Microcokit Training School Program

Methods for detecting and quantifying aquatic microbial communities

Tuesday 4th April

12:30 - 13:30 Registration – Library Room (1st floor)

13:30-17:30 Meeting Room (1st floor):

13:30 Overview of the Marie Curie - MicroCokit Project, Introduction and Course objectives Overview of molecular methods to characterize natural microbial communities

Anna Barra Caracciolo IRSA-CNR, Rome (IT)

14:00 Principles and basic considerations of qPCR/RT-qPCR

Rebecca Sanders LGC, Teddington (UK)

14:30 Metagenomics: a closer look at microbial diversity

Sara Riccardo, Elena Diaconu Bio-Fab Research, Rome (IT)

15:00 - 15:20 Coffee break

15:20 Direct Epifluorescence microscope methods; Developing Cyanobacteria probes for the Fluorescence In-Situ Hybridisation technique (FISH)

Paola Grenni IRSA-CNR, Rome (IT), Gerardo Mengs *NTBC*, Madrid (Spain)

15:50 Microarray

Linda Medlin MBA, London (UK)

16:30 Discussion and Questions

17:00 end of the first day

Wednesday 5th April

9.00 – 12.30 Participants will be organised in small groups for the laboratory demonstrations (Meeting Room)

Lab 02 (Ground floor): Fluorescence *In Situ* Hybridization analysis (FISH)
Maria Ludovica Saccà CREA-CIN Bologna (IT)*, Martina Cardoni IRSA-CNR*, Rome (IT)

Meeting Room (1st floor) and Lab 17 (Ground floor): Practical considerations: primer design, assay optimisation and validation, normalisation, data analysis and troubleshooting of qPCR/RT-qPCR
Rebecca Sanders LGC, Teddington UK, *Diana Conduto JRC,* ISPRA (IT)

10.30-11.00 Beak

Lab 02 (Ground floor): Fluorescence *In Situ* Hybridization analysis (FISH)
Maria Ludovica Saccà CREA-CIN Bologna (IT)*, Martina Cardoni IRSA-CNR*, Rome (IT)

Meeting Room (1st floor) and Lab 17 (Ground floor): Practical considerations: primer design, assay optimisation and validation, normalisation, data analysis and troubleshooting of qPCR/RT-qPCR
Rebecca Sanders LGC, Teddington UK, *Diana Conduto JRC,* ISPRA (IT)

12.30 14.00 Light Lunch

14.00 – 17:30 Participants will be organised in small groups for the laboratory demonstrations

Lab 02 (Ground floor): Fluorescence *In Situ* Hybridization analysis (FISH)

Francesca Falconi, Martina Di Lenola IRSA-CNR, Rome (IT)

Meeting Room (1st floor) and Lab 17 (Ground floor): Practical considerations: primer design, assay optimisation and validation, normalisation, data analysis and troubleshooting of qPCR/RT-qPCR

Rebecca Sanders LGC, Teddington UK, *Diana Conduto JRC,* ISPRA (IT)

15.30-16.00 Beak

Lab 02 (Ground floor): Fluorescence *In Situ* Hybridization analysis (FISH)

Francesca Falconi, Martina Di Lenola IRSA-CNR, Rome (IT)

Meeting Room (1st floor) and Lab 17 (Ground floor): Practical considerations: primer design, assay optimisation and validation, normalisation, data analysis and troubleshooting of qPCR/RT-qPCR

Rebecca Sanders LGC, Teddington UK, *Diana Conduto JRC,* ISPRA (IT)

Thursday - April 6th

9.00 - 12.00 Laboratory demo: Microarray Lab 17 (Ground floor)

Linda Medlin MBA, London (UK)

12.00 - 12.30 Conclusive remarks and distribution of certificates Meeting Room (1st floor)

13.30 Tutors are available for specific details and discussion about the techniques used in laboratory demos.

Microcokit Partners

Anna Barra Caracciolo, IRSA-CNR, (Rome, Italy)
Teresa Lettieri, JRC (Ispra, Italy)
Paola Grenni, IRSA-CNR (Rome Italy)
Linda Medlin Marine Biological Association (London, UK)
Rebecca Sanders LGC (London)
Gerardo Mengs NTBC (Madrid, Spain)
Diana Conduto JRC (Ispra, Italy)
Martina Di Lenola, IRSA-CNR (Rome, Italy)
Maria Ludovica Saccà *Microcokit Marie Curie Fellowship* (CREA-CIN Bologna, Italy)

The Training Course is supported by:
Fabio Riccobono
Sara Riccardo
Elena Diaconu

Letizia Ciccone

Microcokit: microbial community-based sequence analysis linked to anthropogenic pressures to address the water quality

Anna Barra Caracciolo (Project Coordinator)
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The MicroCokit Project (Microbial community-based sequence analysis linked to anthropogenic pressures to address the water quality) is a Marie Curie Industry-Academia Partnerships and Pathways (FP7-PEOPLE-2012-IAPP). It is a close collaboration between research centers (IRSA-CNR Rome, MBA London, European Commission Joint Research Centre) and leading private Enterprises (LGC London, NTBC Madrid).

It has been conceived to investigate and identify complex stressor indicators based on microbial communities and to foster the transfer of knowledge among the partners with the final goal to bring to market faster, more sensitive and robust tools as bioindicators of water quality.

The tools have been developing according different kinds of bioindicators, targeting natural microbial community, pathogen and specific microorganisms.

The river Tiber has been chosen as a pilot case study and four sampling sites were selected based on different anthropogenic pressures which they are exposed to. For each site water sample have been analyzed for both microbiological (Microarray, Metagenomic and FISH analysis) and chemical analysis (organic and inorganic compounds, including emerging pollutants). Following the validation, these data could be then integrated in a modeling system to predict, prevent and mitigate the impact of anthropogenic pressure on water management.

The Training School held at IRSA-CNR responds to the task T6 of the Microcokit project: *Dissemination of the knowledge generated during the project.*

The address of the project public website is: **http://www.microcokit.eu/**

Principles and basic considerations of qPCR/RT-qPCR

Rebecca Sanders[1]

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Quantitative PCR is a precise method for nucleic acid measurement. qPCR techniques have the ability to quantify nucleic acids over a wide dynamic range (at least eight orders of magnitude) and are precise (DNA and RNA measurements can typically be optimised to have a coefficient of variation of < 5% or < 10%, respectively (Devonshire *et al*., 2011)). Routine detection of fewer than five target copies make it possible to analyse small samples such as clinical biopsies or miniscule lysates from laser capture microdissection (Vandesompele *et al*., 2002; Valasek *et al*., 2005; Bustin, 2000). But measurements using this precise technique are only as robust as the upstream processes used to sample, store and prepare the nucleic acid. Furthermore, quantification requires calibration to an appropriate standard. However, what is often overlooked is that the whole stepwise procedure contributes to the experimental precision. Here will be discussed processes that need consideration for accurate, reproducible analysis (Nolan *et al*., 2013).

References:

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25 (2):169-193.

Devonshire AS, Elaswarapu R, Foy CA (2011) Applicability of RNA standards for evaluating RT-qPCR assays and platforms. BMC Genomics 12:118.

Nolan T, Huggett J, Sanchez E (2013) Good practice guide for the application of quantitative PCR (qPCR). http://www.lgcgroup.com/our-science/national-measurement-laboratory/publications-and-resources/good-practice-guides/good-practice-guide-for-the-application-of-quantit/#.WM_6Wblvjcs

Valasek MA, Repa JJ (2005) The power of real-time PCR. Adv Physiol Educ 29 (3):151-159.

Vandesompele J, De Preter K, Pattyn F et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3 (7):RESEARCH0034.

Metagenomics: a closer look at microbial diversity

Sara Riccardo[1], Federico Andreani[1], Elena Diaconu[1], Cristina Prisco[1],Giada Galleazzi[1] and Fabio Riccobono[1].

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Metagenomics is the genomic analysis of a mixed population of microorganisms. The development of new, advanced sequencing techniques is of crucial importance to understanding the critical role microorganisms play in many biological processes, as well as the biological diversity of a microbial population in environmental samples.

Amplicon sequencing, in particular that of the small subunit rRNA gene (16S rRNA gene in Bacteria and Archaea or 18S rRNA gene in Eukarya), is a widely applied approach to study the composition, organization and spatiotemporal patterns of microbial communities, due to its ubiquity across all domains of life.

Early bacterial community studies typically sequenced the entire 16S rRNA gene, but their ability to sample the full array of bacterial diversity was limited by depth of sequencing. With the advent of massively parallel sequencing technologies, which generally yield short reads, focus has shifted from sequencing the full 16S rRNA gene to sequencing shorter sub-regions of the gene at great dept.

The Illumina MiSeq platform provides researchers with a scalable, high-throughput and streamlined sequencing platform to survey community composition from clinical and environmental samples. The most widely used 16S rRNA-based MiSeq sequencing strategies include a dual-indexing approach targeting a region of approximately 469 bp encompassing the V3 and V4 hyper variable regions of the 16S rRNA gene.

This region provides ample information also for taxonomic classification of microbial communities from specimens associated with human microbiome studies and wasused by the Human Microbiome Project

However, the approach described could be adapted to any primer pairs.

Bio-Fab Research srl is a Biotech company whose main activity is to produce and develop services with high biotechnological content in order to offer the necessary support to the scientific community.

Through state-of-the-art sequencing and development of tailor-made bioinformatics tools we can offer a wide range of next generation sequencing services based on Illumina MiSeq and HiSeq technological platforms.

Here we will give you an overview of 16S Metagenomic sequencing on the MiSeq Illumina platform, with particular emphasis on the library preparation and secondary analysis using MiSeq Reporter.

References:

Metagenomics: key to human gut microbiota.
Maccaferri S, Biagi E, Brigidi P.
Dig Dis. 2011; 29(6):525-30. doi: 10.1159/000332966. Review.
PMID: 22179207

Metagenomics study of endophytic bacteria in Aloe vera using next-generation technology
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Fluorescent in Situ *Hybridization* (FISH)

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The non-PCR-based methods (without DNA extraction) commonly used in environmental studies for quantifying microbial cell number and their phylogenetic characterization are epifluorescence microscope methodologies, such as total direct counts of microbial abundance (e.g. by DAPI stain), cell viability (Live/Dead assay) and Fluorescence *In Situ* Hybridization technique (FISH). They make it possible to characterize *in situ* microbial populations in their natural ecosystems (Grenni et al. 2009; Barra Caracciolo et al. 2010; Barra Caracciolo et al. 2015) making it possible to detect both culturable and unculturable microorganisms. In particular, FISH combines the precision of molecular genetics with the direct visual information from microscopy, allowing simultaneous visualization, identification, enumeration and localization of individual microbial cells within their natural microhabitat. Because whole cells are hybridized, artefacts arising from biases in DNA extraction, PCR amplification and cloning are avoided (Amann and Fuchs 2008).

FISH is used to detect microbial cells at different hierarchical phylogenetic levels using fluorescent labelled oligonucleotides complementary to 16S or 23S ribosomal RNA (rRNA). Oligonucleotide probes are designed based on signature nucleotide positions in the bacterial 16S or 23S rRNA and may be used to target either a narrow or a broad group of organisms. Probes for kingdoms (*Bacteria*, *Archaea*), families, genera, species or strains can be differentially labelled and used in combination to simultaneously detect the occurrence and distribution of several taxonomic groups within a single environmental sample (Grenni et al. 2009). Fluorescent dyes can be used or multiple probes can be designed to target different regions of the same 16S or 23S rRNA molecule, thus increasing the strength of the signal.

High levels of rRNA (equivalent to high ribosome numbers), which result in high detection signals with the FISH technique, are observed in active populations of bacteria in which protein synthesis is occurring either in non-dividing or dividing cells. The whole-cell identification of microorganisms together with information on morphology of active cells is realised quickly and with highly specificity.

During the past years, different protocols have been developed in order to improve the sensitivity of FISH and to permit the detection of single active cells. Although there are some specific differences in protocols designed for water, soil and sediment (Barra Caracciolo et al. 2005; Barra Caracciolo 2010), the method has the following basic steps: fixation and permeabilisation with the aim of ensuring a proper penetration of oligonucleotide probes inside cells, hybridization of the probe with a rRNA complementary site, removal of non-specifically bound probes and fluorescent detection of cells (Bottari et al. 2006).

There is a constantly growing on-line data base available in public resources (probeBase, http://probebase.csb.univie.ac.at) which contains the specification of more than 1200 probes and their hybridization conditions that can be applied in a variety of environmental samples (Greuter et al. 2016).

References:

Amann R, Fuchs BM, 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. *Nature Reviews Microbiology* 6, 339-348.

Barra Caracciolo A, Grenni P, Cupo C, Rossetti S, 2005. In situ analysis of native microbial communities in complex samples with high particulate loads. *FEMS Microbiology Letters* 253(1), 55–58

Barra Caracciolo A, Bustamante M A, Nogues I, Di Lenola M, Luprano ML, Grenni P, 2015. Changes in microbial community structure and functioning of a semiarid soil due to the use of anaerobic digestate derived composts and rosemary plants. *Geoderma*, 245–246, 89-97.

Barra Caracciolo, A, Bottoni, P, Grenni, P, 2010. Fluorescence In Situ Hybridization in soil and water ecosystems: an useful method for studying the effect of xenobiotics on bacterial community structure. *Toxicological & Environmental Chemistry*, 92(3), 567-579.

Bottari B, Ercolini D, Gatti M, Neviani E, 2006 Application of FISH technology for microbiological analysis: current state and prospects. *Applied Microbiology Biotechnology* 73, 485–494

Grenni P, Gibello A, Barra Caracciolo A, Fajardo C, Nande M, Vargas R, Saccà M L, Martinez-Iñigo M J, Ciccoli, R, Martín M, 2009. A new fluorescent oligonucleotide probe for *in situ* detection of s-triazine-degrading *Rhodococcus wratislaviensis* in contaminated groundwater and soil samples. *Water Research* 43, 2999-3008.

Greuter D, Loy A, Horn M, Rattei T, 2016. probeBase--an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016. *Nucleic Acids Research* 44(D1), D586-9.

Hugenholtz P, Tyson GW, Blackall LL, 2002. Design and Evaluation of 16S rRNA-Targeted Oligonucleotide Probes for Fluorescence In Situ Hybridization. In: *Gene Probes* (Methods in Molecular Biology Series) 179, 29-42.

Application of microarrays for analysis of community diversity by species identification

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DNA microarray technology was introduced in 1995 by Scheena and rapidly became one of the most powerful innovations in microbiology because it allowed a rapid acquisition of copious data through the analysis of a large number of samples using a range of different probes in parallel under a diverse spectrum of applications (Ye et al. 2001). Microarrays were originally used as a functional genomic method for studying gene expression, single nucleotide poly morphism detection and DNA resequencing (Lipshutz et al. 1999, Kauppinen et al. 2003, Ji & Tan 2004, Yap et al. 2004, Al-Shahrour et al. 2005, Broet et al. 2006, Gamberoni et al. 2006) but quickly expanded into species identification, the so-called phylochip (De Sanits et al. 2006). A microarray consists of DNA sequences that are applied to the surface of a glass slide with special surface properties in an ordered array. It is based on a minimized form of a dot blot (Gentry et al. 2006, Ye et al. 2001). A DNA microarray experiment involves microarray production, sample isolation and preparation, hybridization and data analysis. Prior to hybridization, the target nucleic acids are labeled with a fluorescent dye directly or indirectly (Cheung et al. 1999, Southern et al. 1999, Metfies et al. 2006). The hybridization pattern is captured via fluorescent excitation in a special device, the microarray scanner (Ye et al. 2001).

The use of DNA phylochips to identify organisms is a relatively new and innovative application of microarray technology. Specific probes initially developed for other hybridization assays (e.g. whole cell, FISH) have been successfully modified (Kegel et al. 2013a) and employed for the phylochip detection method. Although a large number of species or other taxa can be identified as targets in one experiment (Ye et al. 2001), the techniques have not widely been applied to biodiversity and ecosystem science. A number of European research groups utilize DNA microarrays for the identification of marine organisms. DNA phylochips have been used to identify phytoplankton (Gescher et al. 2008a, Metfies et al. 2010), toxic algae (Barra et al. 2012, Edvardsen et al. 2012, Dittami et al. 2013a, b, Kegel et al. 2013a, b, Metfies & Medlin 2004, Ki & Han 2006, McCoy et al. 2012, 2015, Medlin et al. 2006, Gescher et al. 2008b, Taylor et al. 2013), bacteria (Loy et al. 2002, Peplies et al. 2003, 2004a, b, 2006, Lehner et al. 2005, Loy et al. 2002), fish

(Kappel et al. 2003) and invertebrates (Chitipothu et al. 2014). A commercial phylochip is available for all known prokaryotes (Schatz et al. 2010).

Specific probes can be designed with a variety of software packages of which the probe design and probe match tool of the ARB software (Ludwig et al. 2004) is one of the most powerful. A taxonomical hierarchical strategy to probe design is the best option for ensuring that false positives can be eliminated. Thus for a species to be present, the genus, family, order, class, phylum, and kingdom probes must also be present. In exploring a world where microbial biodiversity is not well known, the hierarchical probe approach will identify the correct target against a myriad of unknown targets. This method will eliminate false positives because the entire taxonomic hierarchy must be present for the target species to be present. Considering the vast unknown biodiversity in aquatic systems, this method is indispensable. Probes can be either in-situ synthesized directly on a glass slide or synthesized separately and spotted and fixed later. Probes for species and higher taxon levels, and various positive and negative controls are spotted in multiple replicates across the array.Hybridisations of the MIDTAL/μAQUA microarray are done in two technical duplicates (two grids on one slide or one grid on two slides). Microarrays are scanned with a fluorescent reader, such as GenePix from Molecular Devices Cooperation (Sunnyvale, USA) and analyzed with their software. This is exported as an excel file and loaded into the GPR analyzer (Dittami & Edvardsen 2013). This program implements the computation of signal to noise ratios (S/N) across the hierarchical probe set, which eliminates false positives. The excell file from the averaging of the two replicated GPR files can be exported into excell for display purposes as bar or line graphs or exported into PermutMatrix for display as a heat map.

The main advantage of the phylochip microarray is the magnitude of probes that can be analyzed at a time. Results from an experiment can be analyzed within three hours of the sample RNA extraction, which is a real advantage over NGS methods. RNA targets rather than DNA ones will highlight only actively growing cells and thus the phylochips can be quantitative. The introduction of any PCR step prior to the hybridization makes the phylochip non-quantitative.

References: See reference list in Kegel et al. 2016