MELiSSA

TECHNICAL NOTE

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Compartment III nitrogen sources instrumentation upgrade

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List of acronyms

CI : compartment I

CII : compartment II

CIII : Compartment III

CIVa : Compartment IVa

CIVb : compartment IVb

CV : Compartment V

FIA: Flow Injection Analysis

HPC: Higher Plant Chamber

MELiSSA: Micro-Ecological Life Support System Alternative

MPP: MELiSSA Pilot Plant

NEDD: N-(1-napthyl)-ethylenediamine dihydrochloride

UAB: Universitat Autònoma de Barcelona

UPS: Uninterruptible Power Supply

1. Context: the MELiSSA Project and the MELiSSA concept

1.1. The MELiSSA Project

Over the last 15 years several Space Agencies (i.e. NASA, JAXA, RSA, CSA, ESA) have been studying the regenerative life support systems needed to sustain long-term manned space missions.

Space exploration constraints dictate that the primary objective of the studies is to reduce the launched mass of metabolic consumables (i.e. water, oxygen, food) by increasing their recycling rates up to, ideally, closure of the gas, liquid and solid loops.

Within Europe, the main part of the work has been performed within the MELiSSA (Micro-Ecological Life Support System Alternative) project by a highly comprehensive European and Canadian scientific and technical network, coordinated by the European Space Agency (specifically the European Space Research and Technology Centre ESTEC).

Within MELiSSA, it is proposed to follow a global approach of Life Support requirements by addressing jointly the main Life Support functions, i.e.:

- Air revitalization,
- Water production,
- Waste management,
- Food production and preparation
- Quality Control and Safety issues
- Ergonomics and Habitability

With regards to the challenge of sustaining Human Life during long-term manned space missions, a stepwise engineering approach is followed in MELiSSA, starting from basic research and development studies, including preliminary flight experiments, up to a comprehensive ground demonstration of the technologies developed.

1.2. The MELiSSA concept

The MELiSSA concept is based on the duplication of the functions of the earth without benefiting from earth's large resources (i.e. oceans, atmosphere..) and from terrestrial comfort.

The goals of the MELiSSA loop are the recovery of food, water and oxygen from wastes, i.e. CO_2 and organic wastes, using light as a source of energy.

From the observation of a lake ecosystem (i.e. the identification of the elementary consumption, degradation and production functions composing this ecosystem), the

MELiSSA loop is conceived as a closed regenerative system, based on five compartments duplicating the lake ecosystem's elementary functions (see below Figure 1, further information is available at http://www.estec.esa.int/ecls).

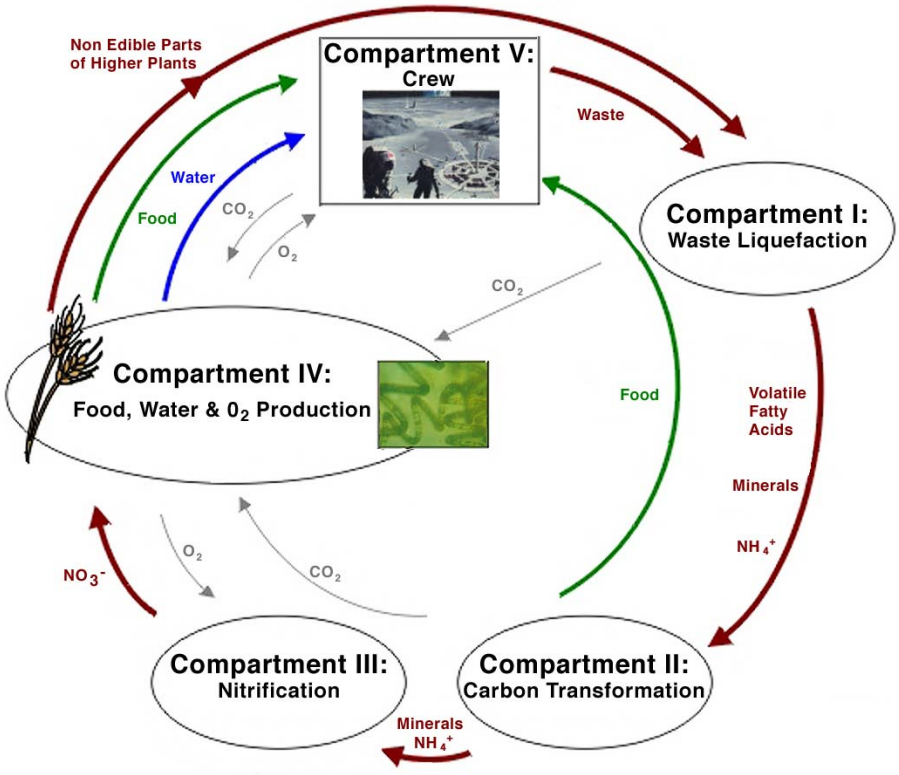


Figure 1: MELiSSA Advanced Loop Concept

Each compartment has a given objective within the complete biotransformation and connections with other compartments.

The basics are the followings:

- In Compartment I, the different waste sources are degraded in an anaerobic thermophilic bioreactor. The wastes include non edible material from plants, excess bacterial material from other compartments, faecal material, etc. The degradation yields a range of volatile fatty acids (VFA) that are transferred in Compartment II.
- Compartment II is photobioreactor where the VFA produced by Compartment I are further converted, basically to CO_2, by the photoheterotrophic growth of the bacteria *Rhodospirillum Rubrum*.
- Compartment III is responsible for the bioconversion of the nitrogen source, i.e. from ammonium NH_4^+, as produced in CI, into nitrate NO_3^-. Compartment III is a

fixed-bed bioreactor, with a co-culture of *Nitrosomonas* and *Nitrobacter* bacteria immobilized onto a solid support (beads).

- The production compartments are Compartment IVa and IVb:
    - Compartment IVa is devoted to the culture of the photoautotrophic cyano-bacteria *Arthrospira platensis* (*a.k.a. Spirulina platensis*), and is used mainly for the production of oxygen from CO_2,
    - Compartment IVb is devoted to the culture of a number of selected higher plants (i.e. wheat, lettuce and beet), for the production of food and oxygen.
    - These compartments are the closing steps for the loop, since they provide with the functions of atmospheric regeneration (converting the CO_2 generated by the crew and other bacterial compartments into O_2) and edible material generation. In addition, higher plants can also provide a way to biologically regenerate potable water through transpiration.
- Compartment V corresponds to the crew (i.e. consumer) compartment. For the first demonstration of the MELiSSA loop, it has been decided to work with laboratory animals.

The development of each individual compartment follows the same engineering logic:

- Technologies characterization in batch and continuous modes,
- Stoichiometry studies,
- Hydrodynamic characterization,
- Static Modelling,
- Dynamic Modelling,
- Control Model (for predictive control),
- Safety issues (chemical and microbiological),
- Maintenance and Dependability.

At the upper level of the complete loop (i.e. closed loop of interconnected compartments), a system approach is mandatory to achieve mass balance closure, a relevant safety of the complete system and its reliability for long term operation. This system approach is supported by a knowledge-based control leading to the development of a predictive control based management of the overall MELiSSA loop.

2. The MELiSSA Pilot Plant

As expressed previously, the challenge of sustaining human life in frame of long-term missions is such that an extensive demonstration of MELiSSA on ground is a mandatory step in the process of its adaptation to space.

Owing to the state of the art at laboratory scale, the five MELiSSA compartments are progressively developed up to a pilot scale, according to a sizing scenario defined by the MELiSSA Consortium as representative of a full scale manned mission **(i.e. production of 1 eq-man oxygen, production of 20% of 1 eq-man daily diet)**.

The European Space Agency (ESA) has entrusted the implementation of the MELiSSA Pilot Plant to the Universitat Autonoma de Barcelona (UAB), with **the challenge to make it the primary European Facility for Life Support Ground-Demonstration.**

The MELiSSA pilot compartments will be integrated (i.e. connection of the gas, solid and liquid phases) within the MELiSSA Pilot Plant, with **the ultimate objective of a long-term demonstration (i.e. around 3 years of continuous operation) of the MELiSSA loop (i.e. 5 compartments interconnected).**

A new MELiSSA Pilot Plant facility has been built by the Universitat Autònoma de Barcelona., in the Departament d'Enginyeria Química, Escola Tècnica Superior d'Enginyeria (ETSE). This new facility of 214 m^2 will be devoted to the location of:

- compartments I, II, III and IVa, three Higher Plants Chambers composing CIVb, the animal compartment (i.e. CV),
- a human waste collection unit,
- a control room,
- Auxiliary equipments.

3. The Compartment III of the MELiSSA Pilot Plant

As mentioned previously, one of the compartments of the MELiSSA loop, Compartment III, is devoted to the nitrification step within the loop. Nitrifying bacteria are required in a life support system to carry out 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 polymeric (expanded polystyrene) substratum (Biostyr®) which has an average diameter of 4.1mm. In order to avoid inhibition by light, the fixed bed was protected with thin foil. The scheme of Compartment III and a

picture during its operation can be observed in Figure 2, and further details on the design of this reactor can be found in Pérez *et al.* 2004.

The operating conditions in the reactor 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.

In regular continuous mode operation the reactor is fed with a given amount of NH_4^+ and is operated at a constant gas flow rate. These are the main process parameters on the reactor performance:

- Compartment III is capable of processing ammonium loads up to 1.4 kg $N\text{-}NH_4^+ \cdot m^{-3} \cdot d^{-1}$.

- The flow rate and concentration of the liquid stream entering this reactor are defined by the requirements of the MELISSA loop. As a reference for design purposes, the range of concentrations and flow rates used during tests with the current reactor design have been used. They were as follows:

Flow rate	Concentration
0.15-0.60 L/h	300-600 mg $N\text{-}NH_4^+$/L.

- Because nitrification is an aerobic process, oxygen is provided to the reactor: an approximate value of oxygen requirement can be obtained from the stoichiometry (1.5 mols O_2 /mol NH_4^+). However this value must be corrected taking into account gas – liquid mass transfer and biofilm thickness, which both have an effect on the oxygen requirements. Previous studies performed with bench scale packed-bed reactors (Pérez, J.; 2001) gave as a result an oxygen ratio of 11 mol O_2 /mol NH_4^+ as the minimum oxygen supply.

- CO_2 is provided to the reactor as carbon source: as an approximate value to be taken into account for design, 5% of the carbon supplied is incorporated to biomass according to stoichiometry of the process.

- Base for pH control purposes is provided: Na_2CO_3 at a concentration of 100g/L.

- Acid for pH control purposes is provided: although the nitrification process usually requires addition of base during stable operation, the addition of CO_2 to lower pH was required from time to time.

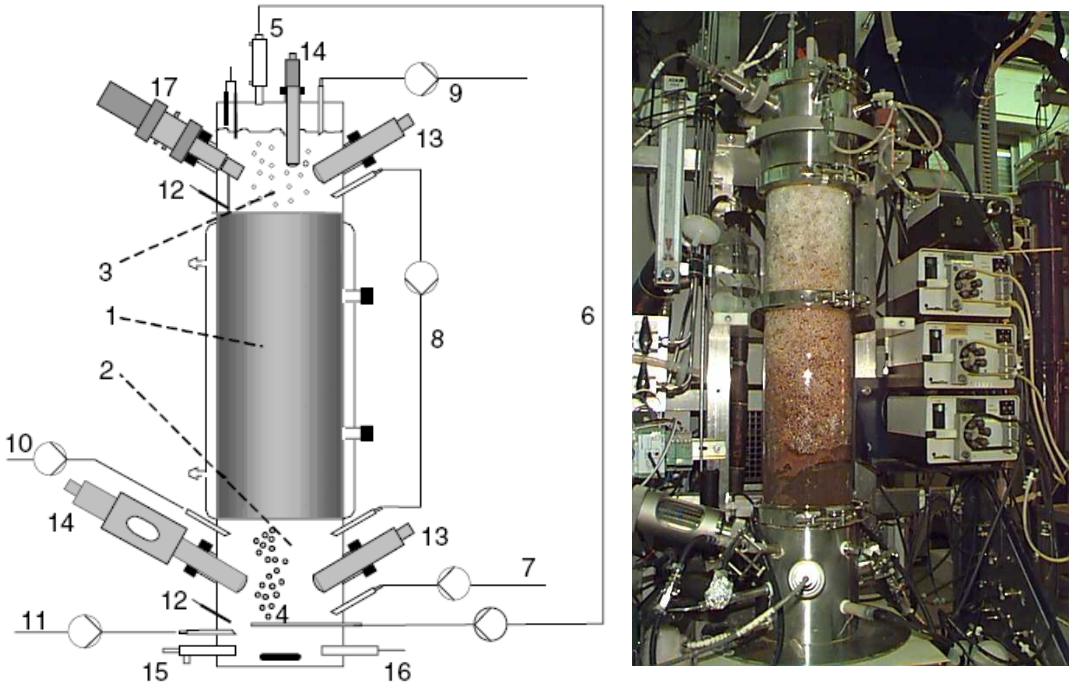


Figure 2: : Schematic overview of compartment III.

General schematic of the nitrifying bioreactors at pilot scale (left) and picture of the hardware (right). (1) Packed-bed section with immobilized culture, (2) bottom section for aeration, liquid distribution and instrumentation, (3) top section for gas disengagement, (4) gas sparger, (5) gas exit condenser, (6) gas loop, connected to oxygen/nitrogen regulated supply to control dissolved oxygen, (7) liquid feed, (8) liquid recirculation, (9) liquid outlet, (10) acid addition, (11) base addition, (12) temperature probes, (13) dissolved oxygen probes, (14) pH probes, (15) cooling system, (16) heating system, (17) sampling device.

4. Scope of the study: need for an on-line measurement of nitrite concentration in Compartment III.

The nitrification process is a two step biochemical reaction in which first NH_4^+ is converted into NO_2^- by the strain *Nitrosomonas europaea* (ATCC 19718) and second NO_2^- is converted into NO_3^- by the strain *Nitrobacter winogradskyi* (ATCC 25391). In a certain number of conditions, such as lack of oxygen or imbalance of the bacterial populations, nitrification can be achieved only partially, which means that nitrite will be present at the outlet stream of Compartment III, as shown in previous studies (TN 43.3, Pérez et al. 2000). Additionally, it has also been demonstrated in experiments of continuous connection of Compartment III and Compartment IVa (TN 47.6, Creus et al., 2001) that nitrite occasionally produced in Compartment III is not further consumed in Compartment IVa, thus potentially it could reach the consumers compartment with *A. platensis* cells used in the food preparation, unless intensive washing of the cells would be done. In general terms, due to the toxic nature of nitrites, their potential accumulation in the MELiSSA liquid loop must be completely avoided to avoid any further complication. As a consequence, one of the main aspects to be controlled in the operation of compartment III is nitrite accumulation, which should be completely avoided. The operation conditions of Compartment III will be controlled in order to keep nitrite concentration bellow the safety limits. Therefore, the development of the control laws of Compartment III is being built on the basis that nitrite concentration will be monitored on-line.

Nitrite analysis had not been widely developed at the time the first decisions were taken regarding monitoring of nitrogen species in the MELISSA loop and only preliminary efforts to implement nitrite analysis on-line had been carried out. Most analysers would determine the total NO_x^- ($NO_2^- + NO_3^-$) concentration, making it impossible to discern between these two species. On the other hand, monitoring of ammonium and nitrate concentrations had been widely studied and commercial solutions were commonly available. Due to this, a first approach was conceived, in order to develop an indirect on-line measurement of nitrite, based on the use of two on-line analysers, one for nitrate and one for ammonium. In addition of the necessary data for on-line concentration of nitrate and ammonium, the proposed rationale was that from the difference between both data, then nitrite concentration could be determined.

With this approach, combining the measured ammonium and nitrate concentrations provided by two online analysers with the nitrite prediction software designed by SHERPA Engineering, it should in theory be possible to estimate the nitrite concentration in the effluent of compartment III (TN 73.1, Leclercq, 2003). This nitrite predictor software is of relevant importance to develop and implement the control law of the nitrifying compartment.

A set of experiments designed by SHERPA Engineering for the validation of the nitrite predictor performance was carried out in the pilot reactor of compartment III, the results

being available in TN 52.4 (Montràs et al., 2004). These experiments performed at the MELISSA pilot plant with the online ammonium and nitrate analysers connected to the pilot reactor of compartment III proved that the nitrite estimator based on the measurements provided by these two analysers was not sufficiently precise to provide a reliable nitrite control.

The main reason for this conclusion was the difficulty to quantify nitrate and ammonium with high precision and repeatability to allow the correct estimation of the nitrite concentration. During normal operation of the nitrifying compartment, the nitrite concentration is very low, and then the intrinsic error of the ammonium and nitrate on-line analysis is too high to be used by the estimator to evaluate the nitrite concentration. Only important perturbations causing important peaks of nitrite concentration could be detected using this approach.

In conclusion, the first working hypothesis for the on-line nitrite concentration analysis proved to be unsuccessful for fine tuning control of Compartment III. As a consequence, the attention was focused again on the identification and selection of an equipment for on-line nitrite analyzer in the nominal operating conditions of Compartment III. It was also expected that new systems would be available since the time the first trade-off for on-line nitrogen species analytical equipment was performed.

The scope of this study is therefore to perform a trade-off among potential on-line nitrite analysers, that should comply with a number of technical requirements described in the next section, and propose a suitable analyser to be incorporated in the MELiSSA Pilot Plant as part of the associated monitoring and control instrumentation of Compartment III.

5. Technical requirements for the on-line nitrite analyzer

The selection of a suitable analyser for on-line monitoring of nitrite concentration in compartment III pilot reactor, should be based on several requirements to be taken into account. Indeed, the results obtained from the experiments performed at the MELiSSA Pilot Plant for the validation of the nitrite predictor software developed by SHERPA Engineering provided some useful information on the requirements for the nitrite measurement. The analysis of these results allowed to fix a number of requirements regarding the accuracy, measurement range and sampling frequency that an adequate analyser should have. These requirements are summarised in table 1.

The range of measurement was selected by taking into account that the analyser should be able to measure nitrite concentration not only under normal operational conditions (low range) but also during irregular operation (high range). The optimal configuration should allow to monitor the nitrite peak indicating a transient state up to the highest possible concentration, that was fixed at 20 mg/L $N\text{-}NO_2^-$.

The sample volume consumed in each analysis should also be minimised, therefore the possibility to recycle the sample after analysis should be considered.

Table 1: Requirements of the on-line nitrite analyser (provided by SHERPA Engineering)

Range of measurement	Accuracy	Response time
0.02-0.5 mg/L N-NO_2^- 0.5-20 mg/L N-NO_2^-	5% - 10%	maximum 12 min.

In addition to the mandatory system requirements, further issues were regarded as additional advantages, such as a low maintenance cost and the autonomy of the equipment.

Potential interferences with the analytical method used by the considered on-line analysers also need to be taken into account. It is important that any potential interference by compounds present in the sample matrix (i.e., components of the feed medium to Compartment III) is known in advance to check the maximum acceptable concentration of these species in the sample.

6. Review of on-line instrumentation for nitrite analysis: pre-selection of analysers.

As a first step in the trade-off performed in this study, a wide search was undertaken in order to obtain as much information as possible on commercial companies that work on the development of process analysers for the detection of chemical ionic species. This first study concluded with a list of a few commercially available analysers that have the capacity to determine nitrite in an aqueous solution at the required level in the specifications. Several companies were offering analysers that were discarded from the beginning of the trade-off due to their features, not fulfilling with the requirements previously established. The most general problem was that a number of the studied systems would only measure NO_x^- instead of NO_2^-, as for example Dr. Lange analysers, well known in this applications, but still presenting such a limitation.

This first step in the trade-off led to a reduced number of potential analysers to be further tested. One important point observed in this process, from the information compiled on the different commercial process analysers that had the capacity to measure nitrite, is that the wide range of nitrite concentrations required in the specifications imposes a high demand on the equipment (wide concentration range and high accuracy). Therefore, in the second step of the trade-off, three equipments were selected, on the basis of the technical specifications of the analyser itself.

The main technical information available for the three analysers selected at that point is summarised in table 2.

Table 2: Characteristics of the three commercial analysers for on-line nitrite measurement selected in the trade-off

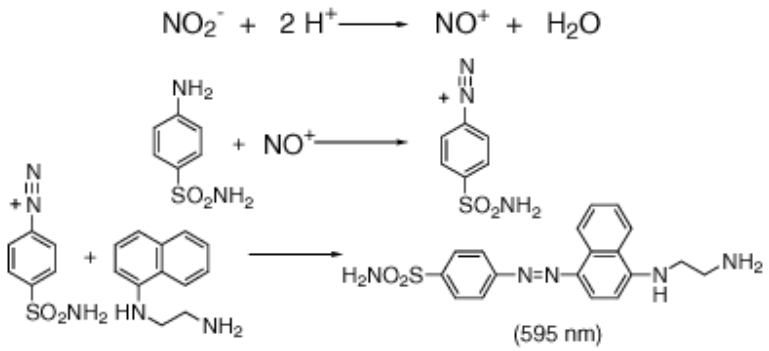
ANALYSER	SA 9000 SKALAR	ADI 2019 HD APPLIKON	FIAlab2500
Range	10-200 ppb NO_2^--N 0.5-10 ppm NO_2^--N	0.01-20 ppm NO_2^--N (with dual configuration)	0.01-20 ppm NO_2^--N (with dual configuration)
Analytical technique	Segmented flow analysis	Batch	Flow injection/ Sequential injection analysis
Detection method	Colorimetry-VIS (540nm)	Colorimetry-VIS (540 nm)	Colorimetry-VIS (540 nm)
Analytical method	Azo-Dye method	Azo-Dye method	Azo-dye method
Precision	better than 5% f.s.d.	1-2% full range	not provided
Repeatability	better than 5% f.s.d.	1-2% full range	not provided
Analysis Time	10 min approx.	8 min	not provided
Calibration	Automatic 2-point calibration	Automatic dilution. 2, 3, 5 or 7 point calibration (Sequential additions of standard)	Automatic dilution
Reagent needs	not available	0.8 mL/analysis	30 µL
Sample requirements	3 mL/min	0.2-2mL/analysis (to be optimized)	30 µL/analysis
Interference	Not expected	Not expected	Not expected
Automatic dilution requirements	$[NO_2^-$-N]>150 µg/L	$[NO_2^-$N]>150 µg/L	$[NO_2^-$N]>150 µg/L
Price	EUR 24500	EUR 23000 (single analyser, excl. filter module and dual wave-length system)	USD 16994 (single analyser, excl. dual wave-length system)

As a final step of the trade-off, the three suppliers of the selected equipment were contacted to investigate their capacity and willingness to perform the changes required to adapt the conventional versions of their process analysers so that they can comply with the given specifications in Table 1, that, as mentioned previously are very demanding in terms of wide range and high precision.

7. Description of the pre-selected analysers

7.1 Standard method for the determination of nitrite in water

The three analysers that were considered for the final trade-off are based on the implementation of the same analytical method, the so-called azo-dye standard method used for the determination of low concentrations of nitrite. The main difference between them lies on the on-line implementation of this method, which will be addressed in detail in the present document. The azo-dye method determines NO_2^- through the formation of a reddish purple azo-dye, produced under acid conditions (pH 2.0 to 2.5), by coupling diazotised sulphanilamide with N-(1-naphthyl)-ethylenediamine dihydrochloride (NEDD), following the reaction:



The applicable range of the method for spectrophotometric measurements is 10 to 1000 µg NO_2^--N/L. Photometric measurements can be made in the range 5-50 µg NO_2^--N/L by using a 5-cm light path and a green colour filter. Beer's law is valid up to 180 µg NO_2^--N/L with a 1-cm light path at 543 nm, with the possibility to measure higher nitrite concentrations by diluting the sample. Nowadays, implementation of an on-line diluting system is not a major problem, therefore it is feasible to use an analyser based on this principle for the measurement of higher concentrations.

7.1.1 Interferences of the method

Due to the principle used by the analysers described above, interference problems are very unlikely to be detected. The main interference is produced by the presence of NCl_3, which imparts a false red colour when the colour reagent is added to the sample.

The following ions interfere because of precipitation under test conditions: Sb^{3+}, Au^{3+}, Bi^{3+}, Fe^{3+}, Pb^{2+}, Hg^{2+}, Ag^{+}, chloroplatinate ($PtCl_6^-$) and metavanadate (VO_3^-), as well as any compound that could alter the colour of the solution and have a negative effect on the absorbance measurement.

Any suspended solids present in the sample should also be removed to avoid interference when the sample reaches the detector by filtration through a 0.45 µm-pore-diameter membrane filter.

None of the species mentioned above which have been found to interfere in the correct determination of nitrite, are present in the feed medium of Compartment III, therefore it is concluded that interferences in the analytical determination of nitrite will not be a major problem. However, this point will be tested experimentally.

7.1.2 Reagents

Colour reagent: An aqueous solution is prepared by adding 100 mL of 85% phosphoric acid and 10 g of sulphanilamide to 800 mL of water. After completely dissolving the sulfanilamide, 1 g of NEDD and water are added to reach a total volume of 1L.

Sodium Oxalate: 0.025M sodium oxalate is prepared by dissolving $Na_2C_2O_4$ in 1000 mL of water.

Ferrous ammonium sulphate: 0.05M ferrous ammonium sulphate is prepared by dissolving 19.607g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 20 mL of concentrated H_2SO_4 in water and diluting it to a total volume of 1000mL

Stock nitrite solution: A stock solution of nitrite must be prepared and standardized. This stock solution is used to prepare standard solutions used to calibrate the analysers.

Preparation and storage conditions:

NEDD colour reagent solution: The solution is stable for approximately one month when stored in a dark container and kept in the refrigerator at 4ºC.

Nitrite standard for calibration: the standard is prepared from a stock of nitrite solution. The stock solution should be prepared with fresh reagent, to ensure the absence of moisture, and should be protected against the access of air. Standardization of the stock solution should be carried out before preparing the standard solution to determine the NO_2^- content.

7.2 SKALAR SA-9000 process analyser

Among their different equipment, SKALAR (Breda, The Netherlands) technical support responsible concluded that to perform an on-line nitrite analysis in the operational conditions required by compartment III, the most appropriate analyser was SA9000,

whose operation was based on the segmented flow technique. The sample and the reagents are pumped in a continuous flowing stream in the required ratio and contamination between samples is prevented by segmentation with air bubbles.

Although the frequency of analysis was acceptable and the concentration range could be adapted to the determination of high levels by implementing a dilution, this alternative presented as a main drawback the difficulty to adapt its operation to low sample volumes, as required. After consultation with SKALAR engineers a potential adaptation of the equipment to handle lower sample volumes, it was concluded that this was not possible, and therefore this alternative had to be rejected.

7.3 Applikon ADI-2019 analyser

Among the different process analysers developed by Applikon® Analytical (Schiedam, The Netherlands) that can measure nitrite, the ADI 2019 HD was the recommended one. This analyser has been adapted to the measurement of higher nitrite concentrations than those allowed by the conventional standard method and it allows an automatic multiple time calibration at pre-set intervals.

The measure of high nitrite concentrations is achieved by replacing the pipette used in the standard version of the analyser with a sample injection loop that makes it possible to inject very small amounts of sample (sampling loops of 0.2 and 2mL were tested). By using this configuration, analysis of nitrite concentrations up to 20 mg/L N-NO_2^- can be achieved. However, the optimisation of the analyser performance at high concentration ranges may results in a decrease on the accuracy of the equipment when working at lower ranges. This will need to be tested experimentally.

The experimental layout of the ADI 2019 HD nitrite analyser is presented in Figure 3. On the right side of Figure 3 it can be observed how the sample is introduced to the analyser by means of an injection loop instead of the standard pipette used for lower concentrations. This configuration reduces considerably the amount of sample that is consumed by the nitrite analyser when compared to other techniques such as Flow Injection Analysis (FIA).

The ADI 2019 HD analyser operates batchwise with the chemical reaction taking place in the colorimetric cell that houses the mixing cuvette, the thermostat, a LED and the detector. This configuration allows high precision as well as low reagent consumption. Batchwise operation makes it possible to keep the sample and reagent consumption low, and does not have a negative effect on the dead time as long as the frequency of analysis is kept high and the distance between the analyser and the reactor outlet is short.

Addition of such a small amounts of sample is made by a micro burette, which makes it possible to minimise the error on the measured concentration. Dilution water can be added either with a micro burette or with a peristaltic pump if the volume of dilution water that needs to be added is larger.

The analyser consists of several modules (Figure 3) that are assembled according to the required application. Reaction and the subsequent colorimetric measurement take place in the cuvette module, which consists of a reaction cell (cuvette) placed in a holder, where the colour reagents and the sample are added by means of different wet part modules such as pumps, pipettes or burettes. A LED is used as a light source. A light with a limited bandwidth is emitted and goes through the reaction solution, where it is partly absorbed. A photo-detector is then used to detect the amount of light that has not been absorbed by the solution, which can be correlated to the nitrite concentration present in the sample.

The sample is provided to the cuvette module by the sampling system using a rotary type valve that has two positions, allowing sample to flush the sample loop or switching a precise amount of sample (a 0.2mL and a 2mL sample loops where used in the configuration presented herein) to the reaction cell. Dilution, when required, is achieved by adding water at this point by means of a peristaltic pump or a pipette (depending on the required precision). The analyser configuration used in the tests presented in this document was adapted to the measurement of nitrite concentration within a wide range (0-20ppm) and hence the selection of a peristaltic pump as an addition system instead of a pipette.

Reagent solution was added to the cuvette by means of a peristaltic pump. Rinsing and draining of the cuvette module in between sample analysis were also carried out by two peristaltic pumps.

The number of standards used to calibrate the analyser can be selected depending on the requirements of the analysis. Standard solutions of different concentrations are prepared by automatic dilution of a single standard by means of a burette module.

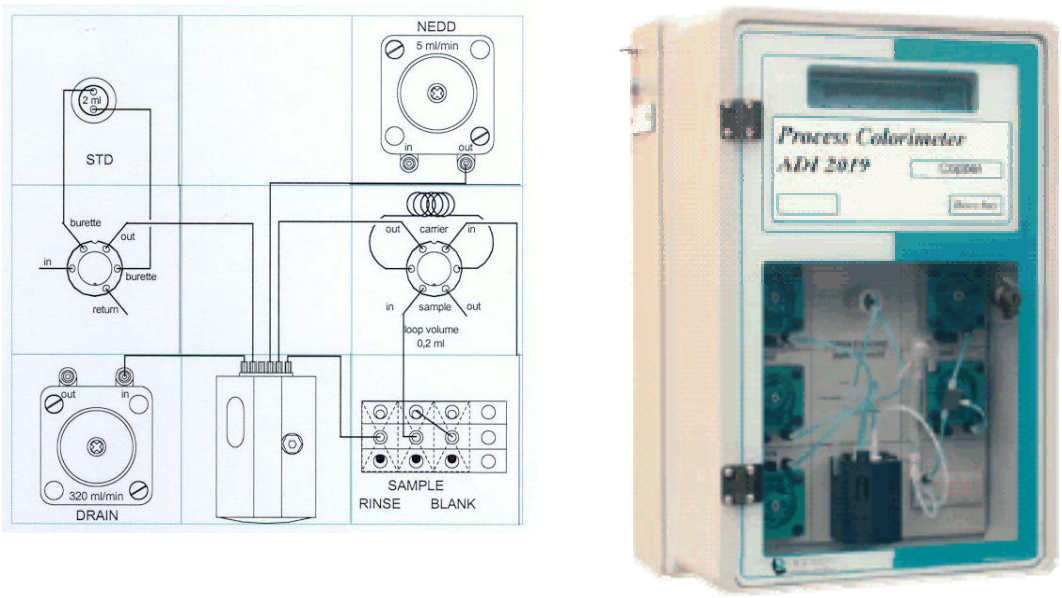


Figure 3: Layout of the ADI 2019 Heavy Duty process analyser. Adapted from Applikon®

7.4 FIAlab-2500 and FIAlab-3500

FIAlab (Bellevue, WA, USA) is a company that specializes on the development of laboratory analysers based on the techniques of Flow injection analysis (FIA) and Sequential injection analysis. The FIAlab-2500 analyser bases its operation on FIA techniques. The sample and the reagents are injected in the carrier flow by means of a six-way valve and absorbance of the sample is measured as it flows across the cell in which the detection system is installed.

After the first interaction with the company, and taking into account not only the range of measurement but also the low sample consumption requirements that apply, the recommended analyser was the FIAlab-3200, which is an analyzer based on a newer technique, sequential injection analysis, which is described by FIAlab as the second generation approach to FIA compatible assays. The main advantage of this configuration is the lower sample and reagent consumption requirements and, as a consequence, the lower amount of waste produced. In a SIA analyzer the system is filled with the carrier solution, and the sample and reagents are then inserted leading to three different zones, mixing and reaction takes place in a holding coil and then the reagents and sample are propelled to the flowcell in which the measurement takes place.

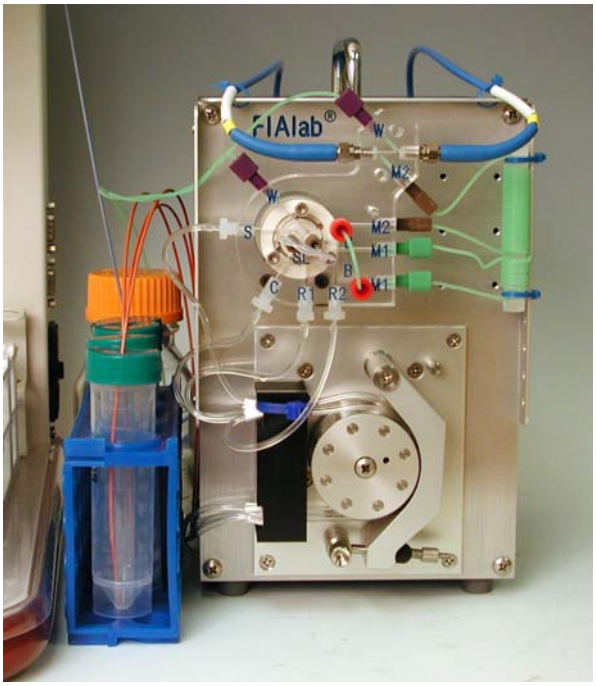


Fig 4.A: **FIAlab-2500 , FIA analyser**

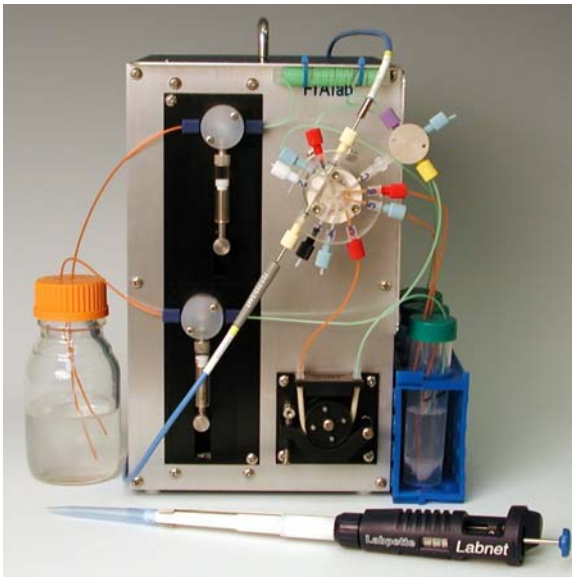


Fig 4.B: **FIAlab-3200 , SIA analyser**

8 Interface reactor-analyser

In addition to the aspects related to the analyser properties, other aspects need also to be considered. One of the most important ones is to keep sample consumption to a minimum, and to guarantee that the sample to be treated by the analysers is free of any potential cells in suspension, that could interfere with the nitrite analysis. One practical aspect to take into account is that when one sample is analysed through the system, the previous sample must be first displaced out of the system. Normally, in order to ensure this, and to avoid cross contamination between two samples, a certain amount of the new sample is flushed in the analyser, thus an additional volume of sample is required.

Therefore, the design of the correct interface that allows the coupling of the analyser to the reactor is a key issue on the implementation of the nitrogen analysis loop. The filtration system should be able to provide sample for the nitrite analysers and at the same time, the amount of sample that is used to flush the tubing so that the any previous sample still present in the sample loop can be replaced. Potentially, if this system works under full axenicity, the amount of sample used during this flushing step could be fed back to the bioreactor, thus keeping the liquid volume used for sampling at the minimal

value. However, this practice could be intrinsically risky in terms of compromising the reactor axenicity, and therefore it would be safer to discard the sampling liquid volumes.

One of the suppliers, Applikon, has developed the filter module A-SEP (shown in Figure 5). It is a system specifically designed for on-line analysis in biotechnology whose operation is based on a cross-flow technique, significantly reducing the dead time in the analysis loop. The culture broth is pumped out of the bioreactor in a continuous mode, forced through the channel in the bottom plate of the system. The flow rate required by the analysis loop flows through the membrane while the remaining sample is recycled to the bioreactor or discarded. The filtration system has an internal volume lower than 1 mL and consists of a filter membrane placed between two plates, so that the bioreactor and the recycled sample that is not injected to the analysis loop are isolated from the environment. A description of the filtration system is provided in Figure 5.

This module should be considered here as an example, and further investigation should be made in order to select a final interface reactor-analyser. This selection in fact has to consider other additional aspects, also considered in other TN within this CCN7, such as the re-design of Compartment III. Indeed, in this scope, the filtration of the outlet liquid medium previous to its feeding to Compartment IVa will be required, and a clear possibility is to take the sample for nitrite analysis right after this filtration step.

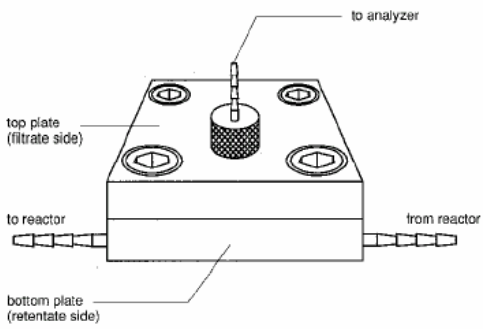


Figure 5. Scheme of the A-SEP filter module from Applikon*:* Flow of the circulation pump adjustable from 10 mL/min up to 100 mL/min. Filtrate pump has a fixed flow of 0.36 mL/min. Different pore-sizes between 0.1 and 5 μm are available for the filter membrane (adapted from Applikon technical data)

9 Final trade-off based on performance of experimental analysis

Out of the three analysers originally selected in the trade-off (Table 2), one of them (SKALAR) was discarded, as mentioned before, after first contacts with the supplier, since the necessary adaptations to limit the necessary amount of sample could not be provided. Therefore, the trade-off was reduced to two analysers (FIAlab and Applikon being the supplier companies).

In view of the potential criticality of this on-line measurement within the MELiSSA loop control, the highly demanding specifications fixed, and the reduced number of candidates, UAB and ESA accorded to perform an experimental testing of both analysers, in order to reach a final conclusion. The main objectives of the experimental tests performance were:

a) to assess directly the performance of the analysers tested, and the possibility to modify them in order to meet the given specifications

b) to assess the capacity and willingness of the supplier companies to perform adaptations in their hardware to meet the desired performance

The two companies offered different solutions for this test campaign, partially due to their geographical situation. FIAlab (US based company) agreed to test in their premises a number of blind samples, of known composition only by UAB, together with four standards with known nitrite concentration in the desired range (from 0.02 to 20 ppm N-NO_2^-). They had two different systems available already configured to perform nitrite determination (FIAlab 2500 and 3500), and the company would provide report on their performance with the samples provided. Applikon (The Netherlands based company) could not test the samples in their premises but offered as an alternative to configure one of their analysers for the specifications required in this case, and then provide it to the MELiSSA Pilot Plant for being tested directly, with the same samples that were being sent to FIAlab. Although not completely equal for both analysers, this solution was found an acceptable compromise to perform this experimental check-out of the analysers performance in this final step of the trade-off procedure.

10 Sample references for the analytical tests

According to the previous approach, a set of 10 samples were prepared, to be used in the MPP with the Applikon analyser, and to be shipped to FIAlab, to be analysed in the company laboratories. It was ensured that in both cases identical 50 mL aliquots of the same sample were treated, so the obtained results would be comparable.
The samples contained nitrite concentrations within the requested measurement range (0-20 ppm N-NO_2^-) in a matrix with the same composition as the culture medium used in

compartment III plus an excess concentration of either HCO_3^- or NO_3^- with the aim to identify the potential interference by any of these two species that are present at a high concentration in the samples from the nitrifying reactor and might have an effect on nitrite determination at certain levels.

The composition of the different samples is specified in table 3: samples A-F contain 600 ppm NO_3^--N and 580 ppm HCO_3^- in addition to different concentrations of nitrite and a constant matrix composition, allowing to evaluate the effect of high NO_3^- concentrations in the sample. Samples 1-4 contain 300 ppm NO_3^--N and 1160 ppm HCO_3^- to evaluate the effect of HCO_3^-.

Table 3. Composition of the samples used to test experimentally the two selected analysers for the final trade-off.

	CONCENTRATION (mg/L)									
	A	**B**	**C**	**D**	**E**	**F**	**1**	**2**	**3**	**4**
$(NH_4)_2SO_4$	1320	1320	1320	1320	1320	1320	1320	1320	1320	1320
$FeSO_4 \cdot 7H_2O$	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Na_2HPO_4	710	710	710	710	710	710	710	710	710	710
KH_2PO_4	680	680	680	680	680	680	680	680	680	680
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	177	177	177	177	177	177	177	177	177	177
$MgSO_4 \cdot 7H_2O$	52	52	52	52	52	52	52	52	52	52
$NaNO_3$	**3642.857**	**3642.857**	**3642.857**	**3642.857**	**3642.857**	**3642.857**	**1821.429**	**1821.429**	**1821.429**	**1821.429**
$CuSO_4 \cdot 5H_2O$	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
$ZnSO_4 \cdot 7H_2O$	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
$CaCl_2 \cdot 2H_2O$	0.740	0.740	0.740	0.740	0.740	0.740	0.740	0.740	0.740	0.740
$NaHCO_3$	**800**	**800**	**800**	**800**	**800**	**800**	**1600**	**1600**	**1600**	**1600**
$NaNO_2$	**0.000**	**0.099**	**0.493**	**4.929**	**49.286**	**98.571**	**0.000**	**0.099**	**0.493**	**49.286**
$N\text{-}NO_2^-$	0	0.02	0.1	1	10	20	0	0.02	0.1	10
$N\text{-}NO_3^-$	600	600	600	600	600	600	300	300	300	300
HCO_3^-	580.952	580.952	580.952	580.952	580.952	580.952	1161.905	1161.905	1161.905	1161.905

11 Tests with the pre-selected analysers

11.1 Results obtained from FIAlab

The interaction with FIAlab company regarding the experimental analysis was not satisfactory. As agreed beforehand, 10 blind samples (in triplicates) and 4 standards with concentrations of NO_2^--N of 0.02, 0.1, 1 and 20 ppm were sent to the FIAlab headquarters in Bellevue, WA, USA. Samples were sent frozen and kept in dry ice in order to avoid degradation of nitrite, a species that is known for its low stability.

FIAlab performed the analysis of only a few of the 30 samples they had received and the results that were sent to UAB are presented in Figure 6. The results obtained with the first three standards (0.02-1 ppm) are satisfactory, while standard 4 (20 ppm) was out of range. The specific concentration of each sample is provided in Table 4.

Long Path Flowcell

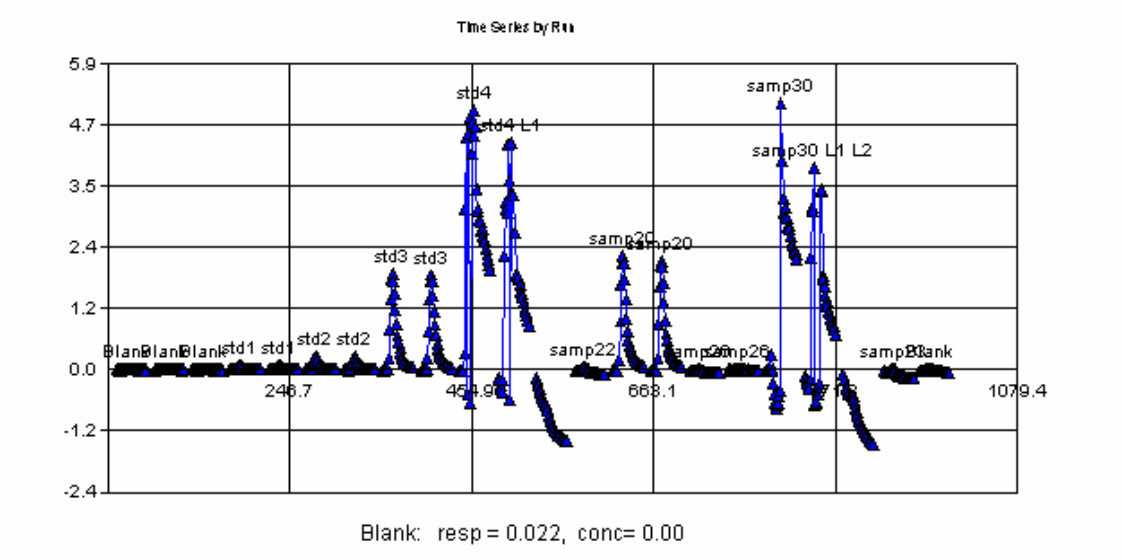


Figure 6A. Experimental results provided by FIAlab on the standards and Samples analysed with their analysers.

Short Path Flowcell

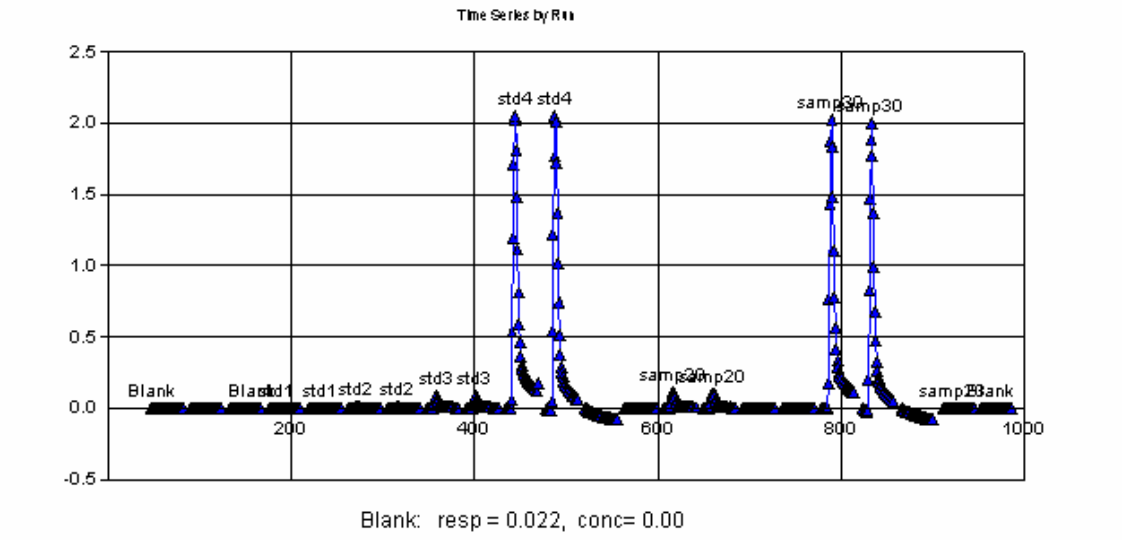


Figure 6B. Experimental results provided by FIAlab on the standards and Samples analysed with their analysers.

Regarding the blind samples, only a few of the samples were analysed by FIAlab and the results received are those shown in Figure 6. The only information that could be obtained from FIAlab was that the concentration range was too wide to perform the analysis correctly, but not detailed information on the performed analysis was provided. FIAlab was indeed not very pro-active at this point, and mainly insisted to suggest the purchase of a dual wave-length more complex analyser, which would allow to switch from the lower range to the high range concentrations when necessary, but no will to perform additional tests that would enable to obtain a more reliable information on the performance of the equipment.

Table 4. Composition of the different samples provided to FIA lab for blind analysis.

Sample ID	Theoretical (mg $N\text{-}NO_2^-\cdot L^{-1}$)
Standard 1	0.02
Standard 2	0.1
Standard 3	1
Standard 4	20
7, 12, 26	0
3, 16, 23	0.02
8, 11, 28	0.1
1, 20, 24	1
6, 15, 21	10
10, 17, 30	20
2, 13, 22	0
5, 19, 29	0.02
4, 14, 25	0.1
9, 18, 27	10

11.2 Results obtained with Applikon ADI-2019

Analysis of samples within the range 0- 20ppm $N\text{-}NO_2^-$

The analyzer provided by Applikon was adapted to nitrite analysis in a wide range (0-20 ppm) as requested, before its shipment to UAB for testing. Therefore, the volume of dilution water added in each analysis was rather high (17 mL dilution water / 0.2 mL sample) and addition was carried out by means of a peristaltic pump. The use of a low precision addition system, coupled with the high dilution explains the lack of accuracy obtained at concentrations below 0.5 ppm $N\text{-}NO_2^-$, as can be observed in Figure 7A (samples prepared in complete Compartment III medium) and Figure 8B (samples prepared in water).

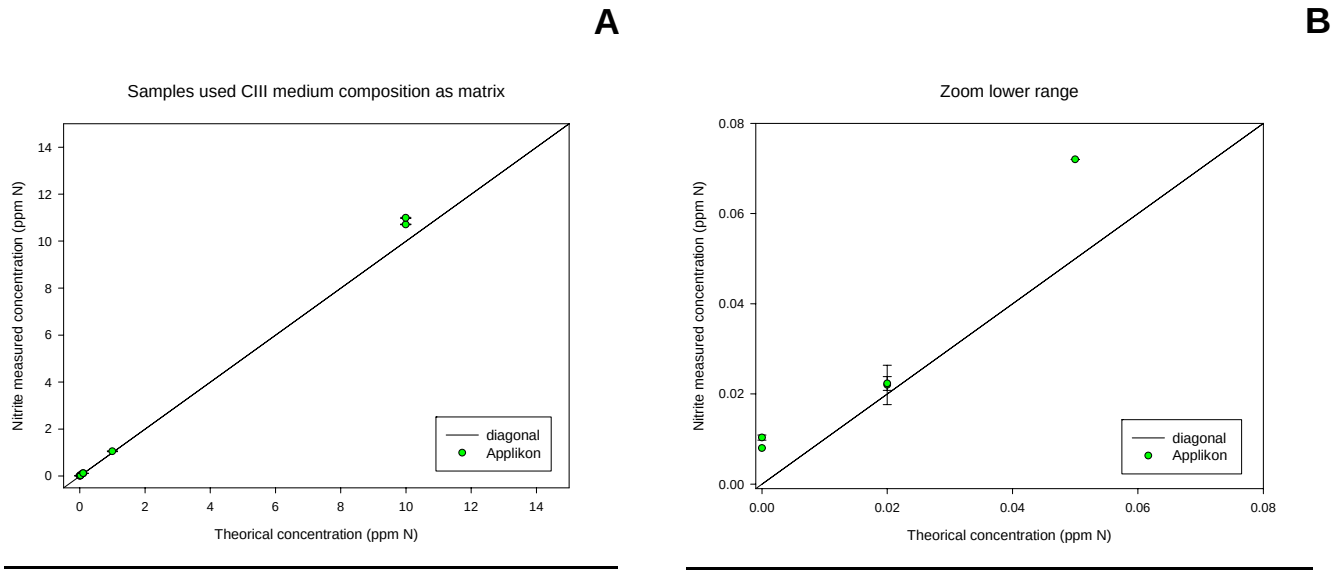


Fig. 7A. Evaluation of the APPLIKON analyzer performance with an analysis range of 0-20 ppm for nitrite samples dilutes in Compartment III culture medium. A: APPLIKON versus theoretical results within the whole analysis range. B: detail for the concentration range below 0.1 ppm.

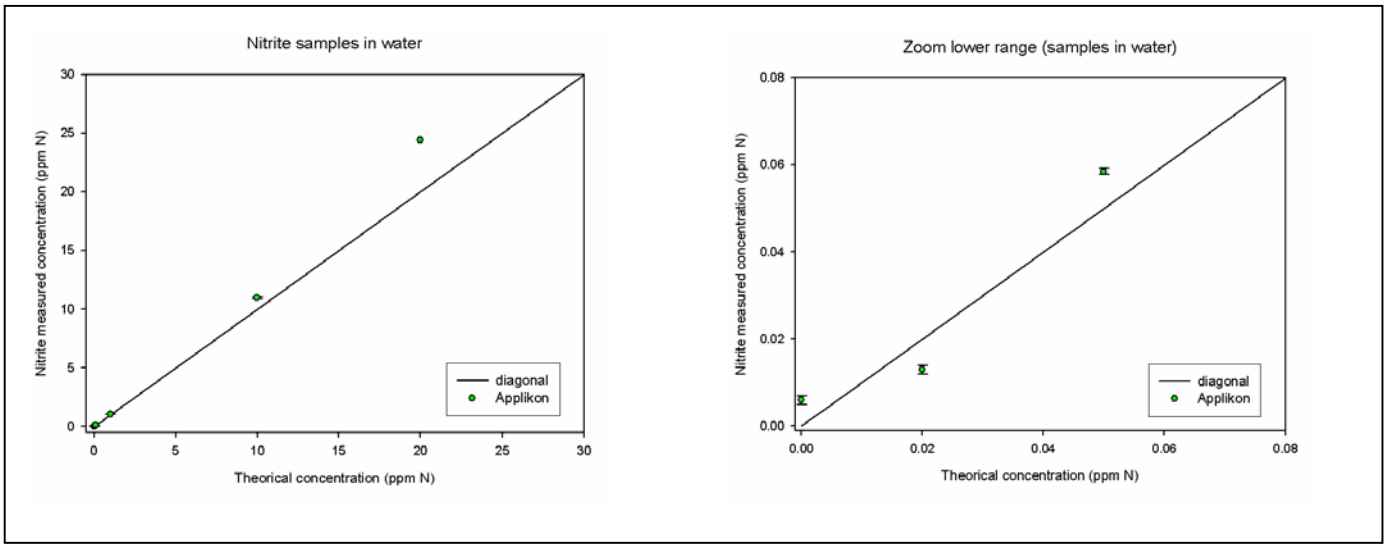


Fig. 7B. Evaluation of the APPLIKON analyzer performance with an analysis range of 0-20 ppm for nitrite samples diluted in water. A: APPLIKON versus theoretical results within the whole analysis range. B: detail for the concentration range below 0.1 ppm.

Analysis of samples within the range 0- 0.5ppm N-NO_2^-

The observed lack of accuracy at the low concentration range was quite probably due to the dilution conditions imposed by the high value of the maximum concentration range that was set as a target. In order to prove a higher accuracy by narrowing the concentrations range, the equipment provided by Applikon analyzer was partially changed in its configuration, but not all the necessary changes could be done, especially those related to hardware improvements (especially, the replacement of the peristaltic pump by a more accurate addition system in the automatic dilution loop).

Finally, a second batch of tests samples containing the same nitrite concentrations were used and the analyzer was calibrated to optimize its performance within the range 0-0.5 ppm N-NO_2^-. To this effect, the system was calibrated using a standard of 0.05 ppm N-NO_2^- and a sample loop with a volume of 0.5 mL was used instead of the 0.2 mL loop used in the previous tests. By implementing these changes, the dilution was reduced 10 times, from 1/100 to 1/10. However, the fact that the dilution water is not added by a high precision device such as a micro-burette, limits the potential improvement on the performance.

The samples were prepared in a solution containing all the components of Compartment III liquid medium and also in water, in order to check the influence of potential interferences on the analysis error, and to discriminate the error due to the dilution and the error due to potential interferences.

The results are provided respectively in Figure 8A (samples prepared in complete Compartment III medium) and Figure 8B (samples prepared in water). It can be observed that although a linearity trends is observed for the complete range, when the data at the lower concentrations are observed in detail, the error increases to a high level. This is still an important drawback, since the expected nitrite concentration at the nominal operation conditions in Compartment III is low. On the other hand, it can be observed that the analysis trends are very similar when a full solution with all the compounds present in C-III medium are used in the preparation of the samples, or when only water is employed, thus suggesting that the observed error at low concentrations can not, in principle, be attributed to the possible interferences of any compound present in the liquid medium.

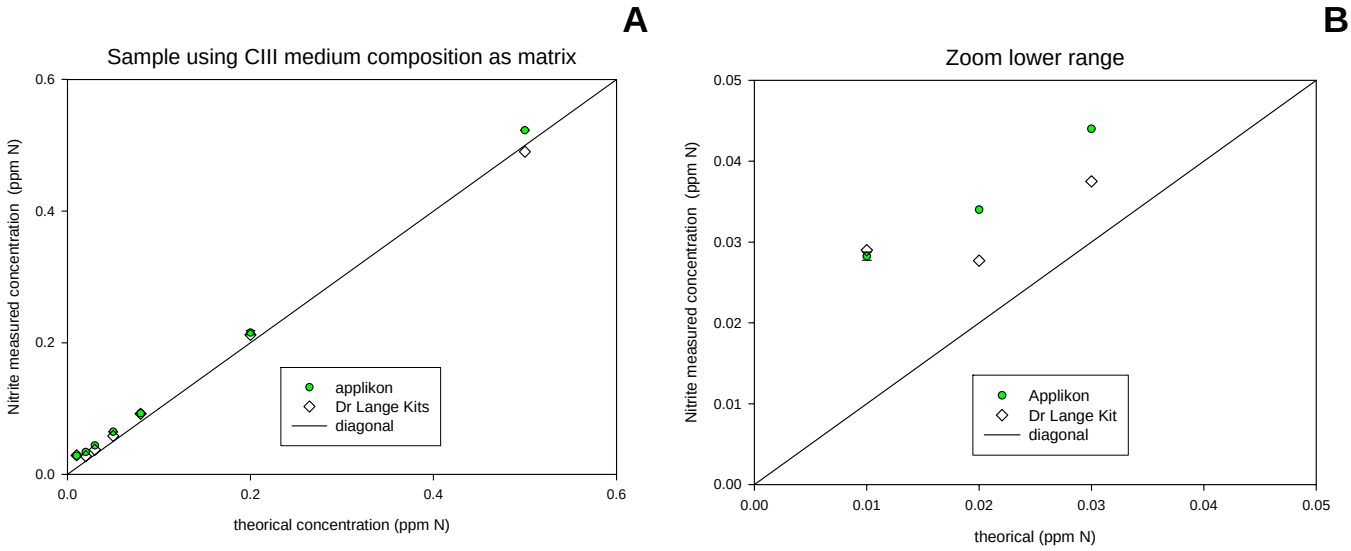


Fig. 8A. Evaluation of the APPLIKON analyzer performance with an analysis range of 0-0.5 ppm for nitrite samples diluted in compartment III culture medium A: APPLIKON versus theoretical results within the whole analysis range. B: detail of the concentration range below 0.05 ppm.

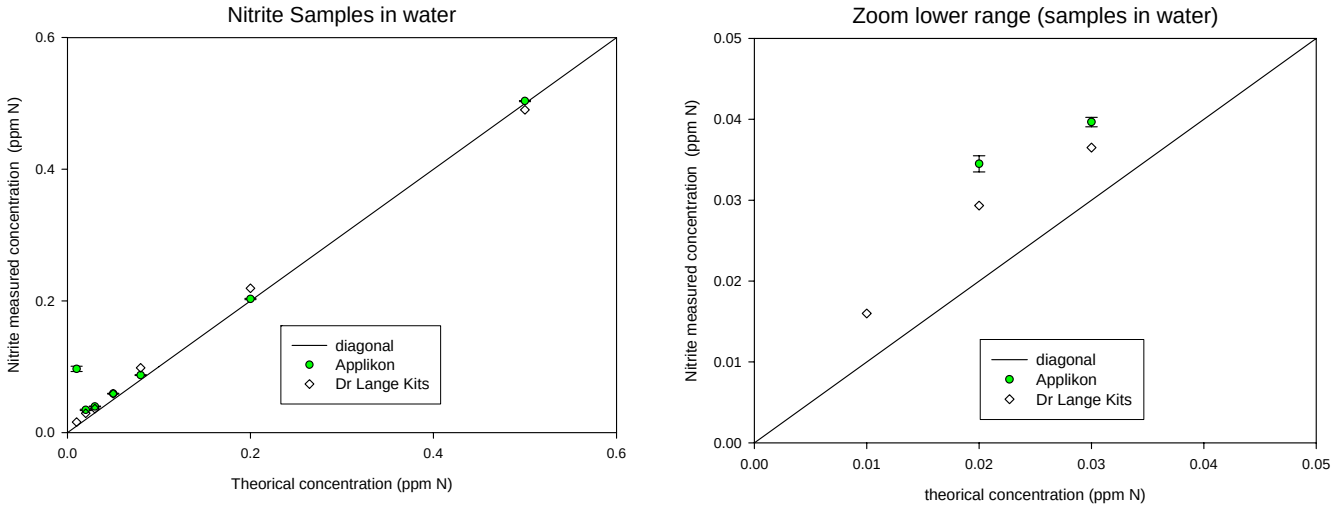


Fig. 8B. Evaluation of the APPLIKON analyzer performance with an analysis range of 0-0.5 ppm. for nitrite samples diluted in water. A: APPLIKON versus theoretical results within the whole analysis range. B: detail of the concentration range below 0.05 ppm.

Analysis of the results: deviation between experimental and theoretical concentrations.

The evolution of the relative error of the results obtained with the Applikon analyzer is shown in Figure 9A for the range between 0-20 ppm and Figure 9B for the range between 0.05 ppm. It can be clearly observed how the error increases exponentially at concentrations below 0.1 ppm N-NO2-.

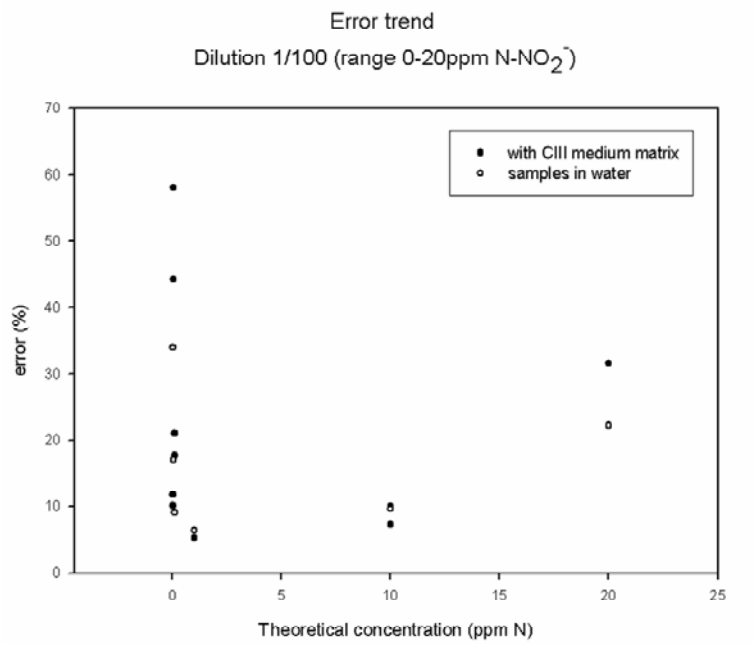


Fig. 9A: Evolution of the relative error of the analysis with Applikon analyzer as a function of the nitrite concentration, in the range of 0-20 ppm.

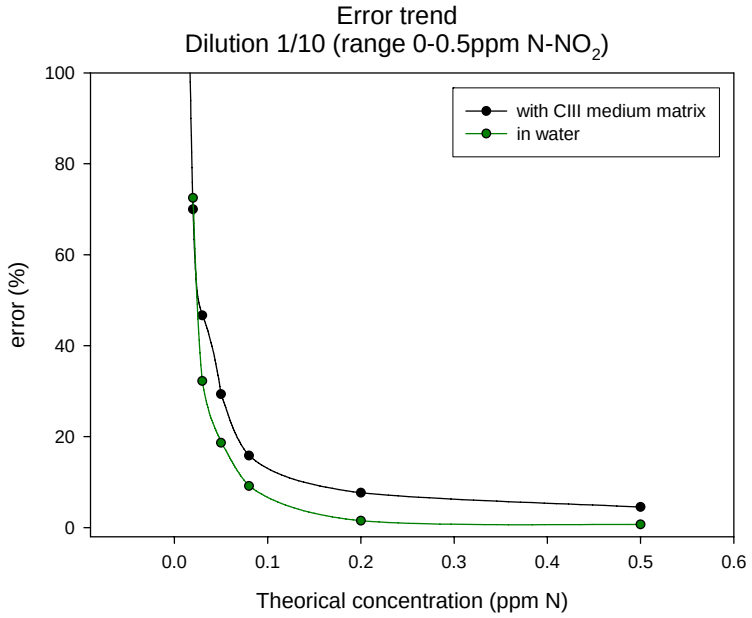


Fig. 9B: Evolution of the relative error of the analysis with Applikon analyzer as a function of the nitrite concentration, in the range of 0-0.5 ppm.

12 Final conclusion and recommendations

After this period of trials, it was concluded that a final decision on the data gathered so far could not be achieved. On the other hand, it can be concluded that it is not affordable to reach in one single equipment a very accurate measurement at very low nitrite concentration, i.e., in the range from 0.005 to 0.1 ppm, and also a very accurate measurement for a peak concentration up to 20 ppm. The main reason for this is the automatic dilution systems that are introduced in the analysers to be adapted to the higher range, increasing greatly the dilution error introduced in the samples at the lower range. Two possible options can be envisaged:

- To use analyzer that would have a high accuracy in the lower range (normal operation of compartment III) and would simply produce an alarm signal when the normal range was exceeded.
- To use a more complex dual analyzer that would switch to a different analysis routine when the lower range of analysis was exceeded. This dual configuration can be attained either by physically switching the sample to measuring cells with a different optical path, or by measuring absorbance at slightly different wavelengths. The optimal wavelength for the determination of nitrite is 540nm,

by performing the measurement at a wavelength 560 or 580 nm, higher concentrations can be measured.

From a strictly analytical point of view both companies consulted in the final part of the trade-off, Applikon and FIAlab assert that they can attain the requested analysis range by using a dual analyser. Considering the experience gained in the interaction with the two companies, it is recommended to follow-up the final tuning of a nitrite analyser only with Applikon company.

This recommendation is based on several criteria. Applikon has developed an interesting element to interface between the bioreactor and the analyser, that could be considered. The company is oriented to develop custom-made analysis systems, and it is certainly committed to develop and provide a successful nitrite analyser. The company is based in Europe (The Netherlands) and is well represented in Barcelona, this favouring the interaction and follow-up of the work, as in fact has been done in this first trade-off.

In summary, the ideal nitrite analyser for Compartment III in the MELiSSA Pilot Plant has still not been completely identified, but it is recommended to undertake further work with the company Applikon, to reach the desired goal. For this new customised hardware will need to be provided, and more test to ensure proper performance will be required.

13 REFERENCES

- **Creus, N. ; Pérez, J. ; Mengual, J.; Montesinos J.L.; Albiol, J.; Gòdia, F. (2001).** Connection of compartments III and IV at pilot scale. ESTEC/CONTRACT13292/98/NL/MV. Technical Note 47.6.
- **Leclercq, J.J. (2003).** Nitrite control of the nitrifying compartment. Robustness study and C software. ESTEC/CONTRACT12924/98/NL/MV. Technical Note 73.1.
- **Montràs, A. ; Pérez, J. ; Gòdia, F. (2004)**. Nitrite predictor complementrary tests. ESTEC/CONTRACT13292/98/NL/MV. Technical Note 52.4.
- **Pérez, J. ; Montesinos, J.L. ; Gòdia, F. (2000)**. Operation of the packed-bed pilot scale bioreactor. ESTEC/CONTRACT11549/95/NL/FG. Technical Note 43.3.

8. ANNEX. Applikon quotation.

8.2.1.1.

D.A.C. (Dpto. Atención al Cliente)

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U.A.B. – ETSE
DPTO INGENIERIA QUIMICA
CAMPUS UNIVERSITARI BELLATERRA
08193 - BELLATERRA
BARCELONA

ATT: SRA ANNA MONTRAS

Barcelona, 25/05/07

OFFER

AUTOANALYZER WITH 2 CHANNELS FOR TWO RANGE NITRITES MEASUREMENTT.

REF: 697ADI2040-NITRITES

SPECIAL PRICE: 44.265 € (VAT included)

CHARACTERISTICS OF THE SYSTEM:

Parameter(s) measured	Nitrite ($N\text{-}NO_2^-$)
Analysis method	Differential Colorimetric determination at 540 nm Automatic sample background color compensation
Number of sample streams	1
Tag	-
Range(s)	0 – 0,5 mg/l $N\text{-}NO_2^-$ 0 – 20 mg/l $N\text{-}NO_2^-$ (ranges are field adjustable)
Analysis time	10 min.
Accuracy	1 – 2 % relative at F.S.R.
Repeatability	1 – 2 % relative at F.S.R.

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8.2.1.2.

Detection limit	typical 1 ppb
Sample volume	0,2 ml 2 ml
Validation/Calibration	Automatic using a Standard solution
Reagents	NEDD $NaNO_2$ Standard D.I. Water
Utilities	Power supply 110/120 - 220/240 V, 50/60 Hz D.I. Water for Carrier & Rinse, max 4 Bar. Drain atmospheric.
Sample pressure	max 0,5 bar.
Sample Temperature	4 - 60 °C
Sample flow	approx. 5 ml/min. during sampling
Solids	Max. size 40 microns, amount less than 0,1 g/l

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8.2.1.3.

Item 1	***ADI 2040 Process Analyzer – Nitrite***
	ANALYZER CONFIGURATION
Analysis module	Cuvette Module with White LED and 540 nm filter Stirrer speed : 0 – 1600 rpm (programmable)
Sampling system	Sampling Loop system with 0,2 ml loop Sampling Loop system with 2 ml loop
Burette system	10 ml Burette system for Nitrite standard
Pump/Valve	Sample pump (5 ml/min) Carrier pump (5 ml/min) Carrier pump (5 ml/min) Rinse pump Drain pump NEDD pump (5 ml/min)
Reagent containers	2.5 liter for NEDD 5 liter for $NaNO_2$ Rinsing Water Reservoir
Analog Output	1 pcs. 0/4 - 20 mA
Digital Output	1 pcs. I/O Card Digital Output with 8 pcs. relays 24V ac/dc, 0.5A each
Serial Output	1 pcs. I/O Card RS232/422/C.Loop
Digital Input	1. Pcs I/O Card Digital Input with 8 pcs. input contacts
Analog Input	-
Software	Basic Software ADI 2040 (including Direct Measurement Module)

D.A.C. (Dpto. Atención al Cliente)

Grupo Instrumentación Científica

TEL.: 902 20 30 80
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Miscellaneous	1 pcs 8 Mb Flash Card with Analyzer software 1 pcs 8 Mb Flash Card for back-up of Analyzer program & data Leak Detector Wetpart Cabinet Purge Assembly Reagent Cabinet with Stand
Classification	General Purpose Area
Ingress Protection	IP66

D.A.C. (Dpto. Atención al Cliente)

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TEL.: 902 20 30 80
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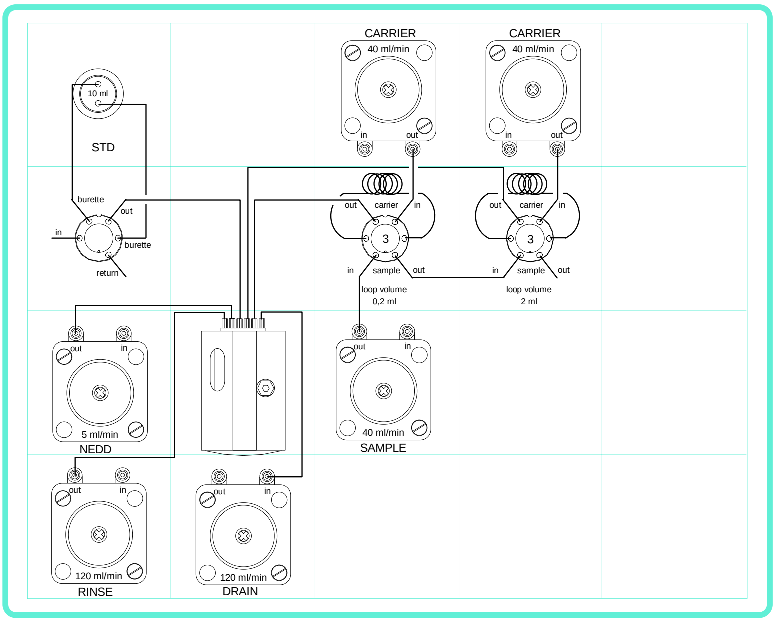

Item 1 — ***ADI 2040 Process Analyzer – Nitrite***

WETPART LAYOUT

Description

The Sample pump is activated and sample flows through the Sampling Loop systems until a representative sample can be taken.
Depending the expected concentration, one of the Sampling Loop system is activated and a precise amount of sample is dosed into the Cuvette Module by means of a Carrier.
Measurement of the initial absorbance.
Addition of 0,8 ml NEDD reagent and after a waiting time for color development, the final absorbance is measured.
Calculation and result output.
Drain & Rinse of the Cuvette Module.

Application

See ADS 1251

9. Comments

Page/paragraph	Comment
11/4	Today, it is not foreseen to supply the consumers with water coming from CIVa, but most probably with evapo-transpiration water from the HPC; nitrite could reach the consumer compartment if A. Platensis is used in the diet without relevant washing step. *OK, the sentence has been changed accordingly*
15/7.1.1	Couldn't we expect some problems of discrimination with nitrates? *In principle, no.*
16/7.1.2	Are there any specifications for storage or follow-up of the reagents solutions? **NEDD colour reagent solution*: The solution is stable for approximately one month when stored in a dark container and kept in the refrigerator at 4ºC. **Nitrite standard for calibration*: the standard is prepared from a stock of nitrite solution. The stock solution should be prepared with fresh reagent, to ensure the absence of moisture, and should be protected against the access of air. Standardization of the stock solution should be carried out before preparing the standard solution to determine the NO_2^- content.
17/7.3	Any value to be given as an indication for "very small amounts of sample"? *…[by replacing the pipette used in the standard version of the analyser with a sample injection loop that makes it possible to inject very small amounts of sample]* (sampling loops of 0.2 and 2mL were tested).
20/8	The interface reactor-analyser has to be discussed. You can leave the option mentioned as a proposal but we are not fully convinced. Recycling the volume of sample, used for flushing, back to the reactor is most probably too risky with regards to contamination. *OK, the sentence has been changed accordingly*
23/11.1	We do not consider that results are satisfactory for standard 3; can you please mention the legend on the diagrams? Can you provide the correspondence between sample numbers and your own references? *See Table below*
27/analysis of the results	Could you please provide the same type of diagram for concentrations higher than 0.5 ppm? *See new figure 7(2) and 7(3)*
28	Same remark as previously on the interface element. However, we agree to pursue the collaboration with Applikon, modalities of this collaboration to be defined. *OK, the sentence have been changed*