MELiSSA

TECHNICAL NOTE

Departament d'Enginyeria Química
Escola Tècnica Superior d'Enginyeria
Universitat Autònoma de Barcelona
Tel.: 93.581.10.18 Fax: 93.581.20.13
08193 Bellaterra Spain

STUDIECENTRUM VOOR KERNENERGIE
CENTRE D'ÉTUDE DE L'ÉNERGIE NUCLÉAIRE

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Biofilm studies in compartment III pilot reactor: experimental set-up and technical description

Prepared by/Préparé par Anna Montràs, Larissa Hendrickx, Julio Pérez
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1. INTRODUCTION

Nitrifying bacteria are required in a biological life support system to perform the oxidation of ammonium to nitrate. In the MELISSA loop, nitrification is carried out in an upflow cocurrent packed bed reactor where the two selected strains, *Nitrosomonas europaea* (ATCC 19718) and *Nitrobacter winogradskyi* (ATCC 25391), are immobilized on a polymeric substratum (Biostyr®).

The biofilm developed in compartment III is not homogeneous along the fixed bed vertical axis. The biomass concentration is visibly higher at the bottom of the reactor, while there is a clear decrease of its concentration towards the upper parts of the packed bed [26, 23]. Previous studies performed in the nitrifying pilot reactor provided data that were used to validate a mathematical model that was capable of predicting a profile of biomass concentration as well as the relative distribution of the two strains (*Nitrosomonas europaea* and *Nitrobacter winogradskyi*) along the vertical axis of the packed bed [27]. The information pointed out by observations and previous studies can be backed up by performing an extensive analysis of the biofilm developed in the reactor. To carry out an exhaustive analysis of the biofilm it is necessary to stop the reactor operation and dismantle it so that biofilm samples can be obtained. Information provided by the biofilm analysis can be used to experimentally validate the results predicted by the mathematical model.

The biofilm from the nitrifying pilot reactor of compartment III was analyzed after a long period of continuous operation (4 years) by visualisation using Fluorescent In Situ Hybridization (FISH) techniques followed by detection with Confocal Laser Scanning Microscopy (CLSM) and subsequent image analysis, with the final aim to obtain experimental evidence about the distribution of the two initial strains (*N. europaea* and *N. winogradskyi*) within the biofilm and along the reactor vertical axis. In addition, CLSM and lectin staining were used in parallel on some fresh samples from the reactor in order to estimate Extracellular Polymeric Substances (EPS) content.

FISH is a highly effective technique that enables researchers to detect specific bacterial cells and to analyze and quantify the spatial distribution of organisms or groups of organisms within bacterial communities. The application of *in situ* hybridization using 16S rRNA-targeted oligonucleotide probes labelled with a fluorescent compound makes it possible to detect bacterial cells at very low concentrations and even at a single-cell level. [3, 4]

FISH has been widely used for the characterization of complex bacterial communities such as biofilms. [2]. Previous studies have proved this technique adequate to analyze the composition of biofilms [34] and to estimate the proportion of ammonia oxidizing bacteria and nitrite oxidizing bacteria in the total bacterial community that constitutes a biofilm [28, 24].

In this technical note an extensive description is given on the techniques and protocols used to study the biofilm developed in the compartment III pilot reactor after four years of continuous operation. A report on the main results of this study as well as an outline of general trends will be presented in this document, while a full account of the quantification tools used and the final results obtained is to be presented in TN 78.92.

2. QUANTIFICATION OF ORGANISMS RESIDING IN A BIOFILM USING CONFOCAL LASER SCANNING ANALYSIS

In this study, biofilms were monitored with the confocal laser scanning microscope (LEICA DM IRE2, LEICA Microsystems, Germany). Confocal laser scanning microscopy (CLSM) allows direct investigation of microbial biofilms visualised with fluorescent probes without outside disturbance [6]. The possibility to observe 3D biofilm morphogenesis *in situ* was accomplished with the development of CLSM by which it was possible to eliminate interference from out-of-focus objects and to create digital reconstructions of the investigated biofilm [12]. Elimination of out-of-focus objects is accomplished by restricted light to a certain confocal plane when the image is formed.

This results in obtaining an optodigital thin section parallel to the microscopic cover glass (x-y plane) with a thickness approaching the theoretical resolution of the light microscope [6]. Consequently a series of 2D (x-y) images perpendicular to the attachment surface (z-direction) can be formed from the scanning object. This series of 2D images can be used to create a 3D reconstruction of the investigated specimen [6]. Afterwards, gray scale images can be enhanced by transformation of the gray level distribution, filtration operations, image calculation operation, object erosion and object dilatation. In a next step the image is converted into a digitized image and ready to be automatically processed [6, 17].

After automated image acquisition, image processing and digital image conversion, investigations based on confocal laser scanning microscopy allow automated quantification of areas occupied by biofilm cells as a stack of 2D images in biofilms divided into Z-sections to obtain the biovolume of the investigated sample. The calculation of the accumulating biovolumes is obtained with a numerical integration algorithm [17], which is integrated in the macro routine of the image analysis software system (LEICA confocal software). The combination of in situ microscopic measurement of the biofilm with the programmed quantification of the obtained data hence produce an objective picture of the investigated biovolume, the biofilm architecture and the microbiological events occurring in the biofilm.

To visualise labeling with fluorescent protein has been performed [7]. For environmental samples, fluorochrome tags such as fluorescent lectins, general nucleic acid stains [13] or oligonucleotide probes are used [10]. Fluorescent lectins are solely used to detect the presence of exopolysaccharides. General nucleic acid dyes are available with differing cell permeability, fluorescence enhancement upon binding nucleic acids, excitation and emmission spectra, DNA/RNA selectivity and binding activity. While some nucleic acid stains differentiate between live and dead cells, others do not. Fluorescently labeled oligonucleotide probes can be obtained with varying degrees of selectivity and can be conjugated to any desired fluorochrome. When biofilm samples are to be stored for future

investigation or hybridized with gene probes, the biofilm needs to undergo paraformaldehyde fixation. If the biofilm will be investigated immediately without hybridization, no fixation would be required.

3. COMPARTMENT III PILOT REACTOR

3.1. Reactor description and operation

The compartment III pilot reactor has a total volume of 8.1L and biofilm was developed on spherical expanded polystyrene (Biostyr®) beads (4.1 mm average diameter). Liquid mixing in the reactor was improved by liquid recirculation. In order to avoid inhibition by light, the fixed bed was protected with thin foil. A detailed description of the reactor hardware, including specifications of the reactor can be found in previous technical notes referring to the design and characterization of this reactor [38-41].

The operating conditions were as follows: pH 8.1, magnetic stirring at the bottom at 400 rpm and temperature controlled at 28.0 ± 0.1 ºC. Air was supplied to the reactor by means of a sparger while oxygen enriched air was added by the control system to maintain a dissolved oxygen set point of 80%. Dissolved oxygen in the culture medium, pH and temperature were measured by means of two on-line probes located at the top and at the bottom of the reactor, whose measurements were weighed by the control system. Dissolved oxygen concentration was controlled by adding pure oxygen or nitrogen to the input gas, a solution of Na_2CO_3 was used to increase pH when necessary, and CO_2 was added when pH needed to be decreased. The total gas flow-rate (3 dm^3 min^{-1}) and as well as liquid flow-rate (0.0028 dm^3 min^{-1}) were kept constant. Exhaust gas was recirculated and an on-off valve regulated pressure in the loop. Oxygen partial pressure in the culture medium was regulated by using oxygen-enriched air if necessary. The optimal temperature proved to be 30°C, coinciding with maximal nitrifying activity. Ammonium conversion ranged from 95 to 100% when the oxygen concentration was maintained above 80% saturation. The maximal surface removal rates were measured as 1.91 gN-

NH_4^+ m^{-2} day^{-1}. Good stability and reproducibility were observed for four years [26]. After four years, the pilot reactor still performed ammonium conversion with high efficiency, with the effluent containing 296 mg $N\text{-}NO_3^-$/l, 0.073 mg $N\text{-}NO_2^-$/l, and <0.169 mg $N\text{-}NH_4^+$/l, thus indicating an ammonium conversion of 99%. The composition of the medium with which the reactor was fed is indicated in table 1.

Table 1: Composition of the feeding medium of the nitrifying fixed bed reactor

Compound	Concentration (g/L)
$FeSO_4 \cdot 7H_2O$	$2.5 \cdot 10^{-3}$
KH_2PO_4	0.680
$NaHCO_3$	0.800
$MgSO_4 \cdot 7H_2O$	0.052
$CaCl_2 \cdot 2H_2O$	$7.4 \cdot 10^{-4}$
$(NH_4)_2 \cdot SO_4$	1.320
$CuSO_4 \cdot 5H_2O$	$4.0 \cdot 10^{-6}$
Na_2HPO_4	0.710
$ZnSO_4 \cdot 7H_2O$	$4.3 \cdot 10^{-6}$
$(NH_4)_6Mo_7O_{27} \cdot 4H_2O$	0.177

The results of the nitrogen compounds analysis before the operation of the reactor was stopped are shown in table 2:

Table 2: composition of the effluent from compartment III prior to reactor dismantling

COMPOUND	Concentration (mg N/L)
Ammonium (inlet)	300 ± 3
Ammonium (outlet)	0.17± 0.02
Nitrite	0.07 ± 0.01
Nitrate	296 ± 3

4. BIOFILM SAMPLING AND PREPARATION

An axenic mixed culture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, immobilized by surface attachment on polystyrene beads, was used for nitrification in a packed-bed reactor at pilot-scale. Sterile COC medium was prepared and fed to the reactor. Although originally intended, optimal axenic reactor processing conditions could not be guaranteed due to difficult sterile valve handling and the use of large media influent volumes. Therefore, it can be assumed that other bacteria may have entered the reactor and have settled in the biofilm community. However, the high nitrifying performance of the pilot reactor offered the opportunity to investigate a functionally stable nitrifying reactor, which had run without decreasing nitrifying efficiency for a period of four years.

For the analysis of biofilm structural and microbial analysis the reactor was dismantled in eight parts and divided using sterile gloves, dishes and instruments (Figure 1). Positions F_1 and F_2 originated from an identical section, position F_1 was the influent-attached compacted biofilm, and position F_2 was biofilm foam-attached biofilm. However, the operating room was not sterile. Hence, it is possible that airborne microorganisms could contaminate the samples (Figure 1) [30]. To limit the chance of contamination, the dismantling was done quickly and the samples were immediately prepared for storage.

Fifty mL of each of the eight fractions were incubated at -80°C in 25% glycerol without any alteration being made on the biofilm samples (i.e. the biofilm was not detached from the support beads), whereas the rest of the biomass of each of the eight fractions was separated from the support beads and freeze-dried in order to perform further analyses (e.g. estimation of total biomass concentration in each fraction).

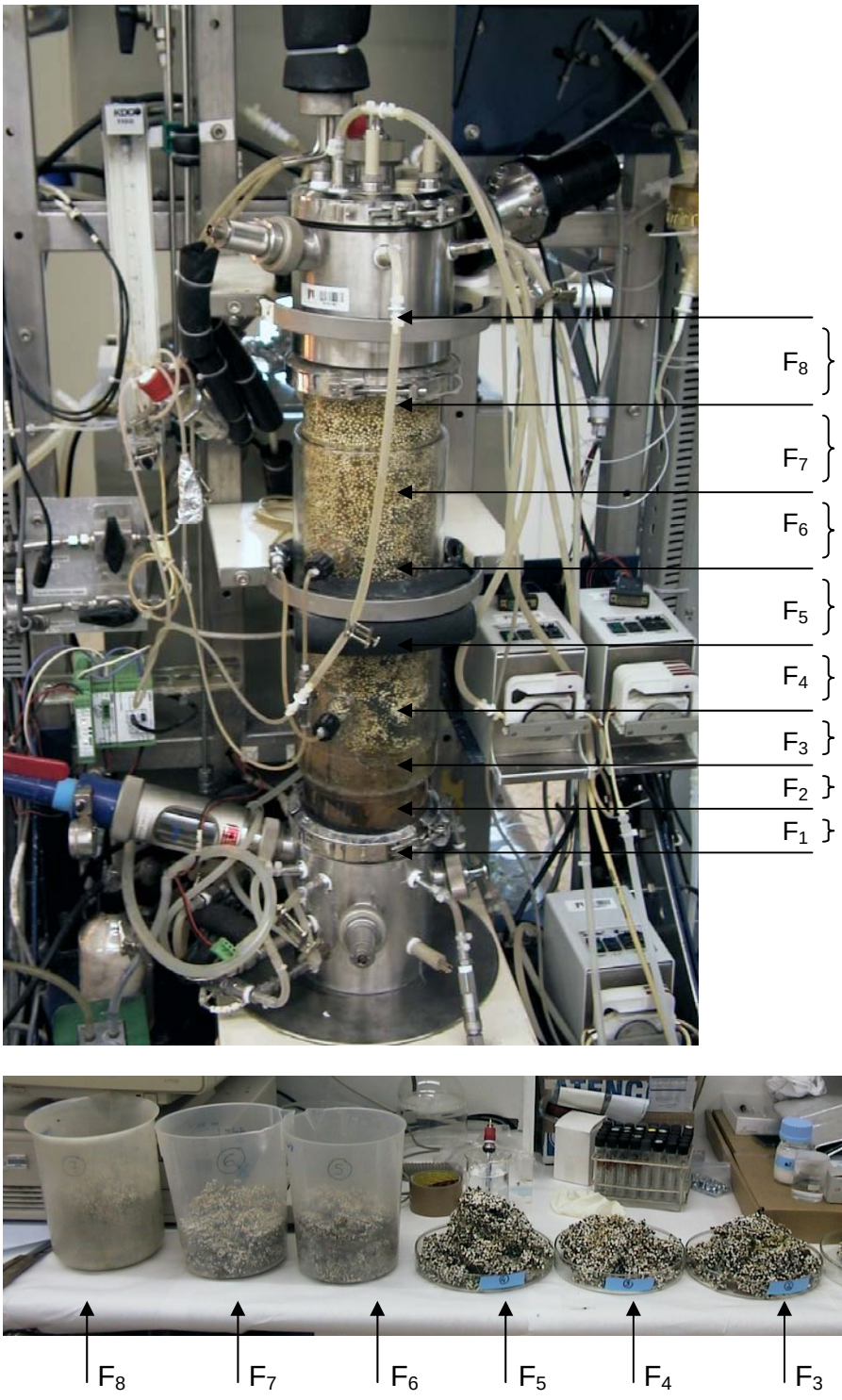


Fig. 1. Scheme of sections (F1-F8) in which the C_{III} bioreactor was divided after dismantling.

The volumes of each of the eight fractions were selected taking into account the concentration gradient along the vertical axis, so that the fractions from the bottom part of the reactor had a smaller volume than those fractions that were closer to the top (figure 1). An estimation of the volume of each fraction was carried out and the obtained values can be found in table 3 together with the total dry weight of the biomass.

Table 3: Estimated volumes of the 8 fractions into which the fixed bed was divided.

Fraction	Approximate fraction	Estimate Volume (mL)	Total dry weight (g)	Relative dry weight (g/ml)
F1	(biofilm below Biostyr® particles)	not estimated	21.2	-
F2	V	250	10.8	0.043
F3	~2.4V	600	24.6	0.041
F4	~2.6V	662	20.5	0.031
F5	~4.4V	1092	20.6	0.019
F6	~4.2V	1056	18.3	0.017
F7	~3.6V	895	13.8	0.015
F8	~5.9V	1477	14.5	0.010

For the biofilm studies with both fresh and fixed samples, the biofilm was not detached from the support beads. On the contrary, it is important that the biofilm remains unaltered in order to obtain as much information as possible on the biofilm structure. Cryosectioning was then performed on small portions of the fixed bed (support beads + biofilm). In the cryosectioning process the beads as well as the attached biofilm were cut obtaining slices with a thickness of 10 μm in which the biofilm structure remained intact.

5. ANALYSIS OF FRESH BIOFILM SAMPLES

In a first part, the general condition of the biofilm was investigated using general nucleic acid stains and EPS targeting fluorescent lectin probes. EPS is used by bacteria to help

them create a matrix on which they can form biofilms. The selection of lectin probes and nucleic acid stains was a result of extensive literature search. In 1998 Lawrence *et al*. [18] found that some lectins with a broad range of carbohydrate specificity were well suited to general staining of exopolymer in biofilms. In recent literature [33, 37] the use of several lectins to target EPS in biofilms is widely discussed and thus we selected the lectins and stains following these recommendations. Concanavalin A (ConA) labelled with Texas Red was used to bind to polysaccharide residues in EPS while nucleic acid was stained by means of DAPI and SYTO13 stains.

A description of the protocol followed to study the general condition of the biofilm is presented in figure 2.

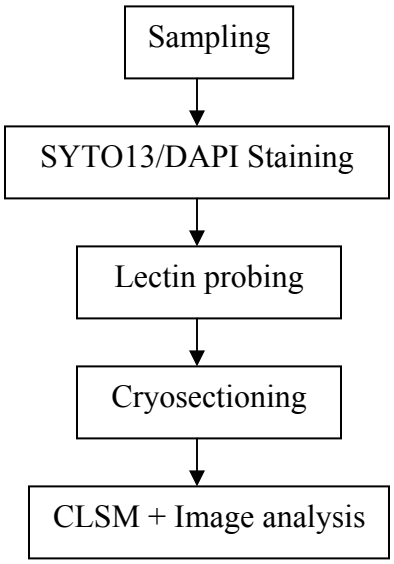


Fig. 2. Scheme of the protocol to analyse the general condition of the biofilm

For the general study of the nitrifying biofilm, a small portion of the biofilm (approximately 5 *Biostyr* beads and the attached biofilm) was collected from each fraction and subsequently washed in a Falcon tube with a 0.01M $MgSO_4$ solution by centrifugation at 6000 rpm for 5 minutes. After washing and within 30 minutes of sampling, cells were stained according an optimized standard protocol with fluorescent acid stain SYTO13 (Molecular Probes, Inc.) at a concentration of 20 µg/mL. Some of the

samples were stained with DAPI nucleic acid stain (Molecular Probes, Inc.) instead at a concentration of 0.3 μg/mL. After 10 minutes at room temperature excess staining was removed by gently rinsing with a 0.01M $MgSO_4$ solution.

Microbial EPS was stained with the lectin probe Concanavalin A (Con A)-Texas red (Molecular Probes, Inc.) at a lectin concentration of 5 mg/mL. Excess staining was removed after 10 minutes at room temperature by gently rinsing with 0.01 M $MgSO_4$ solution. Prior to cryosectioning samples were resuspended in an appropriate volume of Tissue-Tek OCT. embedding compound (Miles Inc., Elkhart Ind., USA) during 15 minutes and immediately frozen at -20ºC for 1 hour to enable slicing of the sample without structural damage of the biofilm. The embedding compound is composed of polyvinyl alcohol (10.24%), polyethylene glycol (4.26%) and the rest and the remaining 85.5% corresponds to non-reactive ingredient whose nature is not revealed by the supplier. Horizontal 10μm-slices of the biofilm were obtained with a cryostat (LEICA Microsystems, Germany) and were subsequently transferred onto gelatine-coated SuperFrost® microscopic slides (Menzel-Gläser, Germany) and immobilized by air drying overnight at room temperature. The microscope slides with the samples were finally stored at 4ºC for up to 2 days .

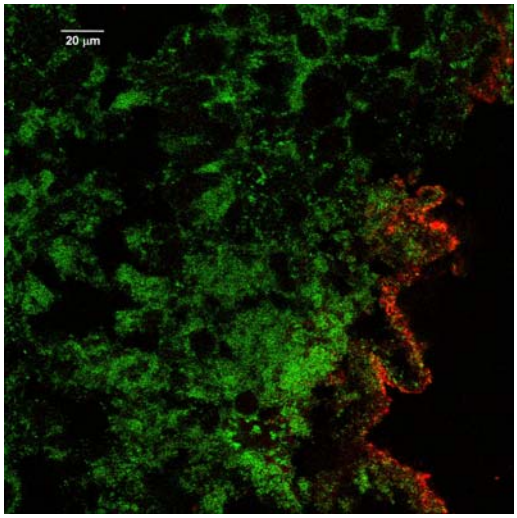
Slides were mounted in citifluor AF3 antifadent solution (Citifluor Ltd., Canterbury, UK) before visualization with a CLSM in order to avoid fading of the fluorescent dyes. One drop of antifadent solution was added to the microscope slide.

In table 4 a list of the samples and the dyes used to stain cell nuclei is presented along with the number of samples that were analyzed for each fraction. Although analysis of a higher number of samples would have been optimal and would have provided us with reliable data regarding abundance of EPS and biofilm morphology, only random samples could be analyzed because of the fact that image visualization had to be performed with a CLSM whose availability was limited. However, the obtained results provided us with relevant information regarding presence of EPS, relative distribution of cells and EPS within the biofilm, general form of the biofilm, etc. As an example two CLSM images

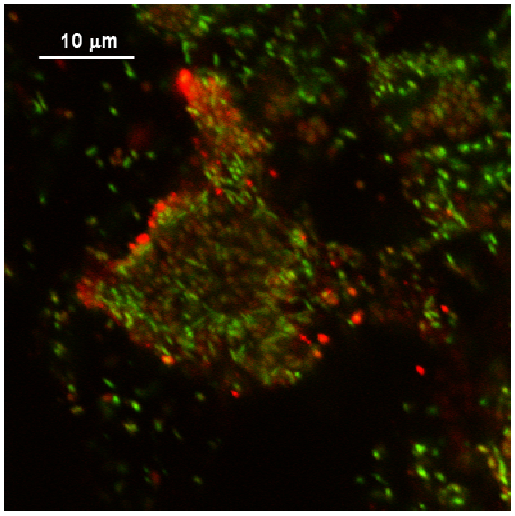
corresponding to fresh samples stained with Syto13 and ConA-(Texas Red) is presented in figure 3.

Table 4: List of samples analyzed with an indication of the dye used to stain cells (DAPI and Syto). A limited number of 6 samples were analyzed (due to limitations in CLSM analysis availability) as indicated in the table. The number of fields that were analyzed by CLSM in each sample is indicated in brackets.

F0	F1	F2	F3	F4	F5	F6	F7
DAPI (1) Syto13 (2)	none	DAPI (1)	none	Syto (2)	Syto (3)	none	Syto (2)



F7 (top of fixed bed), 630x, zoom 1x



F1 (bottom of fixed bed), 630x , zoom 4.5x

Fig. 3: EPS detection by staining with ConA-Texas red (in red). Cell nuclei stained with Syto13 (in green)

6. FLUORESCENT IN-SITU HYBRIDIZATION

The relative distribution of living and active bacteria along the packed-bed was evaluated by performing FISH with previously published rRNA targeted oligonucleotide probes (see table 6). The main target of this study was to obtain experimental evidence on the relative abundance of the two initial strains, but because the maintenance of axenicity in a biofilm reactor, having run continuously for 4 years, is next to impossible (but feasible),

probes targeted against several groups of bacteria that are known to play a role in nitrification in waste water treatment systems were added to the probes solely targeting against *Nitrosomonas europea* and *Nitrobacter winogradskyi*.

A scheme of the protocol for the FISH analysis of the biofilm samples can be found in figure 4.

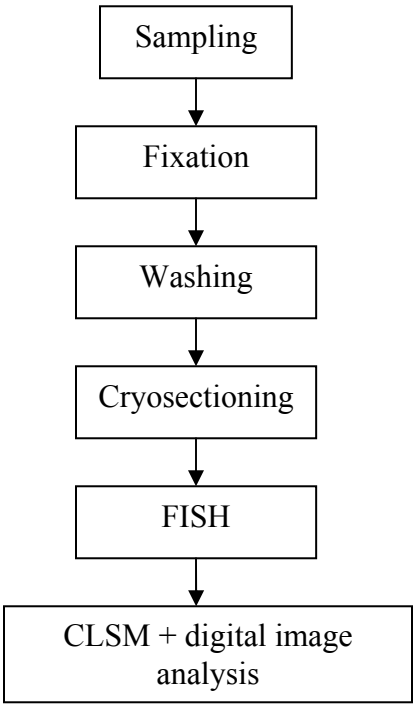


Fig. 4. Scheme of the protocol for the FISH analysis of biofilm samples.

Sampling for the FISH analysis consisted of taking approximately 10 Biostyr beads from each fraction of the fixed bed, which were fixed according to the fixation protocol described in section 6.1. From these 10 beads a 10 µm section was obtained by cryosectioning and immobilized onto a microscopic slide for analysis. Because what we performed is a microscopic analysis, what determines the repeatability and accuracy of

the results is the area of the fields that were finally submitted to CLSM analysis and the thickness of the biofilm slices (which was constant and equal to 10μm). Therefore the number of beads taken from each fraction does not have a negative effect on the accuracy of the final results.

6.1. Cell fixation protocol

Samples were obtained from each fraction of the fixed-bed and fixed with formaldehyde prior to cryosectioning according to the following protocol [2]:

- Samples were obtained and washed in Falcon tubes with a 1X PBS solution by centrifugation at 6000 rpm for 5 minutes.
- 6 mL of a 4% paraformaldehyde solution were added to each sample and held for 3 hours at 4ºC
- Samples were subsequently washed three times with 5mL of the 1X PBS solution
- Samples were then stored in 50% ethanol-PBS solution

The solutions used in the fixation protocol described above are prepared as described below:

Preparation of the PBS (phosphate buffered saline) solution

The composition of the used PBS solution is specified in table 5. All solutions were prepared using bidistilled and must be filter sterilised at 0.45μm. pH was set to 7.0 with NaOH.

Table 5: Composition of the PBS solutions required in the fixation protocol

	1X PBS	3X PBS
NaCl	8 g	24 g
KCl	0.2 g	0.6 g
$Na_2HPO_4 \cdot 2H_2O$	1.78 g	5.34 g
$NaH_2PO_4 \cdot H_2O$	0.23 g	0.69 g
MilliQ Water	1L	1L

Preparation of 4% paraformaldehyde fixative

The fixative solution is prepared by adding 10g of paraformaldehyde to 165 mL of bidistilled water previously heated to 60ºC. After dissolving, 2 or 3 drops of NaOH are added until the solution becomes clear again and finally 3X PBS solution is added up to a total volume of 250 mL and pH is set to 7.0-7.4 using a NaOH solution.

After fixation samples were resuspended in an appropriate volume of Tissue-Tek® OCT compound (Miles, Elkhart, Ind.) during 15 minutes and immediately frozen at -20ºC for 1 hour. The frozen biofilm samples were cut into 10µm-thick horizontal slices with a cryostat (LEICA Microsystems, Germany) at -20ºC. The obtained biofilm slices were then transferred onto gelatine-coated microscopic slides and air dried overnight to attain immobilization of the biofilm slices on the microscope slides.

Samples were finally dehydrated in an ethanol series by being submerged in 50%, 80% and 98% ethanol (3 minutes each), air dried and stored at -20ºC in a moisture-free environment, which was attained by keeping the slides inside a hermetic container until the day the FISH analysis was performed.

6.2. FISH probe selection and hybridization protocol

FISH analysis was performed by selecting probes with different degrees of specificity that allowed us to target nitrifying bacteria in a hierarchical cascade, from the very specific up to the very general (figure 5). In addition, some probes specific to groups other than those of *Nitrosomonas* and *Nitrobacter*, which have been found in association with wastewater treatment and whose presence can be expected in the biofilm from the compartment III packed-bed reactor, were used as well in an attempt to obtain as much information as possible from this study. This approach allowed us to make the probe selection without a previous knowledge on the identity of all bacteria present in the reactor.

Previously published 16S rRNA-specific oligonucleotide probes were purchased from MWG-Biotech AG (Germany) labelled at the 5'-end with the following fluorescent dyes: fluorescein isothiocyanate (FITC) and the sulfoindocyanine dyes Cy3 and Cy5.

The selected probes were used as a combination of two or three probes targeted with different fluorochromes, taking into account the relevance of the information they can provide us when used together. All the samples were hybridized with the probe mixture EUB, EUBII and EUBIII, which is the less specific probe and targets all *Eubacteria*, combined with either one or two more probes of higher specificity as described in figure 5 [24].

The use of group specific probes ALF1b, BET42a and GAM42a allowed us to identify how much of the active biomass present in the sample and targeted by the EUB-mix probes belonged to the *proteobacteria* groups and, more specifically, to the *alfa, beta and gamma* subgroups. Knowing which subgroup *Nitrosomonas* and *Nitrobacter* belong to (figure 5) allowed us to identify these two species and at the same time it was possible to

observe whether any bacterial species other than than the initial *Nitrosomonas* and *Nitrobacter* that also belong to this subgroups are present in the biofilm.

In the case of nitrite oxidising bacteria, *Nitrobacter* belong to the *alfa* subgroup of *Proteobacteria*, whereas other nitrite oxidising bacteria usually found in waste water such as *Nitrospira* do not belong to this subgroup. On the other hand, most ammonia oxidizers belong to the *beta-proteobacteria* subgroup, and thus it is necessary to perform several probe combinations to discriminate between the different species. To this effect probes specific to ammonia oxidizing bacteria (Nso190, Nso1225) were used in combination with probes specific to different known species of ammonia oxidizers (Nse1472, NmV(Ncmob)) so that in the end by combining the information given by the BET42 probe and the more specific probes the abundance of *Nitrosomas* could be established. These probes that are specific to other ammonia oxidising species were used with the aim to obtain information on a potential diversity of ammonia oxidizing bacteria in the biofilm. Hence, using these probe combinations, the presence of other ammonia and nitrite oxidising micro-organisms could be detected. The presence of other nitrifying organisms could have a considerable effect on the modelling of the nitrifying reactor.

Finally, probes specific to *Nitrosomonas* (Nse1472) and *Nitrobacter* (NIT3) were used which allowed us to quantify the relative abundance of these two species.

Because the main goal of this study was to determine the relative distribution of the two initial strains within the packed-bed, the highest priority was given to the combination of the EUB probes with both NEU (specific of *Nitrosomonas* species) and NIT3 (specific of *Nitrobacter* species). Non labelled competitor probes for both NEU and NIT3 probes were also added to the mixture as they anneal on target sites that have been reported to induce false hybridization with the specific probes, avoiding any false positive results. [35, 36]. The relative biovolume of NEU and NIT3 gives an estimation of the relative abundance of these species in each fraction, and by comparing the total biovolume of NEU + NIT3 to the biovolume of EUB we can have an estimation of the total amount of active biomass that is neither *Nitrosomonas* nor *Nitrobacter*.

Despite the relevance of the information provided by the probe combination described above, the use of other probes with different levels of specificity provides complimentary information and makes it possible to back up some of the results obtained with the more specific probes. Therefore probes with high levels of specificity were used together with other combinations [24] as previously stated.

Because the main objective of this study was to evaluate the relative abundance and distribution of the two initial strains, probes specific to heterotrophic bacteria (CF319) were given the lowest priority, followed by the probes specific to other nitrite oxidising bacteria (Ntspa662, Ntspa712) as their present in the reactor is not certain and their quantification is not essential, although it would have been interesting.

At this point, it is important to state that FISH analysis is a very time consuming technique and that the time available to process all the samples was rather limited. This time constraint does not only refer to the fact that samples all were obtained at once (after reactor operation was stopped) and they had to be processed over a period of 4 months so that results were reliable and comparable. It should also be taken into account that FISH analysis strongly depended on the availability of a CLSM for visualization of the samples after hybridization. Image acquisition was performed over a period of 4 months (June-September 2004) at the CLSM facility at UAB, and thus availability was an important constraint. The approximate time required for image acquisition can be estimated at an average of 1 sample per hour.

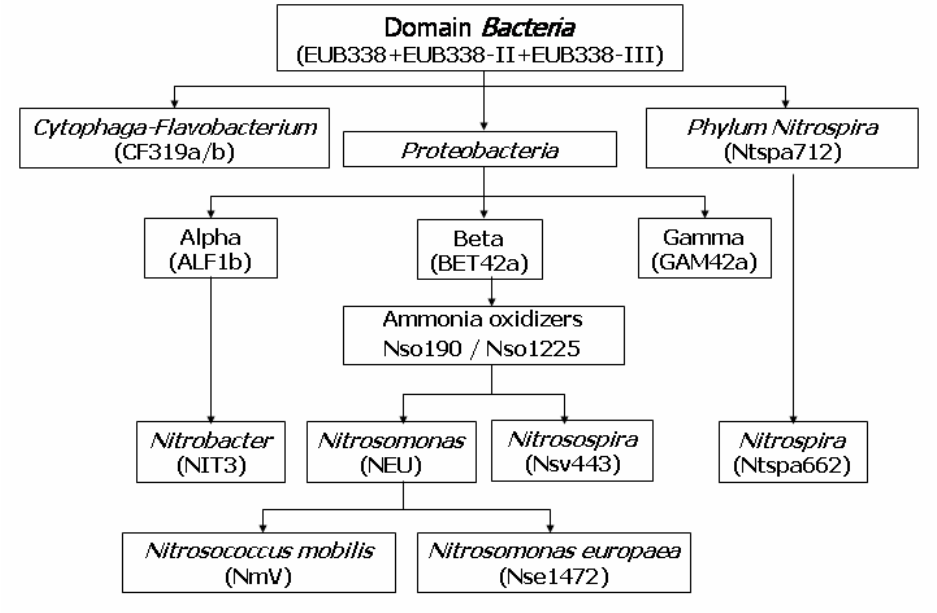


Fig. 5: Specificity of the rRNA-targeted oligonucleotide probes used [23]

All the probes that were used have been listed with all the information that is required for this study in Table 6. The specifity of each probe and the sequence as well as the optimal formamide concentration in the hybridization buffer and the NaCl concentration in the washing buffer have been included in the table. Not so the target site for each probe because we considered this information is not necessary and can be found in the literature [19].

Table 6:
Characteristics of the 16S rRNA-directed oligonucleotide probes used for FISH. (*) probe position according to the E. coli gene numbering.

Name	Probe sequence P[19]	Label	Specificity [19]	Target site (*)[19]	Reference	Formamide (%)	NaCl [mM]
EUB338	**5'-gctgcctcccgtaggagt-3'**	**FITC**	All *Eubacteria*	338-355		0-50%	10-900
EUB338II	**5'-gcagccacccgtaggtgt-3'**	**FITC**	*planctomycetales*	338-355	[1]	0-50%	10-900
EUB338III	**5'-gctgccacccgtaggtgt-3'**	**FITC**	*verrucomicrobiales*	338-355		0-50%	10-900
CF319a	**5'-tggtccgtgtctcagtac-3'**	**Cy5**	*Cytophaga-flavobacterium*-group	319-336		35%	80
CF319b	**5'-tggtccgtatctcagtac-3'**	**Cy5**	*Cytophaga-flavobacterium*-group	319-336	[21]	35%	80
Ntspa712	**5'-cgccttcgccaccggccttcc-3'**	**Cy5**	*Nitrospirae*	712-732		50%	10
+ competitor	**5'-cgccttcgccaccggtgttcc-3'**	**Non**	Competitor for Ntspa712		[9]	50%	10
Ntspa662	**5'-ggaattccgcgctcctct-3'**	**Cy5**	Genus *Nitrospira*	662-679		35%	80
+ competitor	**5'- ggaattccgctctcctct-3'**	**Non**	Competitor for Ntspa662			35%	80
ALF1B	**5'-cgttcgytctgagccag-3'**	**Cy5**	α-*proteobacteria* (some δ-*proteobacteria* spirochaetes)	19-35		20%	225
BET42a	**5'-gccttcccacttcgttt-3'**	**Cy3**	β- *proteobacteria*	1027-1043	[20]	35%	80
GAM42a	**5'-gccttcccacatcgttt-3'**	**Cy5**	γ-*proteobacteria*	1027-1043		35%	80
+ competitor	**5'-gccttcccacttcgttt-3'**	**Non**	β-*proteobacteria*			35%	80
Nso1225	**5'-cgccattgtattacgtgtga-3'**	**Cy3**	β-*proteobacteria* ammonia oxidizers	1224-1243		35%	80
Nso190	**5'-cgatcccctgcttttctcc-3'**	**Cy3**	*β-proteobacteria* ammonia oxidizers	189-207	[22]	55%	
NIT3	**5'-cctgtgctccatgctccg-3'**	**Cy3**	*Nitrobacter*	1035-1052		40%	50
+ competitor	**5'-cctgtgctccaggctccg-3'**	**NO**	Competitor for NIT3		[36]	40%	50
NEU	**5'-cccctctgctgcactcta-3'**	**Cy5**	Most halophilic and halotolerant *Nitrosomonas*	653-670	[35]	40%	50

Name	Probe sequence P[19]	Label	Specificity [19]	Target site (*)[19]	Reference	Formamide (%)	NaCl [mM]
			species.				
+ Competitor (CTE)	**5'-ttccatccccctctgccg-3'**	**Non**	Competitor for NEU			40%	50
Nsv443	**5'-ccgtgaccgtttcgttccg-3'**	**Cy5**	*Nitrosospira spp.*	444-462	[22]	30%	100
NmV (Ncmob)	**5'-tcctcagagactacgcgg-3'**	**Cy5**	*Nirosococcus mobilis*	174-191	[8,1629]	35%	80
Nse1472	**5'-accccagtcatgaccccc-3'**	**Cy5**	*Nitosomonas europea*, *Nitrosomonas halophila* ,*Nitrosomonas eutropha*, isolate Nm103	1472-1489	[5]	50%	10

Fluorescent *In Situ* Hybridization was performed according to Amann [2] and following the protocol described by Hugenholtz *et al.* [15] for the evaluation of 16S rRNA-targeted probes for FISH.

Hybridization was carried out for each set of probes at formamide concentrations in such a way that the stringency requirements were fulfilled.

At the beginning of the process the hybridization oven was prewarmed at 46ºC, which is the optimal temperature for hybridization, while hybridization buffer (table 7) was prepared and mixed with the FISH probes in the adequate proportion to accomplish the required stringency and a concentration of 50ng/µL for each of the probes.

To perform the hybridization, an area of the slide is selected and 90 µL of the probe mixture are extended on it. The slide is subsequently introduced in a moisture chamber, i.e. a 50 mL polypropylene tube containing a folded paper towel (Whatman, 3MM) poured with 2 mL of hybridization buffer in order to prevent evaporation of buffer during hybridization. The tube is then placed into the hybridization oven at 46ºC for 2 h.

Table 7: Composition of the hybridization buffer for the different stringencies used in this study. [2]

Stringency %	5M NaCl (µL)	1M Tris-HCl (µL)	H_2O bidistilled (µL)	Formamide (µL)	10% SDS (µL)
30	180	20	499	300	1
35	180	20	449	350	1
40	180	20	399	400	1
50	180	20	299	500	1

Hybridization was stopped by rinsing the probe from the slide with hybridization buffer prewarmed at 48ºC with a pipet and the slides were then transferred to a new 50 mL Falcon tube containing washing buffer (table 8) at hybridization temperature which was place in a water bath at 48ºC and incubated for 15 min.

Table 8: Composition of the washing buffer for the different stringencies used in this study. [2]

Stringency %	5M NaCl (µL)	1M Tris-HCl (µL)	H_2O bidistilled (mL)	EDTA (µL)	10% SDS (µL)
30	1020	1000	up to 50 mL	500	50
35	700	1000	up to 50 mL	500	50
40	460	1000	up to 50 mL	500	50
50	180	1000	up to 50 mL	500	50

Finally, excess salts were removed by shortly rinsing the slide in ice-cold double distilled water and subsequently the slides were left to dry at room temperature for 2 hours.

Prior to microscopic analysis slides were mounted in citifluor AF3 antifadent solution (Citifluor Ltd., Canterbury, UK) to avoid fading of the fluorescent dyes. A thin film (1 drop) of Citifluor was applied to the slide so that it covered the area on which FISH had been performed and a coverslip was carefully placed on the slide so that the excess of Citifluor could be removed [15].

7. MICROSCOPY AND IMAGE ANALYSIS

7.1. Image acquisition

A LEICA DM IRE2 inverted CLSM (LEICA Microsystems, Germany) was used for image acquisition. The microscope was fitted with a TCS SP2 AOBS confocal laser scanning system.

The excitation and emission wavelengths of the fluorescent stains used for the fresh samples as well as the fluorescent dyes used to label the FISH probes are shown on table 9.

Table 9: fluorescent dyes and their absorption and emission wavelengths

Fluorescent dye	Absorption wavelength (nm)	Emission wavelength (nm)
DAPI	358 (405nm laser)	461
SYTO13	488 (488nm laser)	509
ConA-Texas Red	595 (561 nm laser)	615
FITC	488 laser	518
Cy3	550 (561 laser)	570
Cy5	649 (633 laser)	670

The images obtained by CLSM are 256 gray-scale-level images need to be converted into binary images by selecting a threshold level below which all gray levels are considered as background. All thresholding was performed manually using the Leica Confocal Software (LEICA, Heidelberg, Germany) as the tool for image analysis.

7.2. Qualitative results and general trends

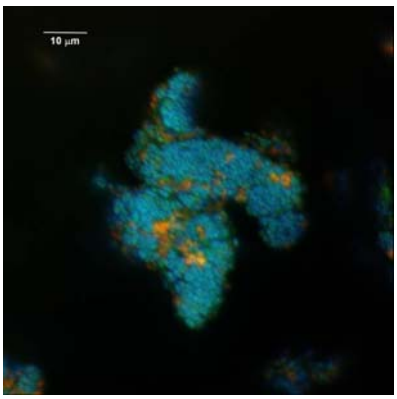
Qualitative analysis of the images obtained by CLSM clearly shows the area covered by EPS and the area occupied by the cells (figure 3). Figure 3 shows two different images obtained at different heights of the fixed bed and the relative distribution of EPS and bacterial cells. Quantification tools will make possible to quantify the relative amount of EPS and cells in the nitrifying biofilm from compartment III.

Regarding the FISH analysis, in table 10 a complete list of the samples on which FISH was performed together with the probe combination applied in each case is shown together with the date on which the hybridization was performed. Successful analyses, i.e. the analyses that produced images good enough to be used for image analysis and subsequent quantification are shown on a green background, whereas unsuccessful analyses are shown on a red background. Due to the fact that image acquisition with CLSM is very time consuming, the remaining samples could not be analyzed within the period of time of 4 months considered as the limit after which samples are no longer well preserved and the analysis performed would not be reliable anymore.

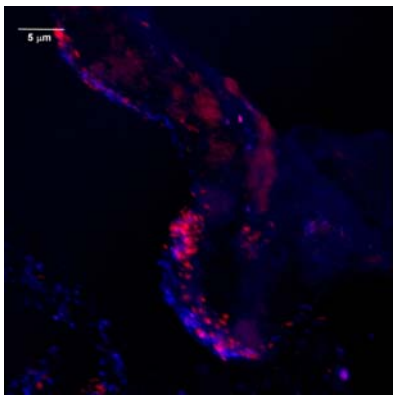
Table 10: List of samples and probe combinations with dates of the performed FISH analysis. Every cell on this table indicates one sample available for FISH analysis, a green background is used for successfully analyzed samples, a red background indicates samples that were hybridized but did not lead to successful results and the remaining samples could not be analyzed due to time limitations.

	F1	F2	F3	F4	F5	F6	F7	F8
NIT3/NEU								
Bet42/Gam42							-	
ALF1B			-	-	-		-	-
Nso190/ Nse1472						-		-
Nso1225/ Nmv(Ncmob)							-	-
Ntspa712	-	-	-	-	-	-	-	-
Nsv443	-	-	-	-	-	-	-	-
Ntspa662		-	-		-	-	-	-
CF319a/ CF319b	-		-	-		-	-	-

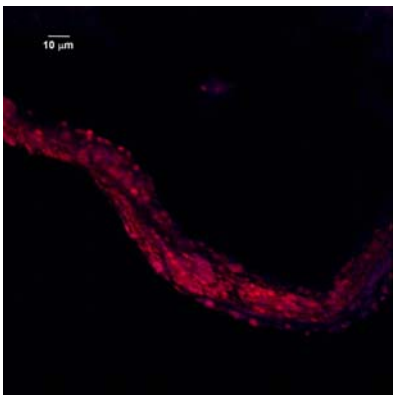
The images in figure 6 serve to illustrate the general trends observed within the packed bed regarding the relative distribution of *N. europaea* and *N. winogradskyi*, whose determination is one of the main goals of this study. As already stated by Pérez *et al.* (2005) [27], qualitative results shown in figure 6 point to an increase in the ratio Nitrobacter/Nitrosomonas towards the upper fractions of the fixed bed.



3.a: Fraction 0 (bottom)



3.b: Fraction 4



3.c: Fraction 6 (top)

Figure 6: FISH images from different heights of the packed bed. *Nitrosomonas* cells (NEU) in blue and *Nitrobacter* cells (NIT3) in red.

8. CONCLUSIONS

A protocol has been developed for the sampling and analysis of the biofilm from the nitrifying pilot reactor using Fluorescent In Situ Hybridization followed by CLSM visualization and image analysis.

The analysis techniques described in this technical note will provide relevant information on the relative distribution of the two initial strains used to carry out the oxidation of ammonium to nitrate in compartment III, providing evidence of the heterogeneous distribution of the two strains along the fixed bed.

Along with the protocol for the application of these techniques to the biofilm samples of compartment III, a method for quantification of the images obtained by CLSM visualizations has also been foreseen. The software selection as well as the different steps of the quantification procedure will be extensively discussed in technical note 78.92.

The potential of the FISH technique as a tool to study the heterogeneous distribution of different strains within the nitrifying biofilm of compartment III has been studied. Further results, including quantification of the information provided by these CLSM images, will be presented in technical note 78.92.

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10. COMMENTS

The key to the different font colours used in this section is the following:

- BLACK: initial ESA comments
- BLUE: first response from UAB
- RED: second ESA comments
- GREEN. second response from UAB

General remark: your answers are very difficult to trace in the TN, as paragraph numbering has been changed and because the page numbers you indicate are not corresponding to the actual TN page numbers

Page numbers have been updated in the present document.

1
Please make clear to which extend FISH combined with CLSM and image analysis can be considered as a quantitative method?

Where are the fresh samples you mention coming from?

- The expression "fresh samples" was used on the text as opposed to the samples used to perform FISH, which had to undergo a protocol of fixation before hybridization and subsequent CLSM inspection. The phrasing has been corrected and replaced by: "In addition, CLSM was used in parallel on some samples from the reactor to visualize Extracellular Polymeric Substances (EPS) and estimate its content". (see page 4)

2.1 (now section 3.1)
Please make the nature of the opaque film, brand name and specifications of bioreactor, sparger, probes, and control system

- The material used to prevent inhibition due to light was thin foil (this information has been added in page 7.
- An accurate description of the reactor, including brand name and specifications, can be found in the technical notes in which the design and characterization of the reactor was presented. The references have been added in the text (see page 7)

3 (now section 4)
Please make clear how the selection of active parts from each fraction of the biofilm was performed

- The paragraph referring to biofilm sampling has been completely rephrased and extended in order to make the procedure more clear (see pages 9- 11)

Please make clear the number of samples taken from each fraction.

A total number of 16 fresh samples were obtained and stained following the protocol presented in figure 2 (8 were stained with DAPI and 8 with Syto13). CLSM inspection of these fresh samples had to be performed within 2 days of sampling and thus it was not possible to analyze all available samples due to a time constraint regarding availability of the CLSM facility. Samples from different fractions were selected taking into account this time limitation, and hence the lack of samples in some intermediate fractions such as F6 or F3. The list of samples that were finally processed is presented in table 4 (page 14)

The same procedure was applied in the case of the FISH samples. 9 samples were taken from each one of the 8 fractions and a fixative was applied on them so that they could be stored, as described in figure 4. FISH analysis was performed on 34 out of the total 72 samples available, and the list of samples that were successfully analyzed by CLSM visualization and that were adequate for subsequent quantification can be found in table 10 (page 27).

Table 4 has been adapted so that the information is presented more clearly (see page 14).

Please make clear the procedure of dismantling, handling and fractioning of the packed bed reactor

- A new paragraph has been added to address the complete procedure, and images have also been added. (see pages 9-11)

Please demonstrate that freeze-drying of the biofilm does not alter the biofilm structure

- Freeze drying does indeed alter the structure of the biofilm. Samples cannot be used to perform further studies regarding biofilm structure or strain distribution after freeze-drying. Biomass was freeze-dried in order to perform further studies, i.e. total biomass concentration in each fraction or analysis of macromolecules. This has been corrected in section 4 (see page 9-11). In the previous TN version, freeze-drying was mentioned (page 3); you confirm it in your comment, however, lyophilization is mentioned instead of freeze-drying on page 6 of the new TN. Please clarify

The two terms refer to the same technique and were used as synonyms, but to avoid any misunderstanding the same term (freeze-drying) has now been used along the whole document.

4 (now section 5)
Please explain why the same portion of biofilm was taken from each fraction while biofilm concentration from each fraction decreases from bottom to upper parts of the packed bed

- The volume of the different fractions was different. An estimate value of the volumes of the different fractions has been included in page 11.

Please make clear the concentration of DAPI nucleic acid stain applied

- 0.3 μg/mL (added in page 13)

Please make clear when the separation of the attached biofilm occurs from the beads

- The attached biofilm was not separated from the bead. On the contrary, it is important that the biofilm structure remains unaltered for the correct study of the biofilm. Cryosectioning was performed on small "pieces of the fixed bed" (beads + biofilm). Both the beads and the biofilm attached to them were cut during the cryosectioning process obtaining 10 μm slices, in which the biofilm structure remained intact.

Please precise it in the TN.

In page 11 a paragraph has been added to clarify the fact that the biofilm structure is not altered in the samples used for the biofilm structure analysis.

Please make clear the nature of the cryosectioning embedding compound

- Tissue-Tek® O.C.T. (optimal cutting temperature) compound (Miles Inc., Elkhart Ind., USA). This information has been included in the document (see page 13).

Chemical Ingredients:
10.24% polyvinyl alcohol
4.26% polyethylene glycol
85.5% non-reactive ingredient

Please make clear why analysis of portions of fraction F1, F3 and F6 of the packed bed was not performed

- CLSM inspection of fresh samples must be performed within 2 days of sampling. It was not possible to analyze all the available samples due to a time constraint regarding availability of the CLSM facility, and thus only a few random samples were analyzed. (see page14)

Please make clear for fraction F0 if the same portion was double-stained or if 2 portions were stained with Syto13 and DAPI strains respectively.

- Two different samples from each fraction were stained, one with DAPI and one with Syto13, but only those indicated in table 4 were finally visualized by CLSM and used for subsequent quantification. The numbers in brackets indicate the number of fields from the same sample that were visualized at the CSLM. In the case of fraction F0 (which has been renamed as F1), one field was analyzed in the DAPI stained sample, 2 different fields were analyzed in the Syto13 stained sample.

Please make clear the total number of samples per fraction analyzed.

- 16 samples were prepared for analysis with CLSM (2 samples from each fraction). Out of these, only 6 samples were eventually analyzed due to time limitations regarding the use of the CLSM facility, together with the fact that fresh samples could not be stored without being damaged. The legend of table 4 has been updated to make it more clear. (see page 14)

Please make clear the staining optimization procedure to determine best concentration of stains

- The protocol was not designed for this application because optimized protocols were already available which were applicable to this analysis. Recommendations from the literature were taken into account (see page 12) Please provide the protocol in annex to this TN

There seems to be some misunderstanding around this point. What is the information requested?

a) if it is the used protocol, it is already described in page 11-13. Do you still want it in an annex?
b) if the information you request is the detailed optimization procedure to reach the analytical conditions (stain optimised concentration, …), then this is not available, as this information is not usually published by researchers.

Please make the brand name and specifications of microtome and gelatin-coated microscopic slides

- The microtome used to perform the cryosectioning was from LEICA Microsystems, Germany

Plese answer the question for the gelatine-coated microscopic slides

The brand of the gelatine coated microscope slides has now been added to the document (see p. 13)

Please make clear the duration of air drying procedure and storage condition before analysis.

- Samples were left to dry at room temperature overnight and were subsequently stored at 4ºC for up to 2 days. (see page 13)

Please make clear the procedure for slide mounted on the AF3 antifadent solution

- A drop of AF3 antifadent solution (Citifluor, UK) is added to the slide on the fields object of analysis previous to CLSM inspection. (see page 13)

Please make clear the status of analysis of sample (1) of fraction F4, sample (1), (2) of fraction F5, and sample (1) of fraction F7

- The caption of table 4 has been modified so that it properly explains the meanings of the numbers in brackets. These numbers indicate the number of different fields in a sample on which CLSM inspection was performed. Two fresh samples were obtained from each one of the eight fractions of the fixed bed. One of the samples was stained with Syto13 and the other one with DAPI. The specificity of the DAPI and Syto13 stains is the same, and thus the results are comparable.

5
Table 5: Please make clear the relevance of the selection of these particular probes for CIII.

- New information including more details on the probe selection has been added (pages 18-20) to complement the information already presented in figure 5 (page 21).

For EUB338, make clear that it target All Eubacteria

- It has been clarified in table 6, pages 22-23

Make clear as well target site for each probe

- This information was not included in the technical note because we think it is out of the scope of this study and is not relevant to understand neither the tools used, nor the results obtained. This answer is not accepted. Please provide the requested information

This information has been added as a new column in table 6, pages 22-23.

Why did not you select/design a probe for *Nitrobacter winograskyi?*

- Because there was a probe available for Nitrobacter and the probability is very low that another Nitrobacter might have out competed N. winogradskyi.

Please write in full name the class of microorganism (specificity) (e.g. gammaproteo=> γ-proteobacteria) and use italic where appropriate.

- This has been corrected along the whole document

Could you be more precise about the specificity of some selected probes (e.g. NEU specific for halophilic and halotolerant *Nitrosomonas spp.* and *Nitrosococcus mobilis)?*

- The specificity of each probe is written in table 6 (page 22), together with the reference in which the probe was described.

Could you make clear the specificity of the Nse 1472 probe? Does this mean that the probe is not specific to *Nitrosomonas europaea* but also *N. halophila*, *N. eutropha* and *Nitrosomonas* sp. Nm103?

- It does indeed mean that this probe is specific to *Nitrosomonas* species, and thus it can also target other *Nitrosomonas* strains such as *N. halophila* and *N. eutropha*.

Which delta proteobacteria and spirochaetes are targeted by ALF1B probe?
What about the selection of targets for *Nitrococcus* and *Nitrospina* genera of nitrite oxidizing bacteria?

- These bacteria have been regularly found in association with nitrifying bacteria. Sorry , there is a misunderstanding, we should have formulated our question in the following way: why didn't you select targets for Nitrococcus and Nitrospina genera of nitrite oxidizing bacteria? Nitrococcus will be targeted with the gamma probe, nitrospira with the nitrospira probe. The occurrence of gamma positive signals in layer N8 could indicate the presence of Nitrococcus. It would indeed be necessary to analyse the samples for presence of Nitrococcus.

As stated, it is supposed to study the distribution of the 2 selected strains over the packed bed. It is a good thing to go further and try to target other strains. However, in order for this approach to be relevant, it would have been valuable to determine first the specific pool of 16S rDNA genes present in the Melissa CIII and design/order afterwards the appropriate probes instead of selecting more or less arbitrarily probes based on analyses performed on biofilms formed in different reactor conditions and different conditions.

- We used probes that are specific to groups of bacteria in a hierarchical cascade, from very specific up to very general. The fact that the pilot reactor of compartment III had been under operation for a 4-year period before this study was performed provided us with a unique chance to go a bit further and try to identify the presence of potential bacteria that may present after such a long time of continuous operation, and thus a few probes designed to target other main groups of bacteria that have been found in association with wastewater treatment were also included.
  This approach can be applied without having any previous knowledge on the identity of the bacteria residing in the reactor (see pages 18-20).

Besides, as far as these probes are reported in your procedure, the associated results shall be reported being negative or not.

- The results obtained with all the probes, including the negative ones, are indeed going to be reported in TN78.92 (Biofilm studies in compartment III pilot reactor: analysis and discussion of the results)

5.1 (now section 6.1)
Please detail the protocol of fixation with formaldehyde.

- A better description of the cell fixation protocol has been included in page 16 and 17.

Please explain the rationale for selecting bigger samples. If ones want to perform a quantitative, ones may need to be precise in the quantity taken and not approximate (cf. approximately 10 Biostyr beads). Could you please clarify?

- This has been clarified in page 15

Please make clear the nature of the cryosectioning embedding compound

- Tissue-Tek® O.C.T. (optimal cutting temperature) compound (Miles Inc., Elkhart Ind., USA)

Chemical Ingredients:
10.24% polyvinyl alcohol
4.26% polyethylene glycol
85.5% non-reactive ingredient

Please make the brand name and specifications of microtome and gelatin-coated microscopic slides

- The brand of the microtome used to perform the cryosectioning was LEICA Microsystems, Germany

Please make clear the duration of air drying procedure and storage condition before analysis.

- Samples were left to dry at room temperature overnight and were subsequently stored at -20ºC in a moisture-free environment (the samples were kept inside and hermetic container) before FISH analysis. (see page 17)

Please make clear the duration and volume of submersion of slides in ethanol.

- Samples were dehydrated in an ethanol series (50, 80 and 96% ethanol). The samples were submerged in each one of the ethanol solutions during 3 minutes. (see page 17)

Please make clear how the moisture-free environment was insured

- The slides were kept inside an hermetic container until the day the FISH analysis was performed. (see page 17)

5.2 (now section 6.2)
Please give comments on the competitor probes used

- Competitor probes were used to avoid false positives. Non labeled competitor probes will anneal on target sites that have been reported to induce false hybridization with the specific probe, resulting in false positive signals. This clarification has been included in the TN text (page 18-20)

Please detail the set-up and optimization of the hybridization steps for each probe

- A more detailed protocol including all hybridization steps has been added (see page 24)

Please modify 'spot' by 'probe'

- The word "spot" is not referring to the FISH probe but to the piece of surface on the slide in which the hybridization is performed. The sentence has been rephrased. (page 24)

Please make clear the composition of the hybridization buffer and volume

- This information has been added in page 24, table 7.

Please make clear the procedure of pre-warning of the moisture chamber.

- The section in which the FISH protocol was described has been rephrased so that all the steps can be followed more clearly (page 24).

Please make clear the number of rinsing step to remove excess/non-hybridized probes after hybridization step?

- Excess salts were removed by submerging the slide in ice-cold double distilled water for 5 seconds and the slides were then left to dry at room temperature. (see page25)

Please make clear the composition of the washing buffer and volume

- See table 8, page 25

Please make clear the procedure for slide mounted on the AF3 antifadent solution

- Please see page 25

The paragraph below Figure 1 + Table 6 does not have its place in this sub-chapter. This should be included in the part dedicated to results and validation.

- This has been modified and the table has been included in the qualitative results and general trends section of this TN (page 26-27)

Table 6: Could you comment the selection of coupled probes? See page 18. Why could not you perform the screening of each fraction with each selected probes? See page 18 Please make clear the rationale for the prioritization of some probes? See page 18 Please report if you included the competitor probes or not and explain their purposes. The competitor probes were indeed included to make sure any false positives were avoided (page 19)

Please make clear the image acquisition duration

- The image acquisition process was carried out over a period of time of 4 months, after which the samples were no longer in perfect condition to obtain reliable results. (see page 20)

Please give the rationale for referring to Yang et al publication. Please make clear how the samples used for calibration of level-to-noise ratio.

- This reference has been removed as all information regarding image analysis and quantification is going to be included in TN78.92

6.1 (now section 7.1)
Please detail the principle of CLSM.

- See pages 5-6

Please give the complete procedure of microscope+ image analysis setting-up performed to minimize signal to noise ratio

- This information is to be included in TN 78.92, where the software analysis and quantification will be presented.

We are at the limit of TN 78.91 and 78.92, as the question is related to procedures.
However, we agree to have the information in TN 78.92, if you think it is more appropriate.
The procedure used for quantification of the FISH images has now been included in TN78.92.

6.2
Please make clear the quantification tools you are referring to.

The software selection and thus the description of the quantification tools used for image analysis will be extensively discussed in TN78.92.

According to the description of work package 78.9:

The work shall be split in two parts. The first part will contain a description of the experimental set-up and the second one the analysis of the results. The specific contents of the TN's to be delivered shall be:

- *TN 78.91, containing the experimental set-up and analysis techniques description*
- *TN 78.92, containing the analysis and discussion of the results "*

- *Software selection for the quantification and a brief description of the software capabilities (for the software finally selected for the quantification of the results)*
- *Quantification of the results through image analysis as well as interpretation of the main results, which will consist in the final estimation of the concentration/ proportion of the different bacterial species and EPS in the biofilm along the bed vertical axis.*

Could you include all the images obtained and this, for each fraction analyzed?
Please present as well all the FISH images obtained and discuss results

In this TN only a few sample images have been included and the general trends outlined. Further discussion and the full quantification results will be presented in TN 78.92, according to the description of work package 78.9, which stated the following:

The work shall be split in two parts. The first part will contain a description of the experimental set-up and the second one the analysis of the results. The specific contents of the TN's to be delivered shall be:

TN 78.91:

- *Objectives of the research*
- *The techniques principles to be used (FISH, staining of fresh samples, cryosectioning, confocal microscopy observation)*
- *Description of the nitrifying pilot reactor operation in the previous period to biofilm analysis*
- *Sample CLSM images to illustrate the results obtained*
- *An initial estimation of general trends*

- *TN 78.92:*

  - *Software selection for the quantification and a brief description of the software capabilities (for the software finally selected for the quantification of the results)*
  - *Quantification of the results through image analysis as well as interpretation of the main results, which will consist in the final estimation of the concentration/ proportion of the different bacterial species and EPS in the biofilm along the bed vertical axis.*

Please make clear in title of Figure 3 the probes used for *Nitrosomonas* cells (NEU) and *Nitrobacter* cells (NIT3)

- This has been corrected (see figure 6).