate, automatic, accurate indexing of sample/reference wells was performed, followed by calibration of the m/z range of the instrument, using the average molecular weights from a standard peptide mixture (Bradykinin, angiotensin, Glu-fibrinopeptide B, renin substrate tetraddecapeptide, ACTH (18-39) all at 1 pmol/ul, bovine insulin, 2pmol/ul and ubiquitin, 10pmol/ul). The acquisition mass range was from m/z 500 to 10000 Da. For maximum throughtput of samples the supernatant mass fingerprints were acquired automatically. Spectra from the reference wells, for lock mass calibration, were also acquired automatically. Twelve target wells were used for each organism to be entered into the database. A 1ul aliquot of matrix was applied to each target spot allowed to air-dry prior to mass spectrometry analysis. Samples were mixed (1/1, v/v) with cyano-4-hydroxycinnamic acid on the target and dried in room temperature.

3.3.3. *DATA ANALYSIS*

Spectral data were exported from M@LDI LR as .TXT files of mass, intensity pairs and to the MicrobeLynx™ search algorithm, which challenges an appropriately selected database from a range of quality controlled supernatant bacterial reference mass spectra. The quality of the replicates was determined by comparing each of the twelve replicate spectra to each other using the root mean square (RMS) value. An RMS rejection value of 3 used to identify significant outliers and an average mass spectral fingerprint obtained from a minimum of ten accepted replicate spectra of an individual bacterium was used to populate the database. The pattern recognition algorithm uses all the mass and intensity data in the mass spectrum to give the best database match with a probability score. A comparative display of the test spectrum and the differences from the best database match is produced in a browser format.

4. ANALYSIS BY FLOW CYTOMETRY

4.1. BASIC TECHNICAL FLOW CYTOMETRIC REQUIREMENTS.

4.1.1. SHEATH FLUID.

Filtered sheath is used and is preferred to distilled water because this latter can induce modifications of the refractive indexes of the cells resulting in changes of the measured forward (FSC) and side (SSC) scatters.

4.1.2. *CALIBRATION OF THE CYTOMETER FLOW RATE.*

Accurate calibration of the sample flow rate is essential for obtaining reliable cell counts. Most flow cytometers do not record the volume of the sample that has been analyzed; they only record the duration of the analysis. Therefore it is necessary, in general, to determine the sample flow rate very precisely. Some instruments can be equipped with automatic sampling devices that deliver known volumes with high reproducibility. Even in this case, the nominal volume must often be calibrated and corrected for a dead volume that must be determined. To calibrate such systems, a suspension of fluorescent beads can be used. After enumeration of the beads suspension by epifluorescence microscopy, 5 to 10 replicates are analyzed by FCM under fixed delivery conditions. The actual volume (V) delivered is given by:

V=A/S

where:
A = number of beads analyzed
S = number of beads per ml determined by epifluorescence microscopy in the initial suspension or in a bead suspension with a well-known bead suspension.

The use of fluorescent microspheres as internal standards at known concentration has sometimes been reported to calculate the analyzed volume.

Another method takes into account a precise method for measuring the calibration without the need of beads and can be applied to the EPICS XL, in which the flow rate remains relatively constant over a large period. Nevertheless, it can be affected by environmental parameters such as room temperature and must be calculated daily at the beginning and at the end of the enumeration experiments. If it is suspected that the rate varies or drifts, it must be determined every 5 or 10 samples, since its determination is critical for abundance estimates. Instability may occur in the flow rate when aggregates or big cells are present in the sample which may clog the flow cell. A pre-filtration through a 10 µm nylon mesh is necessary in such cases.

Protocol :

- Select a rate (Low, Medium, or High).
- Fill a tube with the same liquid as the one containing samples (i.e., sheath Isoton ™).
- Measure the volume of sample (or weigh precisely the tube containing the sample).

- Place the sample in the flow cytometer and wait up to the signal "acquisition starts" appear.
- Simultaneously, start the chronometer running.
- Run the sample for at least 10 min.
- Remove the sample tube and simultaneously stop the chronometer.
- Measure (or weigh) the remaining volume.
- Calculate the rate (R), expressed in microliters per minute, by one of the following two methods.

Volume measurement:

$$R = (V_i - V_f)/T$$

where Vi = initial volume (µl), V_f = final volume (µl), and T = time (min).

Weight measurement:

$$R = (W_i - W_f)/(T \times d)$$

where W_i = initial weight (mg), W_f= final weight (mg), T = time (min), and d = density of the liquid used for calibration (distilled water = 1.00, seawater = 1.03, for example).

4.1.3. *ACCURACY OF MEASUREMENTS*

Any degradation in the accuracy of measurements can be detected if standard beads are used routinely. These fluorescent beads (Immuno-check, Coulter) permit the optical alignment and parameter settings which have to be reproduced before the passing of the samples. Samples are analysed when the fluorescence of beads reach acceptable values of variation coefficient.

4.1.4. *DETECTION AND THRESHOLD.*

When samples are not stained, the best approach is to start with a bacterial suspension. The acquisition is started and the needed photomultipliers (PMT 1 to PMT 4) are increased in order to obtain the main bacterial population in the middle of the screens. In all cases, it is critical to adjust the photomultiplier values in order to use all the dynamic range of the logarithmic scales. Then a flow cytometric tube containing 0.2 µm filtered sheath (Isoton^{TM}) is used. The discriminator set on the size (forward scatter) at the minimum value. The discriminator is increased until the noise can be avoided. The total number of events per second must be maintained below 100. Then the bacterial sample is run again and the discriminator adjusted again. The total number of events per second must be maintained below 1,000 per second.

4.1.5. *DATA ACQUISITION.*

Samples are collected as listmode and routinely 100,000 to 200,000 events are recorded typically during 2 to 10 minutes on an EPICS XL flow cytometer using the medium or high sample flow rate. Rare cells are difficult to study and require the analysis of larger sample volumes.

4.2. FRESH OR FIXED MATERIAL? PRESERVATION AND STORAGE OF EPAS AND UAB SAMPLES.

Ideally, samples should be first analysed fresh. Alternatively, if fresh analysis is not possible, a simple method for the preservation of the samples, that interfere minimally with the cellular properties of cells can be used (see preservation and storage of samples). The combined analysis of the forward and the side scatters can allow the identification of different groups that differ in terms of size and granularity, respectively. Several aspects are critical to succesful analysis of bacterial samples :

- careful sample preservation (if necessary)
- good discrimination of populations from noise
- accurate identification of populations
- careful determination of flow sample rate

4.2.1. *PRESERVATION AND STORAGE OF THE SAMPLES.*

Best results should be obtained on fresh samples run immediately after collection. Fresh samples can be stored at 4°C for up to 12 hours with minimal effect. Fixation will always result in cell loss (about 10%) and in change of scatters signals. The choice of the preservative may depend of the nature of the cells. Glutaraldehyde (0.1 to 1%) and paraformaldehyde (0.5 to 3%) are the most common chemicals used for preserving seawater samples, for example. Glutaraldehyde at high concentrations (> 0.5%) generates crystallisation, inducing noise that emits in the green region of the spectrum. The use of paraformaldehyde is an acceptable alternative to glutaraldehyde since it does not induce autofluorescence of the preserved cells.

Preparation of the fixative solution requires special care. Paraformaldehyde is a polymerised formaldehyde having poor crosslinking properties and cannot be used in that state as a fixative. By heating paraformadehyde in water, the polymer dissociates into formaldehyde which is more water soluble and an efficient fixative. It is very difficult to completely dissolve the paraformaldehyde powder, it must be vigourously mixed in distilled water for 2 hours or more at 70°C. The solution obtained can be clarified, after cooling at room temperature, by addition of small amounts of sodium hydroxide 1N. The pH is then adjusted , but the solution must be always filtered on Whatman paper filters and then through 0.2 µm-filter before aliquoting and storage.
However, after thawing, paraformaldehyde aliquots must be kept at +4°C and should not be used beyond a week.

4.3. OPTIMISATION OF CELL INDIVIDUALISATION FOR *ARTHROSPIRA PLATENSIS* FILAMENTS.

Arthrospira platensis grows as filaments and for most flow cytometrical applications, analyses need to be performed on single cells. Therefore, we will have to optimise the cell individualisation of *A. platensis* filaments. Different methods in order to dissociate cells will have to be performed and compared. In some cases, the cells are loosely associated and mild physical shearing techniques will release the cells from the filaments. However, this is probably not the case for A. platensis. Therefore, a mechanical performed (pipetting, vortex mixer, ultrasound) or chemical (enzymatic digestion) treatment might have to be. Mechanical techniques can cause significant cell damage. In some cases, enzymatic digestion is an absolute necessity. Unfortunately, most enzymes have severe effects on cells. These may be

reversible or they may cause lysis. The lack of success in dissociating *A. platensis* cells from filaments may, at least in part, be due to extreme conditions necessary for dissociation of cells resulting from the age of the culture. The criteria for choice of enzyme or mechanical treatment conditions will need to be established by trial and error. After treatment, a study of the cell membrane integrity by phase contract microscopy and flow cytometry will have to be assessed.

4.4. ESTIMATION OF THE ABUNDANCE OF THE MELISSA STRAINS AND ANY BACTERIAL CONTAMINANTS.

An important parameter to take into account is the absolute (and not relative) abundance of the different cell populations. Unfortunately, most available instruments are not set to deliver well-defined sample volumes. Therefore in all cases it is necessary to precisely estimate the volume of sample analysed. On the EPICS-XL, the most accurate method consists in determining the flow rate very precisely and then recording the time of analysis for each sample.
Another possibility which is more accurate is to use beads at a known concentration (Molecular Probes) that is added to the bacterial sample. By a rule of three, it is then easily possible to calculate the concentration of the various bacterial populations.

The following method could be used for the enumeration of bacteria.

4.4.1. *MATERIALS.*

Bacterial samples or cultures, either fresh or frozen (see preservation and storage of the samples)

Immunocount fluorescent microspheres (Molecular Probes) of known concentration diluted to obtain 1000 beads per ml of suspension.

Sheath (Isoton^{TM})

EPICS-XL flow cytometer

4.4.2. *PROTOCOL.*

If sample has been frozen, thaw at 37°C. Transfer 1 ml of sample to a suitable flow cytometer tube. If the cell suspensions are too concentrated, they can be diluted in sheath previously filtered through a 0.22-µm-pore-size filter.
Add 10 µl of around 10^5 beads/ml suspension of fluorescent microspheres (as an internal reference). Calibrate the flow rate of the cytometer (see Protocol of calibration of the cytometer flow rate).
Set the discriminator to red fluorescence and set all parameters on logarithmic
amplification. Insert the sample, allow around 15 sec for the flow rate to stabilize, and then begin data acquisition. Data for natural samples are typically collected in listmode files for 2 to 4 min with a flow rate of 50 to 100 µl/min.
Record the time of analysis to determine precisely the cell concentrations of each
population. Compute the absolute cell concentration for each population in a given sample as follows:

$$\mathbf{Cpop = T \times N_{POP}/R \times (V_{total}/V_{sample})}$$

where **Cpop** = concentration of population in cells/µl, N_{POP} = number of cells acquired, ***T***= acquisition time (min), ***R*** = sample flow rate (µl/min) as determined for the sample series, **V*total*** = volume (µl) of sample plus additions (fixatives, beads, etc.), and ***Vsample*** = volume of sample (µl).

Report parameters relative to the beads added to the samples:

$$X_{rel} = X_{pop} \times X_{beads}$$

where X_{POP} is the average value of a cell parameter (scatter or fluorescence) for a given population and X_{beads} the same parameter for the beads.

Before calculation of the ratio, X_{pop} and X_{beads} must be expressed as linear values (not numbers of channels) after conversion from the logarithmic recording scale.

4.5. DETERMINATION OF SIZE VARIATIONS OF MELISSA STRAINS AND ANY BACTERIAL CONTAMINANT

There is a relationship between the forward scatter (size) and cell mass. In addition, the scatter signal from bacteria depends on its shape and on the range of scatter angles detected. As bacterial populations are morphologically heterogeneous, we will investigate to which extend results may vary according to the composition (possibly taking into account age and density) of reference populations. Furthermore, a study will be performed in order to analyse whether a change in the bacterial culture (due to viral infection, specified stress,..) could be correlated with a change in size and granularity. Finally, a possible relationship between the side scatter (SSC) and bacterial morphology will be investigated. Visual evaluation is to be performed with phase contrast microscopy. All the changes will be quantified by the use of calibrated microspheres of known diameter size (0.1, 0.2, 0.5, 1, 2, 5 and 10 µm diameter) and a calibration curve of the mean channel versus the size will be established. If there is heterogeneity of the bacterial size, it should be reflected by a high variation coefficient.

4.5.1. *FLOW CYTOMETRY PROTOCOL*

Fresh bacteria will be passed on an EPICS XL flow cytometer at a low rate (1000 events per second). Forward and side scatters corresponding to the size and granularity will be measured and detection of variation in size and granularity monitored and compared.

4.6. DETERMINATION OF GENOME SIZE OF MELISSA STRAINS AND ANY BACTERIAL CONTAMINANTS

In general, estimation of genome size is best assessed on fresh material on isolated nuclei. However, when a complex membrane composition prevents the release of nuclei from algae or when isolated nuclei are not stable over time, these analyses can be performed on fixed cells. Little information is available on the genome size and the base composition of the MELISSA strains. In addition to being an important item of information by itself, genome size can be used to discriminate bacterial contaminants of MELISSA strains that have similar morphological features. Determining ploidy level in cultured populations is also critical for assessing the sexual cycle of eukaryotic cells.

The best way to quantify the genome size of bacteria is to use a hypotonic treatment in order to work on nuclei alone. The nuclei are released by hypotonic shock.

Depending on the species of interest, the composition of the hypotonic solution may need to be optimized. For example, addition of extra citrate gives isolated nuclei with more condensed chromatin. Increasing concentrations of detergent may help to remove membranes or cytoplasmic material attached to nuclei, which induce background fluorescence. Examination of nuclei by epifluorescence microscopy will help us to determine the best conditions.

N.B. With some eukaryotic algae, isolated nuclei cannot be obtained by this method because of the complex composition of membranes (e.g., for diatoms) or because nuclei are not stable and degrade very quickly. In such cases, the DNA content can be estimated on whole cells after treatment with alcohol (ethanol or methanol) to remove fluorescent pigments, followed by incubation with RNase. However, the presence of contaminating DNA (mitochondrial and chloroplastic) often leads to inaccuracy of genome measurement. For prokaryotes, which have no nuclei, DNA content can be assessed after fixation. The use of paraformaldehyde is recommended.

4.6.1. *MATERIALS*

The hypotonic citrate solution contains 50 µg/ml of propidium iodide (Sigma) in water supplemented with Tris (0.01M), $MgCl_2$ (5mM) and RNase (1 mg/ml).

4.6.2. *PROTOCOL*

To obtain bacteria that contain only fully replicated chromosomes, different substances such as rifampicin (around 150 µg/ml) or cephalexin (around 10 µg/ml) will have to be added to the culture grown to an optical density of 0.1 to 0.3. The molecular mechanism of rifampicin is an inhibition of bacterial RNA polymerase that forms a complex with rifampicin. Rifampicin prevents the ribosomes from binding to messenger RNA, and thus proteins are not produced. Bacterial nuclei are obtained from 50 µl bacterial cell suspension incubated in 1 ml of hypotonic citrate solution at 37°C. The nuclei will be released by hypotonic shock and will remain stable in the solution. If isolated nuclei can not be obtained, fix the culture with 0.5 % paraformaldehyde. Add 20 µl internal reference (E. Coli for example). Mix the sample by vortexing. After at least 30 minutes of staining, the DNA contents of up to 15,000 cells are measured with a flow cytometer EPICS XL. Measurement of DNA content is performed with both linear and logarithmic amplifications.

The measurement of DNA content for an uncharacterized or contaminating population can be obtained by comparing the mean fluorescence of this species with that of a species for which the genome size is known (Fig. 1 **)**. This reference must be added before staining. If one wants to compare two strains to determine whether they have the same genome size, it is critical to work with a mixture of the strains as well as with each strain separately.

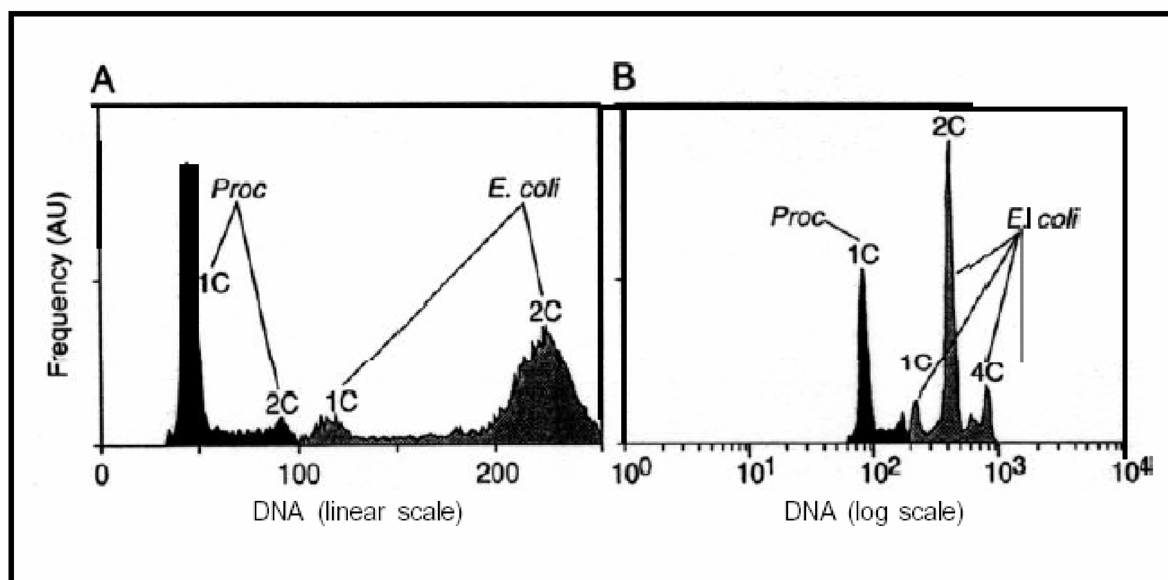


Figure 8 : Estimation of the genome size of the photosynthetic prokaryote *Prochlorococcus* (Proc), using a culture of *E. coli* pretreated with rifampicin as internal reference. Data are presented on both linear (A) and logarithmic (B) scales.

Use of internal reference.

The DNA content is measured by comparing the mean DNA fluorescence of the species to that of a standard. Many parameters can influence staining of isolated nuclei, such as the temperature of incubation, the stage of the culture or the concentration of chemicals like detergent or citrate. The staining of the internal reference will be affected by these parameters in a way similar to that of the sample. This precludes the use of inert material such as fluorescent microspheres as the standard.
The choice of the internal reference will also depend on the species to be measured. If the species and the standard have genome sizes that are too different, this leads to inaccuracy in the determination of the DNA content. However, genome size is known precisely only for a small number of microbial strains that have been entirely sequenced (e.g., *Synechocystis* FTC6803 or *E. coli*). If one needs a precise estimation of the DNA content of cells of interest, this constitutes a major drawback.

When the genome size difference between the sample and the reference is large, one should use the data acquisition on logarithmic scale with the following formula to calculate the DNA content:

$$D = 10^{\{(F-FR) \times N/C\}} \times D_R$$

where ***D*** = DNA content of the unknown species; ***DR*** = DNA content of the reference species; ***F***= mean channel of the unknown species; ***FR*** = mean channel of the reference species; **N** = number of decades of the logarithmic amplifier; and C = number of channels used for the acquisition (256 or 1024). This formula requires precise determination of the number of decades of the amplifier used for collecting the fluorescence (Durand, 1999). It is critical not to rely on the value given by the manufacturer (2 to 4 decades), which is never accurate. **Figure 8** allowed to detect the genome size of *Prochlorococcus* at 1.9 Mbp by comparing its DNA distribution, after staining with propidium iodide, SYBR-I or YOYO-1, with that of *E. coli* (1 C = 4.66 Mbp) treated with rifampicin (Fig. 1). This antibiotic inhibits RNA synthesis

and blocks the initiation of new replication sites on the DNA, but allows DNA replication to terminate and cell division to occur. As a result, most cells are blocked with a 1C and 2C genome size. The value measured for *Prochlorococcus* by flow cytometry matches very well with that established by DNA renaturation kinetics (1.89 Mbp; Rippka et al., submitted). This constitutes a good illustration of the potential use of flow cytometry to evaluate the genome size of small organisms.

Good internal standards generally give coefficients of variation below 5%. Nevertheless, in some cases, a rapid degradation of the internal standard can be observed, due to the high hypotonicity of the isolation buffer or to some chemical compounds contained in the initial sample.

Isolation of nuclei

With phytoplanktonic cells, it is often difficult to isolate nuclei without damaging them (e.g. for diatoms). The method described above has been refered in order to obtain optimal results on a wide range of photosynthetic cells. However, slight modifications in the composition of the isolation buffer may be required for *Arthrospira platensis*. For example, higher detergent concentrations may help for some difficult samples. Observations with epifluorescence microscopy will help us to optimize the protocol.

4.7. ASSESSMENT OF VIABILITY, SURVIVAL, MEMBRANE PERMEABILITY AND MEMBRANE POTENTIAL AND CELL CYCLE OF MELISSA STRAINS AND ANY BACTERIAL CONTAMINANT.

CAUTION : The following experiments are considered of minor importance since time and manpower will be first concentrated in order to optimise and obtain results for the above protocols (**point 4.1 to 4.6**) with this contract. However, we judge that some of the following protocols (**point 4.7 to 4.10**) might be tested and compared for the MELISSA strains.

4.7.1. *DETERMINATION OF MEMBRANE PERMEABILITY OF MELISSA STRAINS AND ANY BACTERIAL CONTAMINANT*

4.7.1.1. *MEMBRANE PERMEABILITY INDICATORS*.

Propidium iodide (Sigma) and TO-PRO-1 (Molecular Probes) are both impermeant compounds that markedly increase their fluorescence on binding to double-stranded nucleic acid.

4.7.1.2. *MEMBRANE PERMEABILITY ASSESSMENT*.

Membrane permeability will be determined with around 100 nM TO-PRO-1 or 1 to 5 µg/ml propidium iodide (concentrations will have to be optimised). Both TO-PRO-1 and propidium iodide exhibit substantially increased fluorescences on binding to intracellular nucleic acids; both dyes normally bear positive charges and are excluded from cells with intact membranes, while they stain nucleic acids in cells with damaged membranes. Cells killed by heat exposure (conditions to be determined) will be used as controls for TO-PRO-1 and propidium iodide staining.

4.7.1.3. *FLOW CYTOMETRY MEASUREMENTS***.**

Flow cytometry will be performed with an EPICS XL (Beckman Coulter). Propidium iodide and TO-PRO-1 will be excited at 488 nm and will be measured with the Photomultipliers 3 or 4.
The sheath flow rate will be set at 10 µl/min and the sample analysis rate kept below 1,000 events/s.

4.7.2. *DETERMINATION OF MEMBRANE POTENTIAL OF MELISSA STRAINS AND ANY BACTERIAL CONTAMINANT*

4.7.2.1. *MEMBRANE POTENTIAL-SENSITIVE DYES.*

Diethyloxacarbocyanine iodide {$DiOC_2(3)$}; bis-(1,3-dibutylbarbituric acid) trimethine oxonol {$DiBAC_4(3)$} and Rhodamine-123 will be obtained from Molecular Probes. Rhodamine-123 will be added to a final concentration of 1 to 10 µg/ml from a stock solution of 1 mg/ml in ethanol. For $DiOC_2(3)$ and $DiBAC_4(3)$, between 1 and 30 µM final concentration will be tested.

4.7.2.2. *DETERMINATION OF MEMBRANE POTENTIAL.*

Bacteria will be incubated with 1 to 30 µM $DiOC_2(3)$ for 4 mins, and membrane potential was estimated from the ratio of red to green fluorescence, due primarily to emission from single dye molecules, varies with the size of the bacterial cell or clump but is largely independent of membrane potential, while the red fluorescence, due to emission from dye aggregates, is dependent on both size and membrane potential. The ratio therefore provides a cell size-independent measure of membrane potential. For comparison purposes, 1 to 30 µM $DiBAC_4(3)$ instead of $DiOC_2(3)$ will also be tested as a putative membrane potential indicator.

4.7.2.3. *FLOW CYTOMETRY MEASUREMENTS.*

Flow cytometry will be performed with an EPICS XL (Beckman Coulter). $DiOC_2(3)$ will be excited at 488 nm; its green fluorescence will be detected with the photomultiplier 1, and its red fluorescence will be detected by photomultiplier 3 or 4.
The sheath flow rate will be set at 10 µl/min and the sample analysis rate is kept below 1,000 events/s.

4.7.3. *DETERMINATION OF METABOLIC ACTIVITY (ESTERASE ACTIVITY) OF MELISSA STRAINS AND ANY BACTERIAL CONTAMINANT*

The measurement of fluorescein diacetate (FDA) hydrolysis has been applied to estimate metabolic activity in particular esterase activity, and to help differentiating between live and dead/unhealthy cells. Bentley-Mowat (1982) first reported that the intensity of fluorescence

derived from the cleavage of FDA appeared to depend on the "metabolic vigour" of the cells. Dorsey et al. (1989) optimized the FDA technique for application in flow cytometry; their protocol will be adopted.

FDA is a nonpolar, nonfluorescent substance which enters the cells freely. Inside the cell, nonspecific esterases, among them lipase and acylase but not acetylcholinesterase (Guilbaut & Kramer 1966), break the FDA molecule into one brightly fluorescing fluorescein and two acetates. Being highly polar, the fluorescein is trapped within cells exhibiting cell membrane integrity and the amount of fluorescence will therefore increase over time depending on the metabolic activity of those esterases.

4.7.3.1. *MATERIALS* :

A stock solution of FDA (Sigma Chemicals F-7378) of 5 mg/ml will be made in dimethylsulfoxide (DMSO) and stored at 4°C. The stock solution will be thawed and diluted 100-fold in distilled water; since FDA is only slightly soluble in aqueous solutions and tends to flocculate at >1 µg/ml, the stock solution will be injected fast into the ice-cold water and mixed quickly. Although the working solution might appear slightly opaque, flocculation is prevented. The working solution will be kept on ice to minimize FDA degradation for max. 3 hrs and prepared fresh daily. Between 20 and 500 µl FDA working solution (concentration to be optimised) will be added to 1 ml of bacterial sample (10^7 cells) in flow cytometer tubes kept at room temperature in the dark until measurement.

4.7.3.2. *EQUIPMENT :*

Cells will be analyzed by an EPICS XL flow cytometer equipped with a 488 nm argon laser. Green fluorescein fluorescence will be measured on photomultiplier 1 on a logarithmic scale. **Figure 9** shows an example of FDA staining.

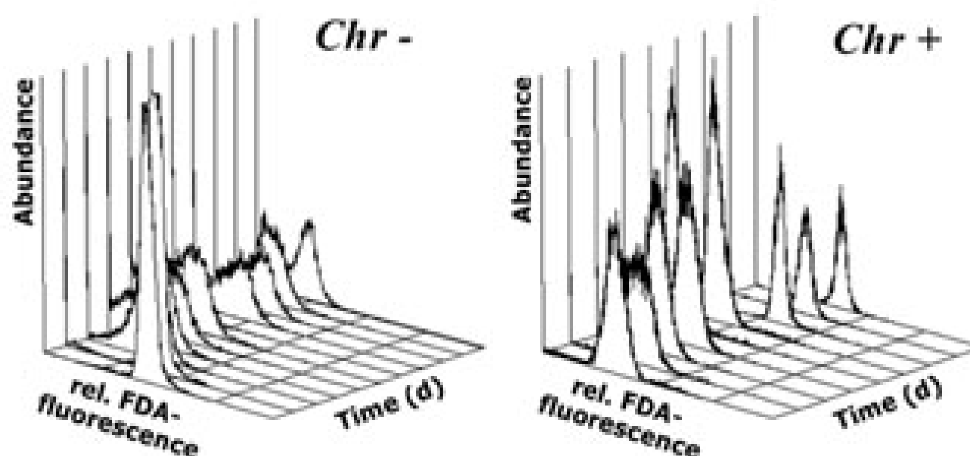


Fig.9: Example of FDA staining: Daily frequency distributions of FDA derived fluorescence (relative units) in *Chrysochromulina hirta* kept in darkness with (Chr+) and without (Chr-) addition of bacteria

4.7.4. *DETERMINATION OF INTRACELLULAR PH VARIATIONS IN MELISSA STRAINS AND ANY BACTERIAL CONTAMINANT*

Carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDAse) is the best reagent currently available in order to measure variations of intracellular pH. Viable and culturable bacteria are able to keep their pH constant when they are incubated at pH = 3 whereas non-culturable cells

can not. CFDAse couples irreversibly to both intracellular and cell-surface proteins by reaction with lysine side-chains and other available amine groups.

4.7.4.1. *CFDASE STAINING PROTOCOL*

A. Chemicals:

- Carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDAse); is dissolved in DMSO to 5 mmol/L, aliquoted in 50 µl and frozen in -20°C until use.
- Bovine Serum Albumin (BSA)
- Phosphate Buffered Saline (PBS)
- PBS + 1% BSA

B. Protocol

- Wash cells 3x in PBS to remove proteins.
- Aliquot cells at a concentration of 1.10^7 cells/ml in a 15 ml tube.
- Dilute CFDAse stock 1 : 10,000 to 0.5 µmol/L in PBS from the 5 mmol/L stock.
- Spin cells down, remove supernatant, and add 1 ml 0.5 µMol CFDAse solution per 1.10^7 cells (concentrations between 0.1 and 5 µMol will be tested). Resuspend cells well. Incubate 5 minutes at RT.
- Wash the cells 2x with PBS / 1% BSA at 4°C to stop the labeling.
- Cells can be used for flow cytometric assay.

NB. Due to high intensity of the signal labeling can be checked best next day.

C. Equipment :

Cells will be analyzed by an EPICS XL flow cytometer equipped with a 488 nm argon laser. Green fluorescein fluorescence will be assessed on photomultiplier 1 on a logarithmic scale.

4.7.5. *DETERMINATION OF RESPIRATORY ACTIVITY IN MELISSA STRAINS AND ANY BACTERIAL CONTAMINANT*

The tetrazolium salt CTC (5-Cyano-2,3-ditolyl tetrazolium chloride) has previously been used as a viability detector in microbiology due to its ability to form intracellular granula of CTF (red fluorescent formazan) when it is reduced by an active respiratory chain.

4.7.5.1. *PROTOCOL :*

The following protocol will be tested and optimised. One ml of bacteria (10^7 cells) will be centrifuged (8000 g, 5 min), the pellet resuspended in 1 ml sterile water at 2°C, incubated at 37° C with 5.5 mM Formate and 0.5 mM CTC for 1 h, and formaldehyde added at 2%. CTF precipitations will be recorded on an Epics XL flow cytometer. The flow protocol will be set up to record the precipitations by log FS, log SS, and log PMT3, with discriminator setting on PMT3 to exclude the non-fluorescent CTF negative bacteria. Gating will be first performed on logFS/logSS scattergrams, and counts obtained as PMT3 positive events.

4.7.6. *CELL CYCLE ANALYSIS OF MELISSA STRAINS AND ANY BACTERIAL CONTAMINANTS*

Flow cytometry has been used extensively in the past to determine the cell cycle of different phytoplanktonic species in culture. In addition to providing a basic understanding of the relationships between cell cycling and environmental factors such as nutrient levels or light (Vaulot et al., 1994), the determination of the percentage of cells within the different phases of the cell cycle also provides a very elegant way to assess the division rate of the MELISSA strains and any bacterial contaminant. Cell cycle analysis of bacterial species is performed on fixed cells.

Nucleic acid stains are useful for assessing the genome size of bacterial cells, but no universal staining procedure exists because of the large number of nucleic acid dyes and the wide taxonomic diversity of bacteria. The intercalary dyes, such as propidium iodide or ethidium bromide, bind with both RNA and DNA and allow precise measurement of nucleic acid content.

Flow cytometric analysis of bacteria, that have generally a very low DNA content, requires the combination of highly fluorescent stains and sensitive instruments. Nucleic acid stains are used for this purpose. However since they stain both DNA and RNA and since bacteria may have a relatively high RNA content when grown under optimal conditions, RNA must be removed enzymatically. For years, only the UV-excited dyes Hoechst 33258, Hoechst 33342 and DAPI could be used for DNA analysis of bacterial cells. Thereafter, propidium iodide was introduced and is nowadays tha traditional DNA dye. Recently, new dyes from Molecular Probes Inc, (Eugene, Oreg.) TOTO-1, TO-PRO-1, YOYO-1, YO-PRO-1 and PicoGreen have been introduced for the detection of small amounts of nucleic acids on electrophoretic gels. TOTO-1 and YOYO-1 are cyanine dyes that are chemically different but possess similar optical properties. They present the advantages of high quantum yield and of excitation at 488 nm, a wavelength available on small flow cytometers equipped with an air-cooled laser. They emit in the green region of the spectra, are cell impermeant and can be used on fixed cells. TOTO-1 seems to exhibit strong affinity for CTAG sequences, while YOYO-1 was found to have two binding modes: at low concentrations it appears to be intercalating and at high concentrations, external binding occurs. PicoGreen has a strong affinity for double-stranded DNA and was designed to quantify this molecule in solution. The fluorescence of these dyes is proportional to DNA concentration and does not depend on the G-C content. The quality of DNA distributions obtained with YOYO-1 or Pico-Green on cultured samples after dilution in a low hypotonic buffer such as Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 7.2), make them useful for culture studies. Very recently, it has been shown that a new dye, SYBR Green I is the most sensitive dye usable for the cell cycle analysis of bacterial cells. It has a much stronger affinity for double-stranded DNA than for RNA (Haugland, 1996; Marie et al., 1997), and is not sensitive to ionic strength.

4.7.6.1. COEFFICIENT OF VARIATION

The quality of the cell cycle analysis is strongly dependent upon the coefficient of variation (CV) of the G1 peak of the linear DNA distribution. A high CV (>l0%) makes it very difficult to estimate the fraction of cells in the different phases of the cell cycle, especially the S-phase. Broad G,-like peaks result from inadequate fixation procedures or from interaction between the stain and some components of the sheath. This can also be due to a concentration of stain

that is either too low or too high, leading, respectively, to nonstoichiometric or nonspecific binding. Moreover, because many bacterial species have a small genome size, the dye concentration must be lowered compared to the quantities used with mammalian or plant cells. The usual practice of filtering stock solutions through 0.2-µm-pore-size filters may induce retention of dye on the filter, thereby changing the concentration of the stock solution.

4.7.6.2. *PROTOCOL.*

Two ways of fixation will be tested : 1) Fix the sample by adding 500 µl of 1% paraformaldehyde (Sigma) to 500 µl of cell suspension or 2) fix the cells with cold 70%-80% ethanol while vortexing. Incubate at 4°C for up to 1 hour. If samples cannot be run immediately after fixation, they must be deep frozen in liquid nitrogen and stored at -80°C for delayed analysis. Frozen samples should be thawed at 37°C. Add 10 µl of 1% RNase (Sigma) for every 1 ml of sample. Incubate 30 min at 37°C. Add 10 µl propidium iodide, SYBR Green I, TOTO-1 or YO-PRO-1 working solution (final concentration corresponds to a 10,000-fold dilution of the commercial solution). Incubate 15 min at room temperature in the dark.

For example, cell cycle analysis of phytoplankton must be performed with both logarithmic and linear amplifications **(*Fig. 10*).** The logarithmic scale is used to discriminate the populations of interest. The linear scale is required to perform cell cycle analysis. On flow cytometers such as the EPICS XL, it is possible to record both signals. Put the discriminator on the red fluorescence. Run the sample for 4 min at low rate (<50 µl/min).

Typically, samples are run at a flow rate of 30 to 50 µl/min and the number of events is kept below 1000 per sec (by diluting samples that are too concentrated).
Cell cycle analysis allows one to determine the percentage of cells in the different phases of the cell cycle : G1, S, and G2 (prokaryotes) or G2/M (eukaryotes).

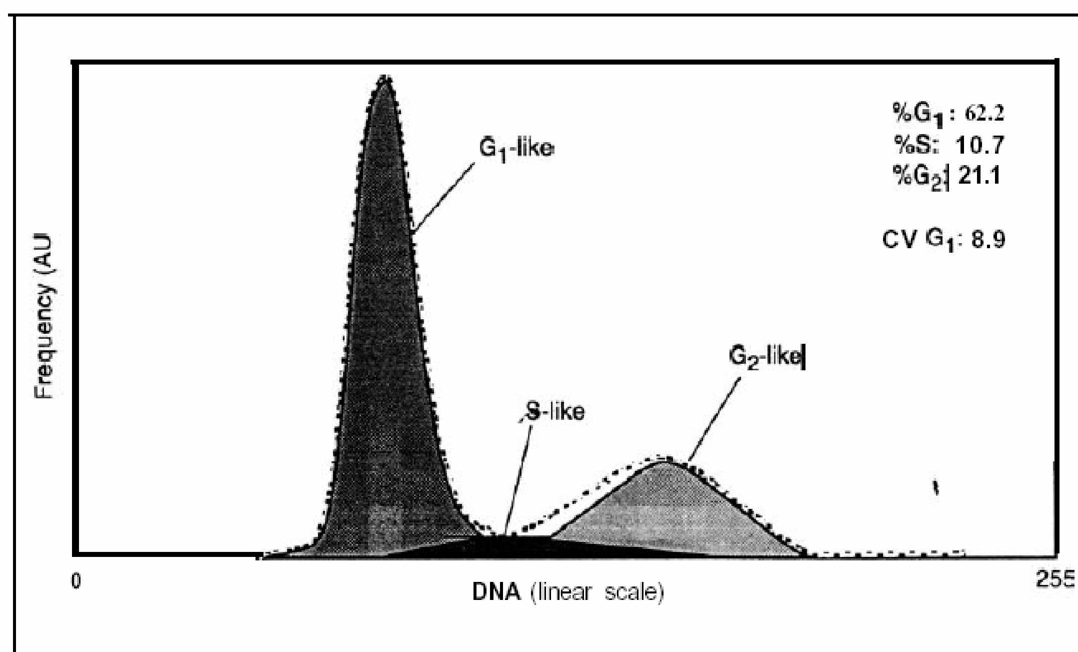


Figure 10 Cell cycle analysis on a natural sample of *Prochlorococcus* collected in the Pacific Ocean (Cast 94, 65 m). The coefficient of variation of the G,-like peak is an indicator of the quality of the DNA staining.

Common problems are excessive noise and broad Gl-like peaks. Noise can result from inadequate fixative solutions. We will have to make sure that all our solutions, including buffers, dyes or detergents are free of contaminating microorganisms. However, dye stock solutions must not be sterilized by filtration through 0.2 µm because some types of membranes can adsorb dyes. Broad Gl-like peaks generally result from too high dye concentration, inducing nonspecific binding.

4.8. FLOW CYTOMETRIC DETECTION OF VIRUS IN MELISSA STRAINS BY DNA STAINING AND FLUORESCENT DETECTION

CAUTION for part 4.8 to 4.10: *As mentioned in TN 70.7 (p.27), no virus that contaminate the MELISSA strains has been detected so far. Therefore, it is proposed for this work to artificially contaminate the MELISSA strains with viruses that are known to contaminate Synechococcus, Anabaena and Nostoc strains. However, we do not know whether these viruses will crosscontaminate the MELISSA strains. Furthermore, the protocols given in the literature concern essentially marine biology and we do not know at present whether there will applicable to the MELISSA strains.*

Highly sensitive nucleic acid-specific stains such as TOTO-1, YOYO-1, and the SYBR Green family (all available from Molecular Probes) have made it possible to detect and enumerate bacteria and, very recently, viruses in marine samples. Flow cytometry has been successfully applied to the analysis of viruses in solution, using the nucleic acid-specific dye SYBR Green I. This has permitted the analysis of viruses with reduced DNA content, down to 40 Kbp. Other dyes, such as SYTOX, PicoGreen, OliGreen, SYBR reen II, SYBR Gold, or RiboGreen (all from Molecular Probes), can be used with the same efficiency as SYBR-I.

Viruses are too small to be discriminated solely on the basis of their side- or forward scatter properties on flow cytometers such as the EPICS XL. Nucleic acid-specific staining is therefore necessary. Because flow cytometry was not designed for the analysis of such small particles, care must be taken in order to obtain reliable data. If samples are too diluted, there will be loss in the emission signal of the nucleic acid-dye complex; if they are insufficiently diluted, coincidence will occur. Analysis of different dilutions of samples has shown that coincidence occurs for viruses above 800 events per second. However, on flow cytometers such as the EPICS XL, for suspensions of beads, bacteria, or small algae coincidence normally occurs above 2000 events per sec. For concentrated suspensions, above 800 objects per second, more virus doublets are observed, which result in an increase of the fluorescence signal due to viruses passing simultaneously through the laser beam. Because all the V-II and 20% of the V-I virus populations can pass through 0.2-µm-pore-size filters, 0.2-µm-pore size-filtered sheath cannot be used to dilute the samples. Reasonable alternatives are 0.05pm-pore size-filtered sheath and buffers, making this the best option.

Viruses contain small amounts of nucleic acids. Another critical point for virus staining resides in the equilibrium between dye concentration and virus abundance. If viruses are too concentrated, a decrease in fluorescence will result. For virus numbers that do not saturate the machine's acquisition capacity, an increase in dye concentration will also result in loss of signal.
Moreover, virus abundance determined by flow cytometry on fixed but unfrozen samples is 3- to 10-fold lower than that found for frozen samples. This suggests that live viruses may have a

structure that prevents access of SYBR-I to nucleic acids. Detergent or heat treatment up to 95°C may be needed to denature the virus capsid and allow the stain to penetrate. For unknown virus material, the concentration of dye as well as the effect of heating must be assessed. In general, it is observed that using half the concentration of SYBR-I used for bacterial staining (to 5 parts in 100,000) and heating between 70° to 80°C are suitable for the majority of viruses analyzed (Marie et al, 1999).

4.8.1. *MATERIALS*

- Natural marine samples or cultures, either fresh or frozen
- 10% paraformaldehyde (Sigma)
- PBS buffer, pH 7.2
- DNA-specific stain(s) such as SYBR Green I or II, OliGreen, or RiboGreen (Molecular Probes)
- Distilled water
- 0.2-µm-pore-size filtration units for plastic syringe
- EPICS XL flow cytometer
- Additional reagents and solutions for flow cytometer calibration

4.8.2. PROTOCOL

- Prepare sample

  For <u>fresh</u> samples: Add 1% paraformaldehyde (0.5% final concentration) and let stand 1 hour.

  For <u>fixed and frozen</u> samples: Thaw samples at 37°C.
- Dilute samples in PBS buffer, pH 7.2, to three different concentrations: typically 100-, 1,000-, and 10,000-fold. Preparation of three different dilutions is necessary because the concentration of viruses is not known beforehand. Analysis must be performed with a suspension of 2×10^5 to 2×10^6 viruses/ml final concentration). To avoid generating large files, samples can be run for 1 or 2 min at a rate ranging from 20 to 50 µl/s. Different buffers have been tested in the litterature for diluting virus samples. PBS buffers give the best result.
- Add SYBR-I at a final concentration of 5 parts in 100,000 and incubate 15 min at room temperature in the dark.
- For virus samples that are freshly fixed (i.e., have not been frozen), or for hard-to-stain material, it is necessary to heat the samples 10 min at 80°C in the presence of detergent (e.g., Triton X-100 at 0.l% final) to improve dye uptake.
- Acquisition of the data

  * Begin the cytometric procedure by calibrating the flow rate (see Calibration of the flow rate).

  * Turn the discriminator to green fluorescence (PMT1).

  * Before starting data acquisition, wait for the sample flow rate to stabilize (this can take up to 20 sec).

  * Run the sample at a rate allowing <l000 events/sec

The single-parameter DNA histograms of the control cultures are analysed by assuming that cells with one genome equivalent corresponds to G1 (postmitosis), cells with two complements of DNA to G2 (cells have replicated their DNA), and those cells in the region that separates the two peaks G1 and G2 are in the process of DNA replication (the S-phase). Infected cells possess not only genomic DNA but also viral DNA. Thus, infected cells will have a DNA content corresponding to G1-like, G2-like and S-like.

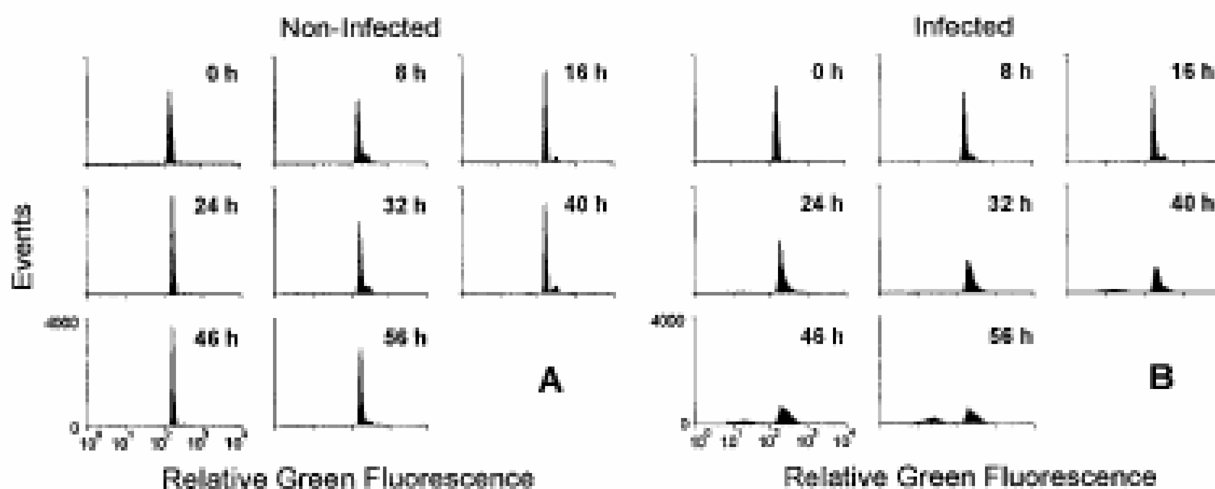


Figure 11. DNA frequency distributions (relative units) in noninfected and virus-infected cultures of *Micromonas pusilla* obtained by flow cytometry using the nucleic acid–specific green fluorescent stain SYBR-I. Note the different scales for *M. pusilla* virusinfected and noninfected cultures.

4.9. FLOW CYTOMETRIC DETECTION OF VIRUSES.

Brussaard and colleagues performed flow cytometric detection of several different virus families (Baculoviridae, Herpesviridae, Myoviridae, Phycodnaviridae, Picornaviridae, Podoviridae, Retroviridae, and Siphoviridae) were stained using a variety of highly fluorescent nucleic acid specific dyes (SYBR Green I, SYBR Green II, OliGreen, PicoGreen) and examined using a standard flow cytometer equipped with a standard 15 mW argon-ion laser. The highest green fluorescence intensities were obtained using SYBR Green I. DNA viruses with genome sizes between 48.5 and 300 kb could easily be detected. The fluorescence signals of the small genome-sized RNA viruses (7.4-14.5 kb) were found at the limit of detection. No significant linear relationship could be found between genome size and the green fluorescence intensity of the SYBR Green I stained virus preparations.

4.9.1. *VIRUSES USED*

For information, the following viruses were studied by Brussaard et al (2000) :

- CeV, PoV and PpV-0 1, which cause lysis of the microalgae *Chrysochromulina ericina, Pyramimonas orientalis,* and *P. pouchetii*.
- Algal viruses MpV-SPl and MpVUF10-38 (both lysing *Micromonas pusilla),* PBCV-1 (lysing *Chlorella* sp.), and S-PM2 (lysing *Synechococcus*
sp. strain WH7803)
- The infectious salmon anemia fish virus ISAV
- The insect *Autographa californica* polyhedrosis virus ACNPV (for simplicity, referred to as Bac), as well as the human immunodeficiency virus (HIV-l), the human herpes simplex virus (HSV- 1), and the human poliovirus (PV-1).

- The bacteriophages Lambda, T2, T4 and T7

See table 1 for the nomenclature of each virus that was studied by Brussaard et al (2000).

In this project, a system based on flow cytometric detection could be used in order to evaluate whether viruses (that could maybe come from compartment I) could be detected.

Virus abbreviation	**Name**	**Family**	**Type**	**Particle size (nm)**	**Genome size (kbp)**	**Tail (nm)**	**Enveloped**	**Reference**
Bac	Insect Autographa *californica* polyhedrosis virus ACNPV virus Bac	Baculoviridae	Circular ds DNA	300 x 50	130	No	Yes	Fields et al., 1996
CeV-B01	Algal *Chrysochromulina ericina,* virus CeV	Phycodnaviridae	ds DNA	140		No	No	Bratbak, unpublished data
HIV-1	Human immunodeficiency, virus HIV 1	Retroviridae	Dimeric ss (+) strands DNA	110	9.2	No	Yes	Fields et al., 1996
HSV-1	Human Herpes simplex, virus HSV type 1	Herpesviridae	Linear ds DNA	100-110	152	No	Yes	Fields et al., 1996
ISA-V	Infectious salmon anemia, virus ISAV	Orthomyxoviridae	Ss RNA	100-130	14.5	No	Yes	Mjaaland et al., 1 9 9 7
Lambda	Coliphage Lambda	Siphoviridae	Linear ds DNA	55	48.5	150 x 8	No	Murphy et al., 1 9 9 5
MpV-SPI	Algal *Micromonas pusilla,* virus MpVSPl	Phycodnaviridae	Linear ds DNA	115	200	No	No	Suttle, personal communication
MpVUF10-38	Algal *Micromonas pusilla,* virus MpVUFl0-38	Phycodnaviridae	ds DNA	100-140		No	No	Sahlsten, personal communication
PBVC-1	*Paramecium burscaria-Chlorella* virus PBCV-1	Phycodnaviridae	Linear ds DNA	160-190	300	No	No	Van Etten et al, 1985
PoV-B01	Algal *Pyramimonus orientalis,* virus PoV	Phycodnaviridae	ds DNA	200		No	No	Bratbak, unpublished Data
PpV-01	Algal *Phaeocystis pouchetii,* virus Ppv-01	Phycodnaviridae	ds DNA	130-160		No	No	Jacobsen et al, 1996
PV-1	Human Poliovirus PV	Picornaviridae	Ss (+) strand RNA	30	7.4	No	No	Fields et al., 1996
S-PM2	Cyanobacterial *Synechococcus* sp., virus S-PM2	Myoviridae	Linear ds DNA	90	90-100	165 x 20	No	Wilson et al, 1993
T2	Bacteriophage T2	Myoviridae	Circular ds DNA	85 x 110	158-205	110 x 85	No	Murphy et al., 1 9 9 5
T4	Bacteriophage T4	Myoviridae	Circular ds DNA	78 x 111	171-214	113 x 16	No	Murphy et al., 1 9 9 5
T7	Bacteriophage T7	Podoviridae	Linear	60	39.9	17 x	No	Murphy et al.,

			ds DNA			8		1 9 9 5

Table 1: Characteristics of the viruses used in Brussaard *et al.* study (2000).

Table 1 : Characteristics of the viruses used in Brussaard et al (2000).

4.9.2. *VIRUS PREPARATION AND STAINING PROCEDURE (ACCORDING TO BRUSSAARD ET AL, 2000) TO OPTIMISE*

Virus samples were taken from crude cell lysates, with the exception of HSV-1, HIV-l, and PCBV-1, which were partially purified by lowspeed centrifugation. All samples were fixed in glutaraldehyde (0.5% final concentration) for 0.5 - 2 h at 4°C, except for HIV-l, which was fixed with 2 % glutaraldehyde for 24 h. Because of safety concerns, the fixation procedure for HIV-l was modified. Viruses were fixed in order to obtain better staining results (Marie et al., 1999). The samples were frozen in liquid N, and stored at - 70°C for no longer than 1 month before analysis. The bacteriophage samples were diluted in TE buffer (10 mM Tris, 1 mM ethylenediamine tetraacetic acid; pH 8.0) prior to fixation and freezing in order to minimize interference from the medium with the fluorescence signal when staining the samples.
The staining procedure with SYBR Green I (Molecular Probes Inc.) was modified from Marie et al. (1999). Briefly, viruses were diluted in TE buffer to an event rate of 100- 1000 viruses s^{-1} to avoid coincidence. Preliminary results (Marie et al., 1999) have shown that dilution in TE buffer improved the flow cytometric distributions of the viruses. The virus samples were diluted in TE buffer at pH 8 because we generally obtained better green fluorescence signals at this pH, compared with pH 7.5 described by Marie et al. (1999). Diluted virus samples were stained with SYBR Green I for 10 min in the dark at 80°C. Samples were heated in order to improve the staining (see also Marie et al., 1999). The final concentration of SYBR Green I was a 10^{-4} dilution of the commercial stock solution. Other dyes used were OliGreen, PicoGreen and SYBR Green II (all from Molecular Probes Inc.) at final concentrations of 10^{-2}, 10^{-3} and 10^{-4} of the commercial stock solutions, respectively. Samples were examined using a flow cytometer equipped with a 15 mW 488 nm air-cooled argon ion
laser and a standard filter set-up. The discriminator was set to green fluorescence.
A total of at least 10,000 events were recorded for each sample. The data were converted to linear values and normalized to fluorescent latex microspheres (yellow-green beads of 0.95 µm; Polysciences Inc., Warrington, PA), which were added as an internal reference for all samples.

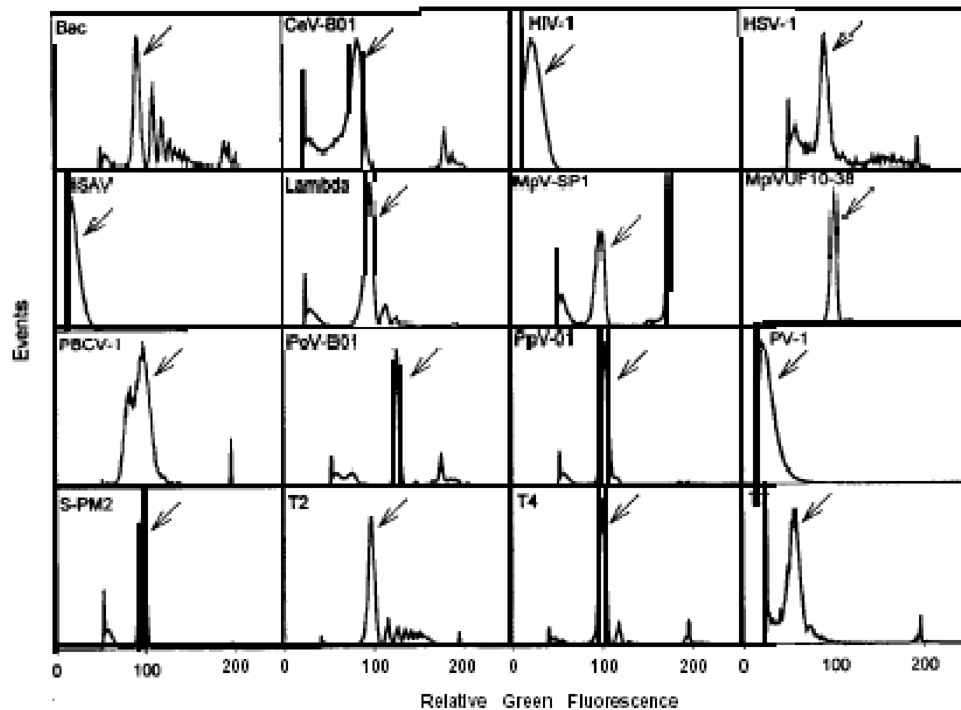


Figure 12. Histograms of green viral nucleic acid fluorescence of different viruses (see Table 1 for details concerning nomenclature and characteristics of the viruses) after staining with 1 x 10^{-4} of the commercial stock solution of SYBR Green I. Each histogram contains 10 000 events. Main peaks showing viruses are indicated with an arrow. Other peaks represent virus aggregates (e.g. Bat sample), bacteria (e.g. second large peak in MpV-SPl sample) and 0.95 µm fluorescent beads (e.g. clearly seen in the PBCV-1 sample).

4.10. FLOW CYTOMETRIC ANALYSIS OF MELISSA STRAIN VIABILITY FOLLOWING VIRAL INFECTION.

In a recent paper, Brussaard and colleagues performed two flow cytometric assays using physiological probes on the phytoplankton species *Phaeocystis pouchetii* and *Micromonas pusilla* to examine the assays' utility in detecting viral infections (2001). Dead cells were detected using the membrane impermeant nucleic-acid dye SYTOX-Green (**see figure 13**), which stains algal cells that have lost their membrane integrity. Live cells were detected using the membrane permeant dye Calcein-AM (**see figure 14**), which is hydrolyzed by intracellular esterases into a green fluorescent charged form. They found that both assays are easy to use, are reproducible and can indeed be used as markers of the viability of individual phytoplankton cells following infection by viruses.

4.10.1. *PROTOCOL (ACCORDING TO BRUSSAARD ET AL, 2001) TO BE OPTIMISED*:

4.10.1.1. *LIVE/DEAD CELL STAINING.*

Calcein-AM and SYTOXGreen were purchased from Molecular Probes Inc. The commercial stock solution of Calcein-AM (1 mM in dimethyl sulfoxide) was diluted in 0.2 µm pore size

(Suppor membrane Acrodisc syringe filters) filtered, autoclaved in PBS to a working stock concentration of 100 µM. SYTOX-Green (5 mM in dimethyl sulfoxide) was diluted in Milli-Q water to a working stock solution of 50 µM. Working stocks were stored frozen at –20°C until use.

Before the experiments, the staining assays were optimized by testing dye concentration, incubation time and temperature. Cells had been killed by fixation with paraformaldehyde (0.5% final concentration for at least 1 h) were used for the optimization procedures. The samples for the SYTOX-Green assay were stained with 0.5 µM dye (final concentration) and incubated in the dark at room temperature for 10 min. As the fluorescent dye is light-sensitive, the samples were incubated in the dark. Samples for the Calcein- AM assay were incubated for 1 h after addition of the dye at a final concentration of 10 µM. Because the basic parameter of this assay is enzyme activity, samples were incubated under culturing conditions. Neither stain had a negative effect on the basic flow cytometric parameters (forward and side scatter, red autofluorescence) or abundance of the cells over a period of 24 h when the optimized final dye concentration for each stain was used.

4.10.1.2. *EXPERIMENTAL SET-UP.*

At the start of the experiment comparing virus-infected with bacterial populations, batch cultures were split into subcultures of 300 to 400 ml. One of the batch subcultures was infected with fresh lysate of the respective virus, whereas the other served as the noninfected control.

For information : the lytic virus (*M. pusilla* virus) isolate MpV-UF10-38 (MpV, E. Sahlsten pers. comm.) was added to *M. pusilla* cultures at a virus to algal cell ratio of 10. The lytic virus (*P. pouchetii* virus) isolate PpV-01 (PpV, Jacobsen et al. 1996) was added to *P. pouchetii* cultures at a virus to algal cell ratio of 40 to 80.

Subcultures were incubated under conditions identical to those of the original algal cultures. The experiments were performed in duplicate. Samples were taken regularly at intervals of generally 2 to 6 h for 52 to 56 h. The total cell count and the viability assays were performed immediately after sampling on unfixed material using flow cytometry. Subsamples for virus count were fixed with paraformaldehyde at a final concentration of 0.5% for 30 min at 4°C, after which the samples were frozen in liquid nitrogen and stored at –70°C until analysis.

4.10.1.3. *BACTERIAL AND VIRUS COUNTS.*

Cells were enumerated on fresh, unfixed material directly after sampling using flow cytometry according to Brussaard et al. (1999). For virus enumeration, fixed frozen samples were thawed at room temperature and then diluted in TE buffer (Tris 10 mM, EDTA 1mM, pH 7.5) to obtain event rates between 100 and 1000 s–1. For virus enumeration, after addition of the green fluorescent nucleic acid specific dye SYBR Green I (final concentration 0.5 · 10–4 commercial stock) and incubation for 15 min in the dark at 80°C (Brussaard et al. 2000), samples were analyzed by flow cytometry according to Marie et al. (1999).

4.10.1.4. *FLOW CYTOMETRY.*

A flow cytometer equipped with an air-cooled argon laser (excitation 488 nm, 15 mW power) was used. Analyses were triggered on red autofluorescence and run for 1 to 4 min at a

delivery rate of 50 µl min–1. To avoid coincidence, bacterial cells were enumerated using an event rate between 100 and 400 cells s^{-1}. Flow cytometric parameters of algal cells were collected as logarithmic signals. For analyses of the collected data, the discrimination between various subpopulations was set for a typical example and kept constant for all samples.

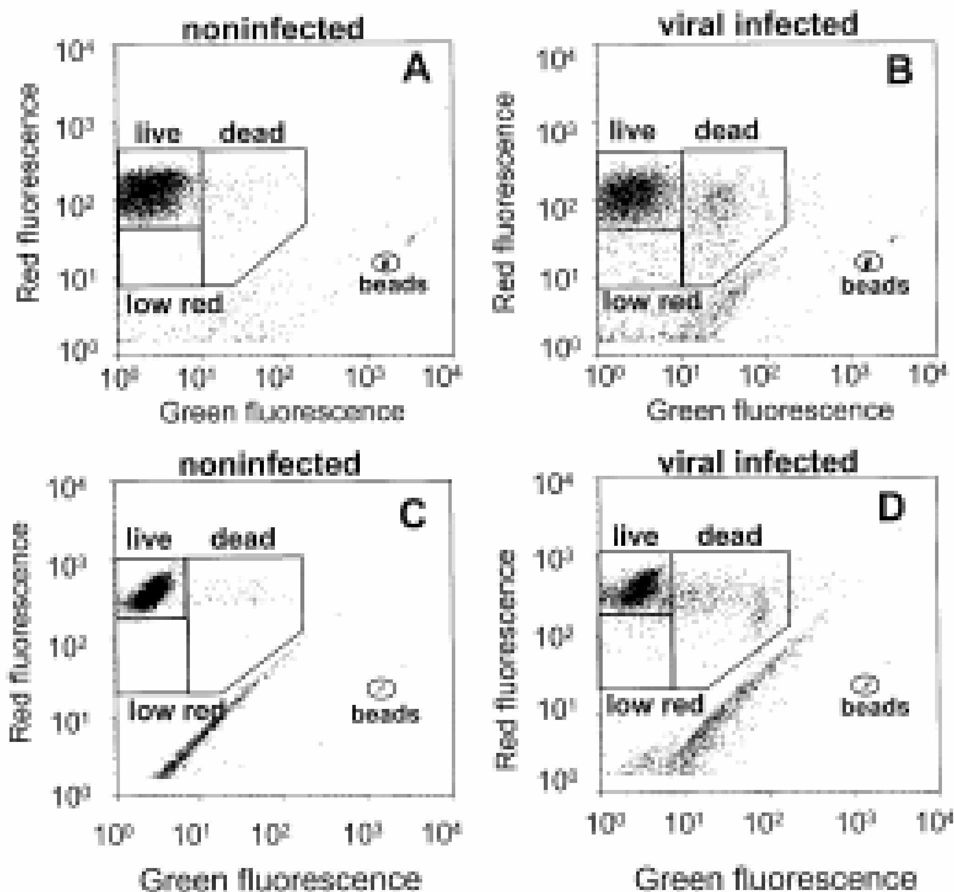


Figure 13. Representative biparametric plot of green fluorescence versus chlorophyll red fluorescence representing axenic algal populations after staining with SYTOX-Green for 10 min in the dark at room temperature. The cytograms show noninfected and virus-infected cultures of (A,B) *Micromonas pusilla* and (C,D) *Phaeocystis pouchetii.* The regions represent populations of algal cells with normal red fluorescence and low green fluorescence ('live'), normal or reduced red fluorescence but enhanced green fluorescence (SYTOXGreen stained, 'dead'), and low red and low green fluorescence ('low red'). The discrimination between the 3 subpopulations was kept similar for all samples

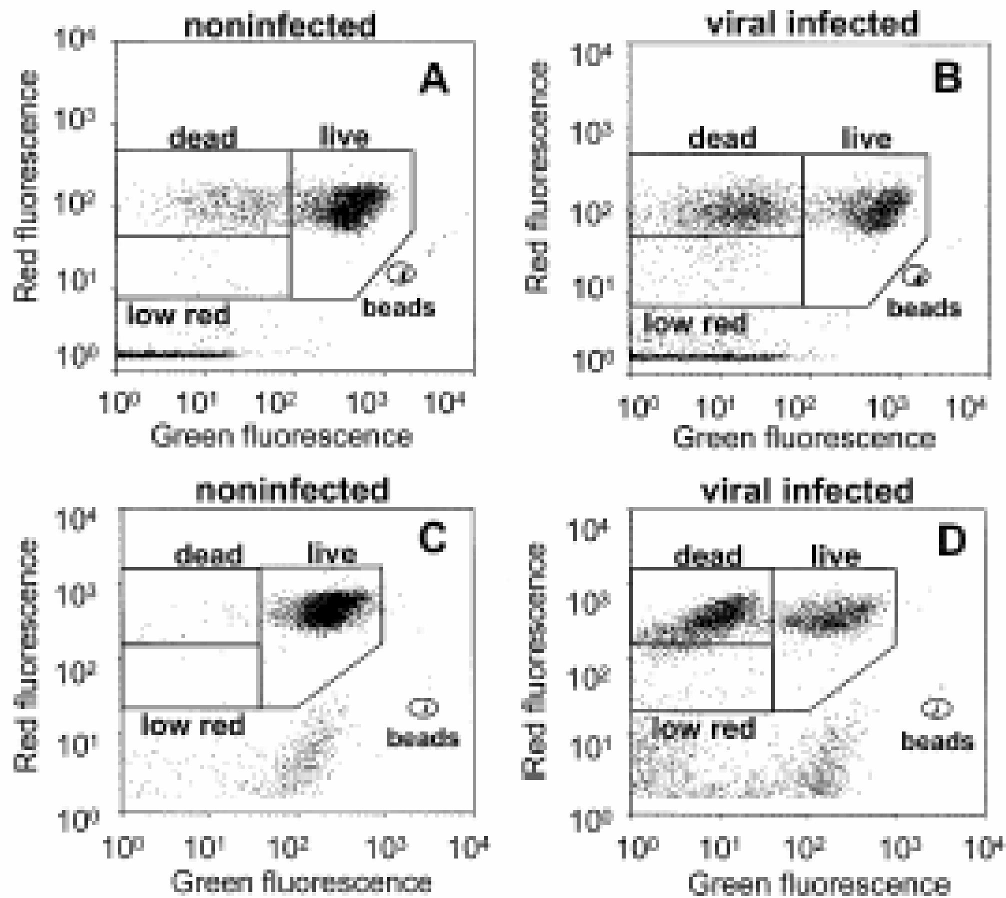


Figure 14. Representative biparametric plot of green fluorescence versus chlorophyll red fluorescence representing axenic algal populations after staining with Calcein-AM for 1 h under culture conditions. The cytograms show noninfected and virus-infected cultures of (A,B) *Micromonas pusilla* and (C,D) *Phaeocystis pouchetii*. The regions represent populations of algal cells with normal red fluorescence and low green fluorescence (Calcein-AM stained, 'live'), normal or reduced red fluorescence but enhanced green fluorescence ('dead'), and low red and low green fluorescence ('low red'). The discrimination between the 3 subpopulations was kept similar for all samples

5. REFERENCES

5.1 REFERENCES OF PCR-BASED GENOMIC ANALYSIS OF MICROBIAL SAMPLES

Scheldeman, P., Baurain, D., Bouhy, R., Scott, M., Mühling, M., Whitton, B.A., Belay, A. & WILMOTTE, A. 1999. *Arthrospira* ('*Spirulina*') strains from four continents are resolved into only two clusters, based on amplified ribosomal DNA restriction analysis of the internally transcribed spacer.FEMS Microbiology Letters 172 , 213-222.

D. Baurain, L. Renquin, P. Scheldeman, A. Belay & A. Wilmotte. 2002. Remarkable Conservation of Internally Transcribed Spacer sequences of *Arthrospira* ('*Spirulina*') (Cyanophyceae, cyanobacteria) strains from four continents and of recent and 30 year-old dried samples from Africa. J. Phycology 38, 384-393.

Pitcher, D.G., Saunders, N.A. & Owen, R.J. 1980.Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8: 151-156.

5.2. REFERENCES OF HORIZONTAL GENE TRANSFER IN THE MELISSA LOOP.

Bale MJ, Fry JC, Day MJ.1987. Plasmid transfer between strains of Pseudomonas aeruginosa on membrane filters attached to river stones. J. Gen. Microbiol; 133, 3099-3107.

Bale MJ, Day MJ, Fry JC. 1988. Novel method for studying plasmid transfer in undisturbed river epilithon. Appl Environ Microbiol. 54, 2756-2758.

Birnboim H.C. and Doly J.1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7,1513-1523.

Couturier, M., Bex F., Bergquist P.L., and W.K. Maas . 1988. Identification and classification of bacterial plasmids. Microbiol. Rev. 52, 375-395.

Lorenz M.G., Gerjets D. and Wackernagel W.1991. Release of transforming plasmid and chromosomal DNA from two cultured soil bacteria. Arch Microbiol. 156,319-326.

Maniatis T., Fritsch E.F., and Sambrook J.1982.Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sander M., and Schmieger H. 2001.Method for host-independent detection of generalized transducing bacteriophages in natural habitats.Appl Environ Microbiol 67,1490-1493.

Taghavi, S. 1996.Un megaplasmide de resistance aux metaux lourds d'Alcaligenes eutrophus: analyse genetique et fonctionelle. Ph.D. Diss., ULB, Brussel.

Top E., De smet I., Verstraete W., Dijkmans R., and Mergeay M. 1994. Exogenous isolation of mobilizing plasmids from polluted soils and sludges. Appl. Environ. Microbiol. 60, 831-839.

5.3. REFERENCES OF PROTEOMIC RESEARCH

Bright JJ, Claydon MA, Soufian M, Gordon DB.2002. Rapid typing of bacteria using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry and pattern recognition software. J Microbiol Methods. 48 ,127-38.

Bumann D, Aksu S, Wendland M, Janek K, Zimny-Arndt U, Sabarth N, Meyer TF, Jungblut PR. 2002. Proteome analysis of secreted proteins of the gastric pathogen Helicobacter pylori. Infect Immun. Jul;70(7):3396-403

Fenselau C, Demirev PA. 2001.Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev. 20:157-71.

Hughes MJ, Moore JC, Lane JD, Wilson R, Pribul PK, Younes ZN, Dobson RJ, Everest P, Reason AJ, Redfern JM, Greer FM, Paxton T, Panico M, Morris HR, Feldman RG, Santangelo JD. 2002. Identification of major outer surface proteins of Streptococcus agalactiae. Infect Immun. Mar;70(3):1254-9.

Kirkpatrick C, Maurer LM, Oyelakin NE, Yoncheva YN, Maurer R, Slonczewski JL. 2001. Acetate and formate stress: opposite responses in the proteome of Escherichia coli. J Bacteriol. 183(21):6466-77.

Rabilloud T. 2002. Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. Proteomics. Jan;2(1):3-10. Review

Rosen R, Buttner K, Becher D, Nakahigashi K, Yura T, Hecker M, Ron EZ. 2002. Heat shock proteome of Agrobacterium tumefaciens: evidence for new control systems.J Bacteriol. Mar;184(6):1772-8

Sabarth N, Lamer S., Zimny-Arndt U., Jungblut P., Meyer T., Bumann D. 2002. Identification of surface proteins of Helicobacter pylori by selective biotinylation, affinity purification and two-dimensional gel electrophoresis. Journal of Biochemistry. (In Press).

Sinha P, Poland J, Schnolzer M, Rabilloud T. 2001. A new silver staining apparatus and procedure for matrix-assisted laser desorption/ionization-time of flight analysis of proteins after two-dimensional electrophoresis. Proteomics. Jul;1(7):835-40.

Wattiez R, Hermans C, Bernard A, Lesur O, Falmagne P. 1999. Human bronchoalveolar lavage fluid: two-dimensional gel electrophoresis, amino acid microsequencing and identification of major proteins.Electrophoresis. Jun;20(7):1634-45.

5.4. REFERENCES OF FLOW CYTOMETRY ANALYSIS

Bentley-Mowat, J.A. 1982. Application of fluorescence microscopy to pollution studies on marine phytoplankton. Bot Mar 25: 203-204.

Bergh, 0. Borsheim, K.Y., Bratbak, G., and Heldal, M. 1989. High abundance of viruses found in aquatic environments. Nature 340:467-468.

Bratbak, G., Heldal, M., Norland, S., and Thingstad, T.F. 1990. Viruses as partners in spring bloom microbial trophodynamics. Appl. Environ. Microbiol. 56: 1400-1405.

Button, D.K. and Robertson, B.R. 1989. Kinetics of bacterial processes in natural aquatic systems based on biomass as determined by high-resolution flow cytometry. Cytometry 10:558-563.

Brussaard, C., Marie, D., Thyrhaug, R., and Bratbak, G. 2001.Flow cytometric analysis of phytoplankton viability following viral infection. Aquatic Microbial Ecology 26: 157-166.

Brussaard, C., Marie, D., Bratbak, G. 2000. Flow cytometric detection of viruses. Journal of Virological Methods 85: 175-182.

Brussaard C., Thyrhaug, R., Marie, D., Bratbak, G. 1999. Flow cytometric analyses of virus infection in two marine phytoplankton species, *Micromonas pusilla* (Prasinophyceae) and *Phaeocystis pouchetii* (Prymnesiophyceae). Journal of Phycology 35: 941-948.

Campbell, L. and Vaulot, D. 1993. Photosynthetic picoplankton community structure in the subtropical North Pacific Ocean near Hawaii (station ALOHA). Deep-Sea Res. 40:2043-2060.

Campbell, L., Nolla, H.A., and Vaulot, D. 1994. The importance of Prochlorococcus to community structure in the central North Pacific Ocean. Lintnol. Oceanogr. 39:954-961.

Cavender-Bares, K.K., Frankel, S.L., and Chisholm, S.W. 1998. A dual sheath flow cytometer for shipboard analyses of phytoplankton communities from the oligotrophic oceans. Limnol. Oceanogr: 43:1383-1388.

Chisholm, S.W., Olson, R.J., Zettler, E.R., Waterbury, J., Goericke, R., and Welschmeyer, N. 1988. A novel free-living prochlorophyte occurs at high cell concentrations in the oceanic euphotic zone. Nature 334:340-343.

Chisholm, S.W., Frankel, S.L., Goericke, R., Olson, R.J., Palenik, B., Waterbury, J.B., West-Johnsrud, L., and Zettler, E.R. 1992. Ptochlorococcus marinus nov. gen. nov. sp.: An oxyphototrophic marine prokaryote containing divinyl chlorophyll a and b. Arch. Microbiol. 157:297- 300.

Courties, C., Vaquer, A., Trousselier, M., Lautier, J., Chrétiennot-Dinet, M-J., Neveux, J., Machado, C., and Claustre, H. 1994. Smallest eukaryotic organism. Nature 3701255.

Dorsey, J., Yentsch,,C.M., Mayo, S., McKenna, C. 1989. Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae. Cytometry 10: 622-628.

Dubelaar, G.B.J., Groenewegen, A.C., Stokdijk, W., Van Den Engh, G.J., and Visser, J.W.M. 1989. Optical plankton analyser: A flow cytometer for plankton analysis, II: Specifications. Cytometry 10:529-539.

Dusenberry, J.A. and Frankel, S.L. 1994. Increasing the sensitivity of a FACScan flow cytometer to study oceanic picoplankton. Limnol. Oceanogr 39:206-210.

Hara, S., Terauchi, K., and Koike, I. 1991. Abundance of viruses in marine waters: Assessment by epifluorescence and transmission electron microscopy. Appl. Environ. Microbiol. 57:273 - 2734.

Hennes, K.P. and Suttle, CA. 1995. Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. Limnol. Oceanogr 40:1050-1055.

Hobbie, J.E., Daley, R.J., and Jasper, S. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Frank J. Jochem F. J., Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. Marine Biology, in press. http://www.jochemnet.de/fda3.html

Hercher, M., Mueller, W., Shapiro, H.M., 1979. Detection and discrimination of individual viruses by flow cytometry. J. Histochem. Cytochem. 27, 350-352.

Jacobsen, A., Bratbak, G., Heldal, M., 1996. Isolation and characterization of a virus infecting *Phaeocystis pouchetii* (Prymnesiophyceae). J. Phycol. 32, 923-927.

Kriss, A.E. and Rukina, E.A. 1947. Bacteriophages in the sea. Dokl. Akad. Nauk SSSR 57:833-836.

Lebaron, P., Catala, P, and Parthuisot, N. 1998. Effectiveness of SYTOX Green stain for bacterial viability assessment. Appl. Environ. Microbiol. 98:2697-2700.

Li, W.K.W. and Wood, A.M. 1988. Vertical distribution of North Atlantic ultraphytoplankton: Analysis by flow cytometry and epifluorescence microscopy. Deep Sea Res. 35:1615-1638.

Li, W.K.W., Jellett, J.F., and Dickie, PM. 1995. DNA distribution in planktonic bacteria stained with TOTO or TO-PRO. Limnol. Oceanogr. 40: 1485-1495.

Marie, D., Vaulot, D., and Partensky, F. 1996. Application of the novel nucleic acid dyes YOYO- 1, YO-PRO-1 and PicoGreen analysis of marine prokaryotes. Appl. Environ,. Microbiol. 62: 1649-1655.

Marie, D., Partensky, F., Jacquet, S., and Vaulot, D. 1997. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green-I. Appl. Environ. Microbiol. 93:186-193.

Marie, D., Brussaard, C.P.D., Thyrhaug, R., Bratbak, G., and Vaulot, D. 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. Appl. Environ. Microbiol. 65:45-52.

Marie, D., Brussaard, C., Partensky, F. and Vaulot, D. 1999. Flow cytometric analysis of phytoplankton, bacteria and viruses. In: Current Protocols in Cytometry. John Wiley & Sons, Inc. 11.11.1-11.11.15.

Monger, B.C. and Landry, M.R. 1993. Flow cytometric analysis of marine bacteria with Hoechst 33342. Appl. Environ. Microbiol. 59:905-911.

Olson, R.J., Chisholm, S.W., Zettler, E.R., and Armbrust, E.V. 1988. Analysis of Synechococcus pigment types in the sea using single and dual beam flow cytometry. Deep Sea Res. 35:425- 440.

Partensky. F., Blanchot, J., Lantoine, F., Neveux, J., and Marie, D. 1996. Vertical structure of picophytoplankton at different trophic sites of the tropical northeastern Atlantic Ocean. Deep Sea Res 43:1191-1213.

Partensky, F., Hess, W.R., and Vaulot, D. 1999. Prochlorococcus, a marine photosynthetic prokaryote of global significance. Mictvbiol. Mol. Biol. Rev 63:106-127.

Porter, J., Daniel, D., Pickup, R., Edwards, C. 1996. Fluorescent Probes and Flow Cytometry:New Insights Into Environmental Bacteriology. Cytometry. 23:91-98.

Porter, J., Pickup, R. and Edwards, C. 1997. Evaluation of Flow Cytometric Methods for the Detection and Viability Assessment of Bacteria from Soil. Soil Biol. Biochem. 29:91-100.

Porter, J., Deere, D., Hardman, M., Edwards, C., Pickup, R., 1997. Go with the flow-use of flow cytometry in environmental microbiology. FEMS Microbiol. Ecol. 24, 93-101.

Proctor, L.M. and Fuhrman, J.A. 1990. Viral mortality of marine bacteria and cyanobacteria. Nature 343:60-62.

Robertson, B.R., Button, D.K., and Kloch, A.L. 1998. Determination of the biomasses of small bacteria at low concentrations in a mixture of species with forward light scatter measurements by flow cytometry. Appl. Environ. Microbiol. 64:3900-3909.

Shapiro, L.P. and Haugen, E.M. 1988. Seasonal distribution and temperature tolerance of Synechococcus in Boothbay Harbor, Maine. EstuarineCoastal Shelf Sci. 26:517-525.

Spencer, R. 1955. A marine bacteriophage. Nature 175:690. 11.11.14 Supplement 10 Current Protocols in Cytometry

Spencer, R. 1960. Indigenous marine bacteriophages. J. Bacteriol. 79:614.

Van Etten, J.L., Burbank, D.E., Schuster, A.M., Meints, R.H., 1985. Lytic viruses infecting a *Chlorella* -like alga. Virology 140, 135-143.

Vaulot, D. 1989. CYTOPC: Processing software for flow cytometric data. Signal Noise 2:8.

Vaulot, D., Courties, C., and Partensky, F 1989. A simple method to preserve oceanic phytoplankton for flow cytometric analyses. Cytometry 10:629-635.

Waterbury, J.B., Watson, S.W., Guillard, R.R.L., and Brand, L.E. 1979. Widespread occurrence of a unicellular, marine planktonic, cyanobacterium. Nature 277:293-294.

Weinbauer, M.E. and Suttle, C.A. 1997. Comparison of epifluorescence and transmission electron microscopy for counting viruses in natural marine waters. Aquat. Microb. Ecol. 13:225232.

Wilson, W.H., Joint, I.R., Carr, N.G., Mann, N.H., 1993. Isolation and molecular characterization of five marine cyanophages propagated on *Synechococcus* sp. Strain WH7803. Appl. Environ. Microbiol. 59, 3736-3743.

6. ADDENDUM

Addendum A: lists of candidate probe sequences

```
Rhodospirilum rubrum (DSM107 and ATCC11170)
Probe design Parameters:
Length of probe      18
Temperature        [45.0 -65.0]
GC-Content         [40.0 -60.0]
E.Coli Position    [   0 -100000]
Max Non Group Hits     0
Min Group Hits        50%
Target              le apos   ecol grps  G+C 4GC+2AT |Decrease T by n*.3C -> probe matches n
non group species     Probe sequence
GUCUGAUUAGUCAGAGGU  18 A=13185  587    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 2; 2; ACCUCUGACUAAUCAGAC
GUGCGGAAUAAUCUUUGG  18 B=1864  141    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 2; 3; CCAAAGAUUAUUCCGCAC
GGAGUGCGGAAUAAUCUU  18 B-   6  138    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 1; 1; 2; AAGAUUAUUCCGCACUCC
GACAUCCCGUGACACUUC  18 C=28181  992    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 2;12;12; GAAGUGUCACGGGAUGUC
GUACCUUGGAGUGCGGAA  18 D=1846  131    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 1; 1; 2; 2; UUCCGCACUCCAAGGUAC
UACCUUGGAGUGCGGAAU  18 B-  17  132    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 1; 1;17;35; AUUCCGCACUCCAAGGUA
AACGUACCUUGGAGUGCG  18 D-   7  128    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 1; 3; 3; 4; 6; CGCACUCCAAGGUACGUU
AUCCCGUGACACUUCCAG  18 C+   5  995    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 1; 1; 1; 6; 6; CUGGAAGUGUCACGGGAU
UCCCGUGACACUUCCAGA  18 C+   7  996    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 1; 2; 4; 8;88; UCUGGAAGUGUCACGGGA
UGGAGUGCGGAAUAAUCU  18 B-   7  137    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 1; 1; 1; 5; 6; AGAUUAUUCCGCACUCCA
ACCUUGGAGUGCGGAAUA  18 B-  15  133    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 2; 4; 5; 7; 7; UAUUCCGCACUCCAAGGU
UCUGAUUAGUCAGAGGUG  18 A+   3  588    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 1; 1; 1; 4; 5; 8; CACCUCUGACUAAUCAGA
CAUCCCGUGACACUUCCA  18 C+   3  994    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 2; 2; 2; 5; 9;10; UGGAAGUGUCACGGGAUG
ACAUCCCGUGACACUUCC  18 C+   2  993    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 1; 2; 5; 7;10;12; GGAAGUGUCACGGGAUGU
CCGUGACACUUCCAGAGA  18 E=28206  998    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 4; 4;25;27;32;1513; UCUCUGGAAGUGUCACGG
CCUUGGAGUGCGGAAUAA  18 B-  12  134    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 1;
1; 1; 1; 3; 3; 5; 5; UUAUUCCGCACUCCAAGG
GGUCUGAUUAGUCAGAGG  18 A-  16  586    3  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 1; 1; 1; 1; 1; CCUCUGACUAAUCAGACC
GAACGUACCUUGGAGUGC  18 D-  11  127    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 1; 3;
3; 3; 4; 6; 6; 7; 9; GCACUCCAAGGUACGUUC
UCUUUGGAAACGAGGACU  18 F=2061  152    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 1; 2;
4; 5; 6; 8;39;44;47; AGUCCUCGUUUCCAAAGA
CGUGACACUUCCAGAGAU  18 E+   7  999    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 1; 1; 1;
2; 6; 7;10;13;20;42; AUCUCUGGAAGUGUCACG
GUGACACUUCCAGAGAUG  18 E+   8 1000    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 1; 2; 2;
5; 7; 7; 9;31;44;67; CAUCUCUGGAAGUGUCAC
UGACAUCCCGUGACACUU  18 C-   2  991    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 1; 1; 2;
3; 3; 4; 6;11;22;27; AAGUGUCACGGGAUGUCA
CUUUGGAAACGAGGACUA  18 F+   3  153    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0;
2;28;28;30;62;66;70;104;108;112; UAGUCCUCGUUUCCAAAG
UUAUCGCUCCAAGAUCGG  18 G=4361  208    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 5; 5;
6;11;11;42;47;51;82;90; CCGAUCUUGGAGCGAUAA
UUUAUCGCUCCAAGAUCG  18 G-   2  207    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 5; 5;
6;11;15;47;88;99;140;190; CGAUCUUGGAGCGAUAAA
UAUCGCUCCAAGAUCGGC  18 G+   1  209    4  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 2; 7;
7;10;15;16;17;24;29;59;67; GCCGAUCUUGGAGCGAUA
CGGUCUGAUUAGUCAGAG  18 H=13167  585    3  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 1; 1;
1; 1; 2; 2; 4; 4; 4; CUCUGACUAAUCAGACCG
CUGAUUAGUCAGAGGUGA  18 A+   5  589    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 1; 1; 1; 4; 5;
8; 8; 9;13;52;63;64; UCACCUCUGACUAAUCAG
CUUGGAGUGCGGAAUAAU  18 B-  10  135    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 1; 1; 1; 1; 1;
2; 4; 6; 7; 7; 9;12; AUUAUUCCGCACUCCAAG
UUGGAGUGCGGAAUAAUC  18 B-   8  136    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 1; 1; 1; 2; 2;
2; 2; 3; 3; 5; 9;11; GAUUAUUCCGCACUCCAA
```

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GUUAGACUAGAAUCCGUG  18 I=13912  644    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 2; 2; 2; 4; 4;
9;21;24;28;54;73;75; CACGGAUUCUAGUCUAAC
AUCUUUGGAAACGAGGAC  18 F-   2  151    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 1; 1; 1; 1; 2; 2;
3; 4; 4; 7; 8; 8;10; GUCCUCGUUUCCAAAGAU
UAGACUAGAAUCCGUGAG  18 I+   5  646    4  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 2; 7;
7;32;49;63;89;96;104;129;161;177;192; CUCACGGAUUCUAGUCUA
AACGUUGGGCACUCUGAA  18 J=32185 1136    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 3; 3; 3;
9;32;49;67;121;147;199;282;734;1399; UUCAGAGUGCCCAACGUU
UUGACAUCCCGUGACACU  18 C-   3  990    4  50.0  54.0  | 0; 0; 0; 0; 0; 0; 1; 1; 1; 2; 4; 4; 5;
8;10;13;42;47;64;102; AGUGUCACGGGAUGUCAA
AAUCUCCAAAAGGCGUCU  18 K=36239 1278    4  44.4  52.0  | 0; 0; 0; 0; 0; 1; 4; 4; 5; 8; 8;
9;12;12;13;17;17;20;26;31; AGACGCCUUUUGGAGAUU
AGCUAAUCUCCAAAAGGC  18 K-   9 1274    4  44.4  52.0  | 0; 0; 0; 0; 0; 1; 3; 3; 5; 7; 8;
9;554;557;593;1200;1269;1324;1498;2112; GCCUUUUGGAGAUUAGCU
AUCUCCAAAAGGCGUCUC  18 L=36275 1279    4  50.0  54.0  | 0; 0; 0; 0; 0; 1; 4; 4; 5; 8; 8;
9;12;12;12;13;22;22;27;35; GAGACGCCUUUUGGAGAU
CUAAUCUCCAAAAGGCGU  18 K-   5 1276    4  44.4  52.0  | 0; 0; 0; 0; 0; 1; 3; 3; 3; 4; 6; 6; 7;
9; 9;23;26;164;220;399; ACGCCUUUUGGAGAUUAG
CUCCAAAAGGCGUCUCAG  18 L+   4 1281    4  55.6  56.0  | 0; 0; 0; 0; 0; 1; 4; 8;
8;16;24;28;29;42;48;58;62;68;92;98; CUGAGACGCCUUUUGGAG
GCUAAUCUCCAAAAGGCG  18 K-   7 1275    4  50.0  54.0  | 0; 0; 0; 0; 0; 1; 3; 3; 3; 4; 6;
6;18;19;19;39;213;214;270;292; CGCCUUUUGGAGAUUAGC
UAAUCUCCAAAAGGCGUC  18 K-   4 1277    4  44.4  52.0  | 0; 0; 0; 0; 0; 1; 3; 3; 4; 6; 6;
7;10;10;11;15;15;16;25;25; GACGCCUUUUGGAGAUUA
UCCAAAAGGCGUCUCAGU  18 L+   5 1282    4  50.0  54.0  | 0; 0; 0; 0; 0; 1; 9;
9;11;20;25;36;38;49;59;73;77;95;103;105; ACUGAGACGCCUUUUGGA
UCUCCAAAAGGCGUCUCA  18 L+   3 1280    4  50.0  54.0  | 0; 0; 0; 0; 0; 1; 4; 4; 5; 8; 9;
9;13;24;25;30;41;57;72;75; UGAGACGCCUUUUGGAGA
ACGUUGGGCACUCUGAAG  18 J+   1 1137    4  55.6  56.0  | 0; 0; 0; 0; 0;
5;17;26;42;64;84;130;175;229;276;360;502;542;641;687; CUUCAGAGUGCCCAACGU
CGUUGGGCACUCUGAAGA  18 J+   3 1138    4  55.6  56.0  | 0; 0; 0; 0;
0;40;75;115;148;166;193;256;334;409;426;537;648;719;829;862; UCUUCAGAGUGCCCAACG
ACACUUCCAGAGAUGGAA  18 M=28244 1003    4  44.4  52.0  | 0; 0; 0; 0; 1; 2; 5;
6;10;25;26;52;64;74;86;123;307;360;5119;5239; UUCCAUCUCUGGAAGUGU
GACACUUCCAGAGAUGGA  18 E+  14 1002    4  50.0  54.0  | 0; 0; 0; 0; 1; 6; 6; 6;
8;13;53;73;80;95;149;197;253;317;1278;1359; UCCAUCUCUGGAAGUGUC
GGAACGUACCUUGGAGUG  18 D-  12  126    4  55.6  56.0  | 0; 0; 0; 0; 1; 1; 1; 1; 4; 4; 4; 4; 7;
7; 7; 8;10;10;11;13; CACUCCAAGGUACGUUCC
AAUGGCGCCUACAAUGGG  18 N=35736 1237    4  55.6  56.0  | 0; 0; 0; 0; 2;
5;92;96;102;189;195;204;292;301;306;409;418;423;436;444; CCCAUUGUAGGCGCCAUU
CAAUGGCGCCUACAAUGG  18 N-   2 1236    4  55.6  56.0  | 0; 0; 0; 0; 2;
5;92;94;99;102;191;201;206;299;303;318;410;424;452;461; CCAUUGUAGGCGCCAUUG
```

Ralstonia metallidurans CH34

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Probe design Parameters:
Length of probe      18
Temperature        [45.0 -65.0]
GC-Content         [40.0 -60.0]
E.Coli Position    [   0 -100000]
Max Non Group Hits     0
Min Group Hits        50%
Target              le apos   ecol grps  G+C 4GC+2AT |Decrease T by n*.3C -> probe matches n
non group species     Probe sequence
AGUAACGAUACUAACGCG  18 A=23280  851    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0;12;12; CGCGUUAGUAUCGUUACU
AACGAUACUAACGCGUGA  18 A+   6  854    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 4;10;11; UCACGCGUUAGUAUCGUU
ACGAUACUAACGCGUGAA  18 A+   7  855    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 1;28;281;305; UUCACGCGUUAGUAUCGU
UAACGAUACUAACGCGUG  18 A+   4  853    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 2; 4; 4; 7; CACGCGUUAGUAUCGUUA
GUAACGAUACUAACGCGU  18 A+   2  852    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 2; 2; 3; 6;11;17; ACGCGUUAGUAUCGUUAC
CAGUAACGAUACUAACGC  18 A-   2  850    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 4; 7;12;21;118;306; GCGUUAGUAUCGUUACUG
CGAUACUAACGCGUGAAG  18 A+   9  856    1  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 2;
3; 3; 5;19;752;6086;6098; CUUCACGCGUUAGUAUCG
GAUACUAACGCGUGAAGU  18 A+  16  857    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
2;16;285;290;340;948;1318;3822; ACUUCACGCGUUAGUAUC
AUUGGAACGUACCCUGUU  18 B=1827  123    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
6;16;16;18;27;44;75;149; AACAGGGUACGUUCCAAU
CAUUGGAACGUACCCUGU  18 B-   2  122    1  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 7; 7;
7;15;96;184;272;315;547; ACAGGGUACGUUCCAAUG
```

```
AUACAUUGGAACGUACCC  18 B-   7  119    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 8; 9;
9;45;173;275;367;426;704;773; GGGUACGUUCCAAUGUAU
UUGGAACGUACCCUGUUG  18 B+   3  124    1  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0;
6;16;16;17;23;33;35;37;43;56;70; CAACAGGGUACGUUCCAA
UACAUUGGAACGUACCCU  18 B-   6  120    1  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 1; 1;
1;29;147;245;322;371;645;727;867;1103;1123; AGGGUACGUUCCAAUGUA
ACAUUGGAACGUACCCUG  18 B-   4  121    1  50.0  54.0  | 0; 0; 0; 0; 0; 0; 1; 7; 7;
7;13;13;41;159;160;298;391;679;712;865; CAGGGUACGUUCCAAUGU
UGGAACGUACCCUGUUGU  18 B+   5  125    1  50.0  54.0  | 0; 0; 0; 0; 0; 0;
6;16;16;17;23;33;34;35;41;51;55;62;78;89; ACAACAGGGUACGUUCCA
UACUAACGCGUGAAGUUG  18 C=23300  859    1  44.4  52.0  | 0; 0; 0; 0; 0; 0;
8;724;727;735;737;740;1475;1489;1499;2254;2291;2427;3176;3232; CAACUUCACGCGUUAGUA
CUGUAUCCAGCAAUGCCG  18 D=6887  385    1  55.6  56.0  | 0; 0; 0; 4; 4; 4; 4; 8; 8;
8;12;12;12;18;22;26;27;39;54;61; CGGCAUUGCUGGAUACAG
UGUAUCCAGCAAUGCCGC  18 D+   2  386    1  55.6  56.0  | 0; 0; 0; 4; 4; 4; 8; 8; 9;
9;13;18;18;25;40;45;48;1167;2298;2302; GCGGCAUUGCUGGAUACA
UAUCCAGCAAUGCCGCGU  18 D+   5  388    1  55.6  56.0  | 0; 0;
0;1124;2256;2265;2285;3425;3445;3462;3479;4618;4649;4650;4683;5880;5946;5971;7159;7245;
ACGCGGCAUUGCUGGAUA
```

Arthrospira sp. (Spirulina platensis PCC7345^T and PCC8005)

```
Probe design Parameters:
Length of probe      18
Temperature        [45.0 -65.0]
GC-Content         [40.0 -60.0]
E.Coli Position    [   0 -100000]
Max Non Group Hits     0
Min Group Hits        50%
Target              le apos   ecol grps  G+C 4GC+2AT |Decrease T by n*.3C -> probe matches n
non group species     Probe sequence
AGGUCAUCCUUAGGUCGG  18 A=38461 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0; 0; CCGACCUAAGGAUGACCU
CAAACUUUAGGGUUCGUG  18 B=38503 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0; 0; CACGAACCCUAAAGUUUG
GAGGUCAUCCUUAGGUCG  18 A-   1 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0; 0; CGACCUAAGGAUGACCUC
GGGUUCGUGUUAUGGGCU  18 B+   9 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0; 0; AGCCCAUAACACGAACCC
GGUUCGUGUUAUGGGCUA  18 B+  10 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0; 0; UAGCCCAUAACACGAACC
GUCAUCCUUAGGUCGGAU  18 A+   2 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0; 0; AUCCGACCUAAGGAUGAC
GUUCGUGUUAUGGGCUAU  18 B+  11 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0; 0; AUAGCCCAUAACACGAAC
CUUACCUCCACUCUCCUU  18 C=38678 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0; 2; AAGGAGAGUGGAGGUAAG
CUUUUUAGGGAGACCUAC  18 D=38394 1538    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 0;45; GUAGGUCUCCCUAAAAAG
AGGGUUCGUGUUAUGGGC  18 B+   8 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 1; 1; GCCCAUAACACGAACCCU
UGAGGUCAUCCUUAGGUC  18 A-   2 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 1; 1; GACCUAAGGAUGACCUCA
GGUCAUCCUUAGGUCGGA  18 A+   1 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 2; 2; UCCGACCUAAGGAUGACC
UAGGGUUCGUGUUAUGGG  18 B+   7 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 2; 3; CCCAUAACACGAACCCUA
CGGUCAGAGAGCUUUCAA  18 E=38485 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 4;38; UUGAAAGCUCUCUGACCG
GUGUUAUGGGCUAUUAGC  18 B+  15 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 0; 8; 9; GCUAAUAGCCCAUAACAC
AUCCUUAGGUCGGAUGGG  18 A+   5 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 1; 1; 1; CCCAUCCGACCUAAGGAU
CGUGUUAUGGGCUAUUAG  18 B+  14 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 1; 2; 2; CUAAUAGCCCAUAACACG
GGUGUAGGAAAACGUCGU  18 F=38857 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 1; 4; 5; ACGACGUUUUCCUACACC
UUUAGGGAGACCUACUUC  18 D+   3 1541    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 0; 2; 6; 7; GAAGUAGGUCUCCCUAAA
ACCCCAAACUGGGGGUAU  18 G=38605 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 1; 7; 9;14; AUACCCCCAGUUUGGGGU
CAAACUGGGGGUAUAGCU  18 G+   4 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 1; 1; 5; 9; AGCUAUACCCCCAGUUUG
GUUAUGGGCUAUUAGCUC  18 H=38521 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 1; 3; 4; 5; GAGCUAAUAGCCCAUAAC
UUGAGGUCAUCCUUAGGU  18 A-   3 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 1; 1; 1; 3; ACCUAAGGAUGACCUCAA
```

```
CCUUUUUUAGGGAGACCUA  18 D-   1 1537    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 2; 6;17;23; UAGGUCUCCCUAAAAAGG
GCUUACCUCCACUCUCCU  18 C-   1 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 3; 3; 3; 3; AGGAGAGUGGAGGUAAGC
UUACCUCCACUCUCCUUU  18 C+   1 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 3; 8;10;12; AAAGGAGAGUGGAGGUAA
AAACUGGGGGUAUAGCUC  18 G+   5 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 4; 7;10;14; GAGCUAUACCCCCAGUUU
UGGUGUAGGAAAACGUCG  18 F-   1 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 0; 8; 9;10;13; CGACGUUUUCCUACACCA
CUUUGCACGGCAGAAGUC  18 I=38645 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 1; 3; 4; 7; 8; GACUUCUGCCGUGCAAAG
AUGGUGUAGGAAAACGUC  18 F-   2 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 2; 2; 3; 4; 5; GACGUUUUCCUACACCAU
GGUCAGAGAGCUUUCAAA  18 B-  17 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 0; 4; 4;38;38;48; UUUGAAAGCUCUCUGACC
GGAAAACGUCGUAAAGAC  18 F+   6 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 1; 1; 2; 2; 4; 4; GUCUUUACGACGUUUUCC
UCAUCCUUAGGUCGGAUG  18 A+   3 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 1; 2; 2; 5; 6; 6; CAUCCGACCUAAGGAUGA
UCCUUUUUUAGGGAGACCU  18 D-   2 1536    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 1; 1; 1; 2; 5; 6; AGGUCUCCCUAAAAAGGA
UUAGGGUUCGUGUUAUGG  18 B+   6 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 1; 2; 2; 2; 2; 2; CCAUAACACGAACCCUAA
CAUCCUUAGGUCGGAUGG  18 A+   4 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 2; 2; 2; 3; 4; 4; CCAUCCGACCUAAGGAUG
GUGUAGGAAAACGUCGUA  18 F+   1 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
0; 7; 7;61;62;70;74; UACGACGUUUUCCUACAC
CCAAACUGGGGGUAUAGC  18 G+   3 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 3; 3; 3; 5; 5; 5; GCUAUACCCCCAGUUUGG
CCCAAACUGGGGGUAUAG  18 G+   2 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 1; 1; 2; 4; 6; 7; CUAUACCCCCAGUUUGGG
CGUCGUAAAGACAAUUCC  18 F+  12 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 1; 1; 1; 2;25;25; GGAAUUGUCUUUACGACG
CUUGAGGUCAUCCUUAGG  18 A-   4 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 1; 1; 1; 2; 3; 3; CCUAAGGAUGACCUCAAG
GCGGUCAGAGAGCUUUCA  18 E-   1 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 1; 1; 1; 2; 6; 8; UGAAAGCUCUCUGACCGC
GUCAGAGAGCUUUCAAAC  18 B-  16 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 1; 1;36;37;39;62; GUUUGAAAGCUCUCUGAC
GUUCCAGUCAGAACCUUG  18 J=38811 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 1; 1; 1; 2; 5; 7; CAAGGUUCUGACUGGAAC
UCUUGAGGUCAUCCUUAG  18 A-   5 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
1; 1; 1; 2; 3; 3; 3; CUAAGGAUGACCUCAAGA
CCACAUCCACCCCAAACU  18 G-   8 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
3; 5; 5; 8;12;15;17; AGUUUGGGGUGGAUGUGG
AACUGGGGGUAUAGCUCA  18 G+   6 1542    2  50.0  54.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0;
4; 7;10;15;24;26;28; UGAGCUAUACCCCCAGUU
CACAUCCACCCCAAACUG  18 G-   7 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 1;
3; 4; 4; 7; 8; 9; 9; CAGUUUGGGGUGGAUGUG
CCUUUGCACGGCAGAAGU  18 I-   1 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 1;
4; 4; 4; 6; 7;17;19; ACUUCUGCCGUGCAAAGG
UCCGCUUACCUCCACUCU  18 C-   4 1542    2  55.6  56.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 1;
1; 1; 2; 3; 3; 5; 9; AGAGUGGAGGUAAGCGGA
UUCCAAUGUAGGUCAAGC  18 K=38883 1542    2  44.4  52.0  | 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 0; 1;
3; 4;13;29;51;65;91; GCUUGACCUACAUUGGAA
```

Addendum B: sequence details on 16S rRNA genes and primer binding sites

```
LOCUS       RSP16SRNAK              1406 bp    DNA     linear   BCT 07-FEB-
1999
DEFINITION  Rhodospirillum rubrum gene for 16S ribosomal RNA.
ACCESSION   D30778
VERSION     D30778.1  GI:494951
KEYWORDS    16S rRNA; 16S ribosomal RNA.
SOURCE      Rhodospirillum rubrum (strain:ATCC 11170) DNA.
ORGANISM    Rhodospirillum rubrum
            Bacteria; Proteobacteria; alpha subdivision; Rhodospirillaceae;
            Rhodospirillum.
```

```
REFERENCE   1  (bases 1 to 1406)
AUTHORS     Hiraishi,A.
TITLE       Direct Submission
JOURNAL     Submitted (21-MAY-1994) Akira Hiraishi, Central Research
            Laboratories, Ajinomoto Co., Inc., Basic Research Laboratories;
1-1
            Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210, Japan
            (Tel:044-244-7181, Fax:044-246-2867)
REFERENCE   2  (bases 1 to 1406)
AUTHORS     Hiraishi,A.
JOURNAL     Unpublished (1995)
REFERENCE   3  (sites)
AUTHORS     Hiraishi,A. and Ueda,Y.
TITLE       Isolation and characterization of Rhodovulum strictum sp. nov.
and
            some other purple nonsulfur bacteria from colored blooms in
tidal
            and seawater pools
JOURNAL     Int. J. Syst. Bacteriol. 45 (2), 319-326 (1995)
MEDLINE     95244315
PUBMED      7537066
REFERENCE   4  (sites)
AUTHORS     Kishimoto,N., Kosako,Y., Wakao,N., Tano,T. and Hiraishi,A.
TITLE       Transfer of Acidiphilium facilis and Acidiphilium aminolytica
to
            the genus Acidocella gen. nov., and emendation of the genus
            Acidiphilium
JOURNAL     Syst. Appl. Microbiol. 18, 85-91 (1995)
```

>gi|494951|dbj|D30778.1|RSP16SRNAK Rhodospirillum rubrum 16S rRNA gene

>gi|494951|dbj|D30778.1|RSP16SRNAK Rhodospirillum rubrum 16S rRNA gene

```
GACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGCATCCTTCGGGATGAGTGGCGCACGGGTG
AGTAACACGTGGGAACGTACCTTGGAGTGCGGAATAATCTTTGGAAACGAGGACTAATACCGCATACGCC
CTTAGGGGGAAAGATTTATCGCTCCAAGATCGGCCCGCGTCCGATTAGCTAGTTGGCGGGGTAATGGCCC
ACCAAGGCGACGATCGGTAGCTGGTCTGAGAGGATGGCCAGCCACACTGGGACTGAGACACGGCCCAGAC   EUB338
TCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGCAACCCTGACGCAGCCATGCCGCGTGAGT
GAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCGGGTGTGAAGATGATGACGGTAACACCAGAAGAAGCCCC
GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCAAGCGTTGTTCGGAATTACTGGGCGTAAA
GAGCGCGTAGGCGGTCTGATTAGTCAGAGGTGAAATCCCAGAGCTCAACTTTGGAACTGCCTTTGATACT   RRUB586
GTTAGACTAGAATCCGTGAGAGGGTGGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAGG
AACACCAGTGGCGAAGGCGGCCACCTGGCGCGGTATTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGATGTCGGGGTACATGTACCTCGGTG
TCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGAC
ATCCCGTGACACTTCCAGAGATGGAAGGTTCCCTTCGGGGACACGGTGACAGGTGCTGCATGGCTGTCGT   RRUB999
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCATCTTCAGTTGCCAGCAAG
TAACGTTGGGCACTCTGAAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCA
TGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGCGCCTACAATGGGCAGCGACCTCGCGAGGGGA
AGCTAATCTCCAAAAGGCGTCTCAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATCGCT   UNIV1390
AGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATG
GGAGTTGGTTCTACCCGAAGACGGTACGCTAACCGCAAGGAGGCAGCCGGCCACGTTAGGGTCAGCGACT
```

```
LOCUS       RECH3416S              1493 bp    DNA     linear   BCT 23-MAR-
2000
DEFINITION  Ralstonia sp. CH34 16S rRNA gene, strain CH34.
ACCESSION   Y10824
VERSION     Y10824.1  GI:2058350
KEYWORDS    16S ribosomal RNA; 16S rRNA.
```

```
SOURCE       Ralstonia metallidurans.
ORGANISM     Ralstonia metallidurans
             Bacteria; Proteobacteria; beta subdivision; Ralstonia group;
             Ralstonia.
REFERENCE    1  (bases 1 to 1493)
AUTHORS      Brim,H., Heyndrickx,M., de Vos,P., Wilmotte,A., Springael,D.,
             Schlegel,H.G. and Mergeay,M.
TITLE        Amplified rDNA restriction analysis and further genotypic
             characterisation of metal-resistant soil bacteria and related
             facultative hydrogenotrophs
JOURNAL      Syst. Appl. Microbiol. 22 (2), 258-268 (1999)
MEDLINE      99319350
REFERENCE    2  (bases 1 to 1493)
AUTHORS      Brim,H.
TITLE        Direct Submission
JOURNAL      Submitted (27-JAN-1997) H. Brim, Flemish Institute Of
Technological
             Research, Environmental Biotechnology Department, Boeretang
200,
             B-2400, MOL, BELGIUM
FEATURES              Location/Qualifiers
```

```
>gi|2058350|emb|Y10824.1|RECH3416S Ralstonia sp. CH34 16S rRNA gene, strain
CH34
AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGTATGCCTTACACATGCAAGTCGAACGGCAGCGCGG
ACTTCGGTCTGGCGGCGAGTGGCGAACGGGTGAGTAATACATTGGAACGTACCCTGTTGTGGGGGATAAC
TAGTCGAAAGATTAGCTAATACCGCATACGACCTGAGGGTGAAAGTGGGGGACCGCAAGGCCTCACGCAG
CAGGAGCGGCCGATGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCCACCAAGGCGACGATCAGTAGCTGG
TCTGAGAGGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATTTTGGACAATGGGGGCAACCTGTATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAG
CACTTTTGTCCGGAAAGAAATCGCGCTGGTTAATACCTGGCGTGGATGACGGTACCGGAAGAATAAGCAC
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAA
GCGTGCGCAGGCGGTTTTGTAAGACAGGCGTGAAATCCCCGGGCTTAACCTGGGAATTGCGCTTGTGACT
GCAAGGCTAGAGTGCGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGG
AATACCGGATGGCGAAGGCAGCCCCCTGGGACGTGACTGACGCTCATGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTTCTCAGT
AACGATACTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAACTCAAAGGAATTGAGG
GGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACA
TGCCACTAACGAAGCAGAGATGCATTAGGTGCCCGAAAGGGAAAGTGGACACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGNGCAACCCTTGTCTCTAGTTGCTACGC
AAGAGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCC
CTTATGGGTAGGGCTTCACACGTCATACAATGGTGCGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTA
ATCCCAGAAAACGCATCGTAGTCCGGATCGTAGTCTGCAACTCGACTACGTGAAGCTGGAATCGCTAGTA
ATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTTTGCCAGAAGTAGTTAGCCTAACCGCAAGGAGGGCGATTACCACGGCAGGGTTCATGACTGGGG
TGAAGTCGTAACAAGGTAGCCGT
```

2-27

RMET110

EUB338

UNIV1390

```
LOCUS        AS16S                    1959 bp    DNA     linear   BCT 27-JUL-
2001
DEFINITION   Arthrospira sp. gene for 16S rRNA, strain PCC8005.
ACCESSION    X70769
```

```
VERSION     X70769.2  GI:15028802
KEYWORDS    16S ribosomal RNA; ribosomal RNA; small subunit ribosomal RNA.
SOURCE      Arthrospira sp.
ORGANISM    Arthrospira sp.
            Bacteria; Cyanobacteria; Oscillatoriales; Arthrospira.
REFERENCE   1  (bases 1 to 1958)
AUTHORS     Nelissen,B., Wilmotte,A., Neefs,J.M. and De Wachter,R.
TITLE       Phylogenetic relationships among filamentous helical
cyanobacteria
            investigated on the basis of 16S ribosomal RNA gene sequence
            analysis
JOURNAL     Syst. Appl. Microbiol. 17, 206-210 (1994)
REFERENCE   2  (bases 1 to 1959)
AUTHORS     Wilmotte,A.M.
TITLE       Direct Submission
JOURNAL     Submitted (20-JAN-1993) A.M. Wilmotte, University of Antwerp
            (U.I.A.), Dept of Biochemistry, Universiteitsplein 1, 2610
Wilrijk,
            BELGIUM
REMARK      revised by [3]
REFERENCE   3  (bases 1 to 1959)
AUTHORS     Wilmotte,A.M.
TITLE       Direct Submission
JOURNAL     Submitted (27-SEP-1993) A.M. Wilmotte, University of Antwerp
            (U.I.A.), Dept of Biochemistry, Universiteitsplein 1, 2610
Wilrijk,
            BELGIUM
REMARK      revised by [5]
REFERENCE   4  (bases 1 to 1959)
AUTHORS     Wilmotte,A.M.
TITLE       Direct Submission
JOURNAL     Submitted (25-JUL-2001) A.M. Wilmotte, Laboratory of Algology,
            Mycology and Experimental Systematics Dept of Botany B22,
            University of Liege B-4000, Liege, Belgium
COMMENT     On Jul 29, 2001 this sequence version replaced gi:556876.
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>gi|15028802|emb|X70769.2|AS16S Arthrospira sp. 16S rRNA, strain PCC8005
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APLA1759