CRANFIELD UNIVERSITY

LAURA CARRASQUER VALLS

DEVELOPMENT OF NEW IMMOBILISED SYSTEMS FOR THE BIO-REMEDIATION OF OIL-SPILLS IN SEA WATER

SCHOOL OF ENERGY, ENVIRONMENT AND AGRIFOOD
Environmental Engineering

MSc
Academic Year: 2014 - 2015

Supervisor: Raffaella Villa
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This thesis is submitted in partial fulfilment of the requirements for the degree of MSc Environmental Engineering

ABSTRACT

The large physical impact of marine oil-spills, coupled with the toxic effect of some of the crude oil components, has increased the use of innovative bioremediation methods in combination with traditional ones. The gel beads developed in this project aim to provide a method to immobilise and optimise the environmental conditions of hydrocarbon-degrading microorganisms to increase their effectiveness, in order to offer a rapid response in case of emergency.

Lab scale tests on n-dodecane and crude oil revealed the potential of these microorganisms for further application in oil-spills. However, the main drawback seems to lie in the lyophilisation process, where the vitality of the cells is severely diminished.

Keywords:

Marinobacter hydrocarbonoclasticus, n-dodecane, lyophilisation, dry alginate beads, urea, biosurfactants, vitality.

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LIST OF ABBREVIATIONS

EU	European Union
C_{12}	n-dodecane
CO	Crude Oil
UNIMI	Università degli Studi di Milano
DABs	Dry Alginate Beads
R	Rhamnolipid
S	Sophorolipid
RDABs	Dry Alginate Beads containing Rhamnolipid
SDABs	Dry Alginate Beads containing Sophorolipid
UreaDABs	Dry Alginate Beads containing only Urea

1 INTRODUCTION

Petroleum, in its various refined forms, is still the main source of energy of our modern society and its extended presence in our lifestyle makes its substitution with cleaner energy sources very difficult. Moreover, its price fluctuation can have a great effect on the economy, being able to influence stock markets and money exchange rates. (Ocean Studies Board, et al., 2003) Its extraction, refinement, transport, use and removal represent a great threat to the environment.

The petroleum residues found in marine environments have different origins such as leakages from land deposits, expected escapes from reservoirs and spills from pipes or carrier ships. Apart from the toxic effect of components such as polycyclic aromatic hydrocarbons (PAH's), there is a very large physical impact when petroleum comes to contact with the marine environment. The complex composition of crude oil makes it difficult to understand and therefore remediate oil spills. Initially, the volatile fraction (boiling point below 200ºC) evaporates naturally, removing about 35% of the initial components (Dutta & Shigheaki, 2000). The rest of the many species present, which each have a different behaviour, is slowly degraded by photo oxidation and biodegradation.

Microorganisms with the ability of degrading oil have been found in natural environments where a spill has occurred. However, the rate of degradation depends on many factors such as: (1) oil-degraders availability, (2) nutrients concentration (nitrogen and phosphorus), (3) oxygen levels and/or (4) climatic conditions. (Swannell, et al., 1996). These, together with limited solubility of the oil in water will constrain the effectiveness of a microbial attack. (Yarett, 2010)

Nonetheless, normally oil-spills cause such an environmental catastrophe that a combination of the different available techniques is normally used, depending on how and where the dispersion takes place.

1.1 Oil Remediation Techniques

Large scale oil-spills can be caused by accidents on oil platforms, leaks from submarine pipelines or collision and/or sinking of oil tankers. Each of these case-scenarios is different, and the selection of the most appropriate remediation technique will depend on the type of oil spilled, weather and environment conditions, proximity to the coast and marine life at risk. (Dave & Ghaly, 2011)

Mechanical methods, such as different types of booms and skimmers are usually used as a first response in order to contain the spill. In combination with mechanical methods, chemical methods can help to protect sensitive areas and break down the oil into smaller particles that can be more easily degraded later on. The most commonly substances used include different types of surfactants, solvents and stabilisers.

If the spill takes place at the surface of the water, under very specific secure conditions, thermal remediation has also been found to be a plausible option (Mullin & Champ, 2003)

Bioremediation methods, such as the one discussed in this paper, are those where microorganisms degrade and metabolise the oil chemical components, a natural process that is accelerated by favouring the environment and conditions for these microorganisms to grow and increase their effectiveness. (Dave & Ghaly, 2011)

1.1.1 Bioremediation and immobilisation

Bioremediation can be carried out by introducing oil-degrading bacteria to the site (bioaugmentation) or by adding nutrients (biostimulation). (Nikolopoulou, et al., 2013)

The natural presence of microorganisms that degrade specifically certain components of petroleum has been known for some time, but their kinetics tend to be slow, of a 3% of degradation after an incubation period of 18 days (Atlas & Bartha, 1972). The combined addition of nitrates and phosphates however,

have proven to compensate the nutritional deficiencies in marine water, increasing the biodegradation rate up to values near a 70% in the same period.

Biosurfactants present an environmentally friendly alternative to traditional chemical surfactants, capable of providing an emulsifying and dispersant effect in immiscible systems such as oil and water without the toxic effect associated to traditional ones (Perfumo, et al., 2010) This facilitates the contact between the microorganisms and the contaminating compounds, reducing the time required for biodegradation. (Bao, et al., 2014)

The combined effect of nutrients and biosurfactants such as the ones tested in this work has proven to enhance the biodegradation of the microorganisms. (Banat, et al., 2011) It has also been observed that autochthonous microorganisms present the maximum effectiveness when in presence of fertilizers and biosurfactants, as reported by (Nikolopoulou, et al., 2013).

Microencapsulation is a new and promising technology being tested for improving bioremediation results. Previous work has demonstrated its potential, encapsulating nutrients to provide their slow release aiding in the degradation process, up to a 43.6% in 240 hours. (Reis, et al., 2013) Similarly, (Moslemy, et al., 2002) reported that encapsulated cells were able to degrade up to a 90% of gasoline hydrocarbons at a concentration of 50-600 mg/ L in a period of 10 days.

All this existing work opens the possibility for the encapsulation microorganisms, nutrients and biosurfactants to achieve a fast response, cost effective and efficient bioremediation solution for marine contaminated sites.

1.2 Kill Spill Project

This thesis work sits in a wider project, Kill-Spill “Integrated Biotechnological Solutions for Combating Marine Oil Spills”, which is an EU funded collaborative project started in 2013. The objective of this project is to identify a general protocol that can be presented as the best response in an event of marine contamination by petroleum. The technologies developed within the project aim to be economically sustainable and environmentally friendly. The best

technologies developed at lab scale will then be tested in the field to ensure adaptability to real conditions.

1.2.1 Scope of the Thesis

The main aim of this work was the development of gel-beads, able to co-immobilize hydrocarbon-degrading microorganisms, surfactants and nutrients, to be used in bioremediation experiments (crude-oil or hydrocarbons in sea-water) at lab-scale.

The objectives that were derived from this were:

- Evaluate different oil-degrading strains and select the most successful one
- Test at lab scale, the effectiveness of the selected strain when lyophilised, on n-dodecane and on crude oil.
- Test the degradation efficiency of the immobilised bacteria and identify the most efficient co-metabolising cocktail.

2 MATERIALS AND METHOLOGY

2.1 Evaluation of the degradation ability of different species on n-dodecane ($C_{12}H_{26}$)

The bacterial strains used in this section were isolated from contaminated areas, in the Department of Food, Environment and Nutritional Sciences (UNIMI) following the methodology reported in (Amer, et al., 2015).

The strains analysed were the following:

- *Marinobacter hydrocarbonoclasticus (ANU5)*
- *Alcanivorans jadensis (339)*
- *Alcanivorax dieselolei (293)*

The samples were prepared as follow: 20 mL of Sea water, 1% of n-dodecane and the same number of bacterial cells (10^8), using a Burker Chamber for the count. Single strains, a combination of two or all three were added to sterile 100 mL baffled Erlenmeyer flasks. The flasks were maintained at 28ºC in agitation (150 rpm). The extraction and quantification of the residual n-dodecane was done at time zero, after 3 hours, 3 days and 7 days. For each combination a negative control (without n-dodecane) sample was also prepared. All trials were done in duplicate.

2.1.1 Extraction of n-dodecane

The residual n-dodecane was extracted from the flasks with hexane using a separator funnel. 20 mL of hexane, which absorbs polar molecules, were added to the whole sample, shaken, let to separate, and the organic phase (on the top) decanted. A second wash of the aqueous phase was done with another 20 mL of hexane, to ensure optimal yields. The two organic phases were joined, dehydrated using sodium sulphate and filtered using cellulose paper. The solvent was gently removed using a rotary evaporator, leaving only the residual n-dodecane.

The residual n-dodecane was then solubilised in 1mL of hexane, diluted if necessary and analysed using a gas chromatograph.

2.1.2 Quantification of n-dodecane

The analysis of the samples was done using a FID gas chromatograph (Dani 6500 series) with a capillary column of polydimethylsiloxane (30m * 0.32mm). The carrier used was H_2 at 0.4 bars using the following temperature gradient: 3 minutes at 50ºC, 5ºC increments each minute until the temperature reached 200ºC. At these conditions, the retention time for n-dodecane varied between 14.5 and 15 minutes.

2.2 Evaluation of the degradation ability of *Marinobacter hydrocarbonoclasticus* (ANU5) on n-dodecane and crude oil.

Lyophilised cells of ANU5, the most effective strain, were tested on n-dodecane and crude oil.

Trials were conducted in redistilled 100 mL baffled Erlenmeyer flasks, containing 20 mL of redistilled sea water, C_{12} at a 1% weight and crude oil at a 0.5% weight. The residual oil was extracted at time zero and after 3 and 7 days. The evaluation was done with two concentrations of cells: 2g/L and 4g/L, therefore a total of 12 samples were prepared.

The density of the n-dodecane employed (ρ_{C12}=0.748 mg/dm3) and the density of crude oil (ρ_{CO}=0.862 mg/dm^3) (Snowdon & Stasiuk, 1997) were used to calculate the adequate volumes, of 267µL and 116 µL respectively, which were pipetted into pre-weighted flasks, under the fume hood. The weight was noted before adding the sea water, to be able to compare it to the resulting weight after the experiment

2.2.1 Extraction of oil and n-dodecane

Residual n-dodecane contained in the C_{12} samples was extracted using the same procedure reported in section 2.1.1. For the samples containing crude oil however, the procedure varied slightly. The whole sample was placed in a separator funnel, and extracted with 20 mL of hexane. After separation of the organic phase, the sample was extracted again with 20 mL of dichloromethane and the two organic phases combined. The total organic phase was dehydrated

using sodium sulphate and filtered using cellulose paper. The residual solvent was removed using a rotary evaporator.

2.2.2 Quantification of n-dodecane and crude oil

For the samples containing C_{12} the quantification of the residual oil was done using the same methodology as reported in section 2.1.2. For the samples containing crude oil, the quantification was done gravimetrically. The samples were extracted as described above in 2.2.1 and the weight measured at the beginning and at the end of the project using a pre-weighted flask.

2.3 Encapsulation of ANU5 (DABs) and evaluation of their degradation ability on n-dodecane and crude oil

These set of experiments were done to evaluate the degradation efficiency of ANU5 when encapsulated in Dry Alginate Beads (DABs). The reagent used was alginic acid sodium salt from brown algae (Sigma-Aldrich) at a concentration of 4%.

2.3.1 Preparation of the encapsulating solutions

The encapsulation media was prepared mixing two solutions prepared as reported in table 1, one for each of the two lipids evaluated:

Table 1: Composition of the alginate solutions for DABs

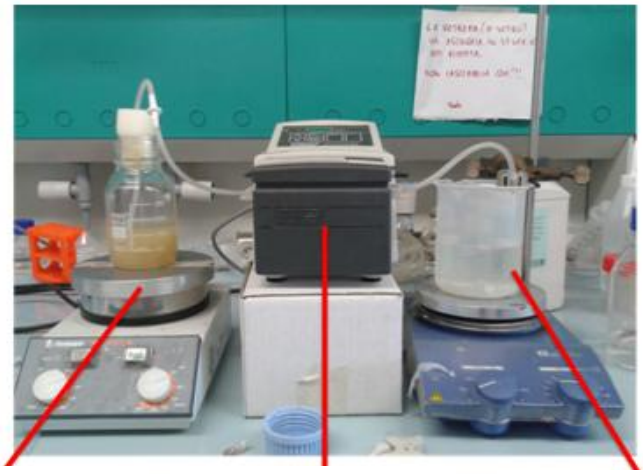
	Solution 1		Solution 2				
	Alginate(g)	H_2O(ml)	K_2HPO_4(g)	Urea(g)	Lipid(µl)	ANU5(g)	H_2O
Rhamnolipid	4,0	100	4,0	0,20	200	0,41	to 100ml
Sophorolipid	4,0	100	4,0	0,20	200	0,40	to 100ml

Solutions 2 were the added to the alginate solutions (solution 1), mixed overnight and left to rest for two hours until there were no bubbles in the mixture.

Curing solution: a 0.2M solution of $CaCl_2$ was prepared to cure/solidify the DABs. 2 litres of this solution were required for each of the two different lipid preparations.

2.3.2 Preparation of the DABs

For the immobilisation of the bacteria in dry alginate beads, the two mixtures prepared in section 2.3 were dropped into the 0.2 M $CaCl_2$ solution (constantly stirred) using a peristaltic pump attached to a pipette tip of 200 µL. The pump must be set to a velocity that allows the mixture to go through drop by drop, to ensure a spherical shape of the DABs



Alginate solution containing bacteria | Peristaltic pump | $CaCl_2$ solution

Figure 1: Laboratory set-up for the preparation of DABs

Once all the mixtures had gone through they were left for another half hour in agitation, drained and washed with redistilled water.

The beads were spread on wax paper, to avoid them sticking to each other and left overnight to dry. It is also possible to spread them on a glass and store them at 37ºC overnight.

2.3.3 Degradation tests

The degradation tests were prepared in 100 mL baffled Erlenmeyer flasks containing: 20 mL of redistilled sea water, C_{12} at a 1% weight or crude oil at a 0.5% weight and 200 mg for each type of DABs, (ones containing S and the others R). The residual oil extractions were done at time zero and after 3 and 7

days. It was calculated that 200 mg of DABs were equivalent to 9.8 mg of lyophilized cells.

2.3.4 Extraction of n-dodecane and crude oil

The procedure was exactly the same as that of section 2.2.1. The DABs were kept and re-dissolved in 15 mL a 0.5 M phosphate buffer solution at pH 7 to solubilise them. Potential C_{12} and CO remaining in the alginate was extracted following the same procedure reported in 2.2.1, but with a volume of 15 mL for each wash, in order to keep a standard final volume

2.3.5 Quantification of n-dodecane and crude oil

CO and C_{12} quantification was done as reported in sections 2.1.2 and 2.2.2. Any remaining CO or C12 in the DABs, extracted in the second procedure was added to the initial weight found.

2.4 Urea release tests on DABs

To evaluate the behaviour of the urea as it is released from the DABs, and the amount that is actually encapsulated, tests were done at time zero, after 3 days and after 7 days in standard conditions of 150rpm and 28ºC.

The samples were done in 100 mL flasks with baffle, containing 20 mL of sterilised sea water and 200mg of RDABs, SDABS or DABs containing only urea.

A sample with DABs containing only urea was solubilised in a 0.5M of phosphate buffer solution and compared to the data obtained during the tests.

The samples were analysed by spectrophotometer, using the Roche Yellow line of R-Biopharm kit for urea/ammonia, which can be found in 4Appendix C.

2.5 Blank tests on CO and C_{12}

To quantify the quantity of contaminant that is actually degraded by the bacteria and that which is lost during the extraction process of the samples, blanks containing only sterilised sea water and CO or C_{12} were prepared. The

extractions were done at time zero, after 3 and 7 days, in triplicate, at the same concentrations and following the same procedure for extraction and analysis as in section 2.2.

2.6 Vitality tests

To evaluate the survival rate of the bacteria after being lyophilised, the following tests were made:

2.6.1 Flow Cytometry

To estimate the amount of cells still alive after the immobilisation process in DABs, flow cytometric analysis was done using an Accuri C6 flow cytometer.

Two samples were prepared, each containing the equivalent amount in mass of lyophilised cells to the ones immobilised in 200mg of each kind of DABs. The weights indicated in Table 2 below where added to 20 mL of sterile sea water.

Table 2: Equivalent weight of lyophilised cells

	Weight of lyophilized cells (g)
Equivalent lyophilised cells in 200mg of RDABs	0,0089
Equivalent lyophilised cells in 200mg of SDABs	0,0098

In parallel, two other samples containing 200mg of each kind of DABs in 20 mL of sterile sea water respectively, were also prepared and analysed to evaluate the amount of cells that were released at time zero, after 24 and 48 hours of incubation.

The colorant SYBR Green, applied at 37ºC for 10 minutes, indicates the total cell count, and propidium iodide, at a concentration of 5 µg/mL also at 37ºC for 10 minutes, shows the dead cells or those with an injured membrane.

Because of the results obtained, described in section Vitality3.4, another analysis was done on a sample containing 10.1 mg of lyophilised cells in 20 mL of sterile sea water, the same sample that was then used to plate, as described in the following section.

2.6.2 Cellular count by plating

The last sample mentioned in the section above was plated into 3 different culture mediums; CYSP, Marine Broth and ONR7a. The methodology for the preparation of these culture mediums can be found in 4Appendix D

From 1 mL of the mother solution, 7 dilutions 1:10, 0.9% NaCl were done in Eppendorf tubes under laminar hood.

100 µl of each sample were plated for each dilution and each medium, meaning a total of 24 sterile Petri dishes to be prepared. After plating, they were left to grow at 28ºC for two days.

3 3. RESULTS AND DISCUSSION

3.1 Degradation capacity of the different strains

To determine the effectiveness of the single and combined strains, residual concentration of C_{12} in the samples was analysed by gas chromatography. Figure 2 shows the average residual C_{12}, for each time of analysis and for all single and combined strains. The grey columns represent the results for the analysis of the blanks.

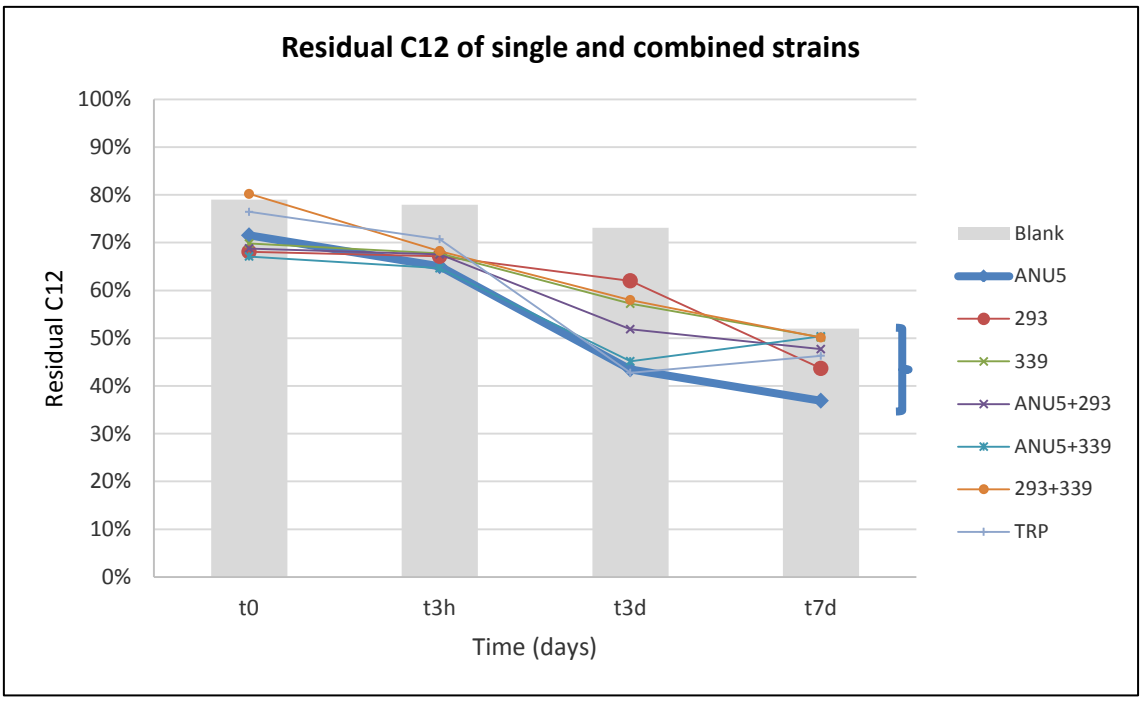


Figure 2: Residual C_{12} for single and combined strains, at the different times of analysis

These results show that even without bacteria present, there is a loss of n-dodecane, quite constant until the third day of incubation and accentuated at the seventh.

All bacterial strains single or in combination, showed some degradation capacity, with a maximum activity up to day three and a slower one until day 7, except for 293.

The strains, isolated from contaminated waters near the port of Genova, proved all capable to degrade C_{12} to some extent. *Marinobacter hydrocarbonoclasticus*

was identified in this study as the most effective one, although it has been known for its weathering capacity for some time now and has been objective of other works (Nicholson & Fathepure, 2003). This bacteria uses compounds present in petroleum as its carbon source, and this work proved further its potential for use in bioremediation techniques.

Although the difference between single strains and combinations was not very wide, there seemed to be no gains from using the bacteria combined, which indicates that for further work, the preparation of experiments can be done with single strains and not loose effectiveness.

Although there is clearly a degradation that occurs with time, in seven days the lowest residual C_{12} achieved is of around 35% in the case of ANU5, which is a relevant result but not close to total degradation. Further experiments could be conducted with a longer incubation time, to assess whether the complete degradation can be achieved.

Also, it is evident that there is a loss of material during the preparation or extraction of the samples, since at time zero none or little degradation was expected, but it was constantly around 20% for C_{12}. The results had to be interpreted bearing this in mind, and for further studies, use more accurate equipment. In the context of the Kill-Spill project, more precise analysis is planned already.

Notwithstanding the extraction and analytical errors reported, ANU5 showed the highest efficiency, with a faster degradation rate. Taking into account the results of the blanks, the net removal for ANU5 after 7 days is of a 15%, the highest one.

All the results, for all single and combined strains and for the blanks can be found in 4Appendix A.

Having ANU5 been identified as the most efficient strain, all further experiments were conducted using this bacteria.

3.2 Degradation capacity of ANU5 in lyophilised form, and in DABs

Several experiments were conducted with the lyophilised ANU5 strain. A negative control (without bacteria) was done for both CO and n-dodecane, to see how much material was actually being lost during the extraction process. Then the same tests were done with lyophilised cells and finally using the DABs containing lyophilised cells, nutrients and lipids.

3.2.1 Blanks

The results obtained from the blank tests showed that there is a more or less constant amount of C_{12} (20%) and CO (50%) that was lost in the during the handling and extraction of the samples. These results are represented, in Figure 4 and Figure 5 of the following section with the results from the degradation tests, so they can be put in context.

This error should be considered in the following sections as a corrective factor for the degradation values, as the material is lost without any bacterial presence, and therefore this decrease cannot be attributed to their action. The complete table with the results for each sample can be found in 4Appendix B

3.2.2 Lyophilised ANU5

In the context of an oil spill, the response has to be as immediate as possible. There is no time for the fermentation process, so the microbial agents need to be readily available. The lyophilisation process allows for the bacteria to be always available for immobilisation if the need arises.

The lyophilised ANU5, initially tested without immobilisation, indicated that there was a decrease in residual $C_{12.}$ In the case of crude oil, the degradation did not clearly increase with time.

Table 3 below, compares the results obtained from the degradation tests with the blanks, to obtain net degradation values:

Table 3: Correction on the degradation tests with lyophilised cells

Average residual C12					
Time	Blank	lyo. Cells 2g/l	lyo. Cells 4g/l	Net Degraded 2g/l	Net Degraded 4g/l
t0	84,80%	51,16%	57,64%	33,64%	27,17%
t3	80,82%	33,21%	36,49%	47,61%	44,33%
t7	80,49%	29,22%	9,96%	51,27%	70,54%
Average residual CO					
Time	blank	lyo. Cells 2g/l	lyo. Cells 4g/l	Net Degraded 2g/l	Net Degraded 4g/l
t0	54,11%	70,30%	44,96%	0,00%	9,15%
t3	52,87%	53,13%	41,48%	0,00%	11,40%
t7	54,31%	50,18%	53,63%	4,14%	0,68%

The results are plotted in Figure 3, where one can clearly see that the process is not working for CO, but it is for C_{12}

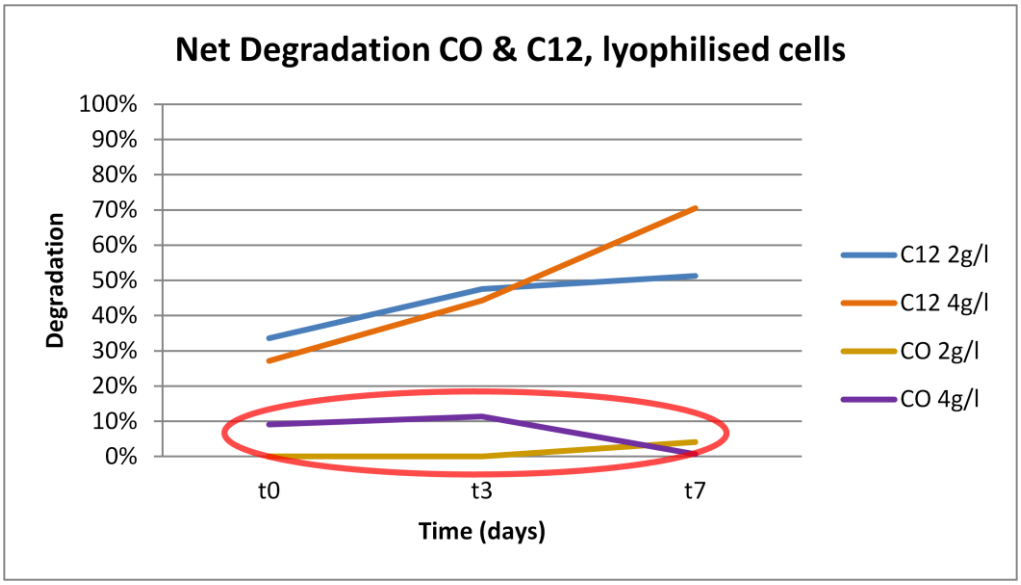


Figure 3 : Net degradation of CO and C12 with lyophilised cells

The microorganisms seem to behave very differently with each contaminant, so the results were also studied separately.

In Figure 4 below, one can appreciate that there is a constant amount of C_{12} lost during the extraction process, but the degradation (darker column still takes place and increases with time.

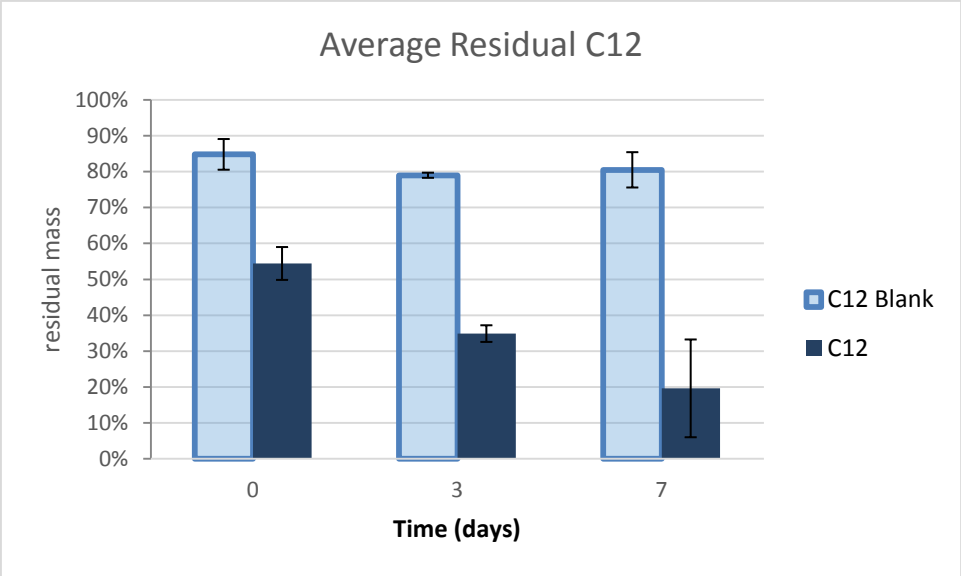


Figure 4: Average Residual C_{12} for lyophilised bacteria

The results for the CO are represented in Figure 5 below. It is clear that no relevant degradation took place, seeing as both the blank column and the residual CO column are almost the same height. Therefore, the process is not working for crude oil.

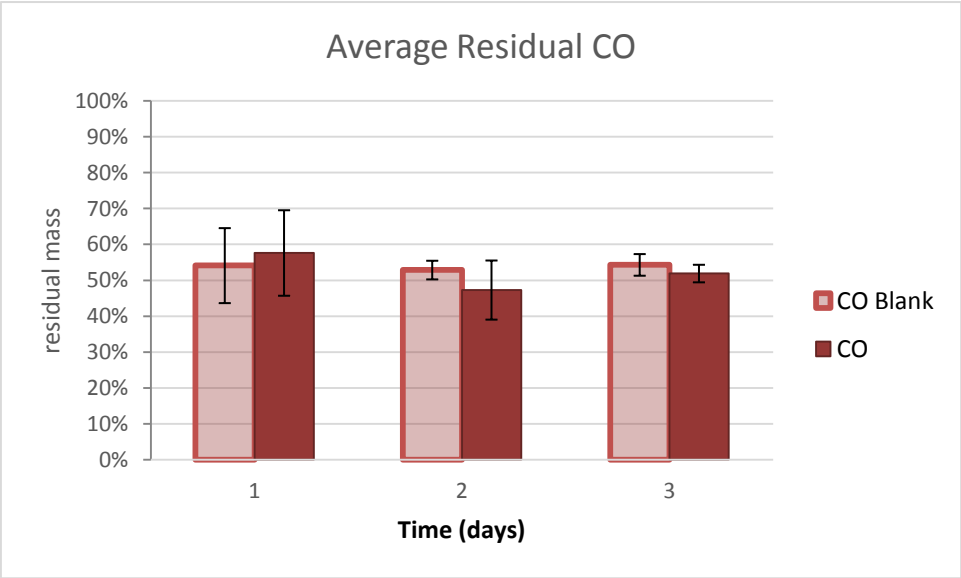


Figure 5: Average Residual CO for lyophilised bacteria

The full set of results, for each contaminant and bacterial concentration can be found in 4Appendix B

Regarding the CO, the results clearly indicate that the experiment is not working. It may be that seven days is not enough time for the more complex combination of species present in the CO to be degraded, or that the bacteria is ineffective for more complex compounds. Before assuming this, however, longer experimentations should be done, and also an analysis of the residual CO using gas chromatography, since in this work the result was only calculated by weight difference due to lack of time. The results of this quantitative analysis would indicate which of the numerous chemical components in CO are being degraded and the ones that are not being affected.

This ineffectiveness could also be a result of the bacteria being damaged during the lyophilisation process. This is why some vitality tests were done later on.

The fact that there is always a constant loss of contaminant at time zero is a constant throughout the experiments done in this thesis. Although the liquid-liquid extraction method used is widely employed, a high error is common when working with two highly immiscible substances like water and oil. (Ray & Engelhardt, 1992). Other studies reported recovery rates of over 60%, but in this study only around a 50% is reached. This is probably due to the lack of precision in the preparation and extraction of samples. Since this work was a preliminary study, the equipment used for the analysis at lab scale was not accurate enough for the small concentrations that are being handled. Furthermore, the low boiling point of some of the chemical components in petroleum, and C_{12} in particular (214 to 218°C), makes it probable that some material evaporates simply during the handling of samples, adding o the error. A solution to this may be weathering crude oil by distillation to remove the volatile fraction before carrying out the experiments. (Nikolopoulou, et al., 2013)

3.2.3 Lyophilised ANU5 immobilised in DABs

The next step and most relevant one to this thesis was the encapsulation of the bacteria to form Dry Alginate Beads. This technology is supposed to allow the bacteria to be stored in a convenient way, already combined with nutrients and other additives such as lipids to maximise their effectiveness on a contaminated site. Other studies (Simons, et al., 2013) have already demonstrated the

increased effectiveness of bacteria when immobilised on a carrier material with nutrients and biosurfactants. (Mulligan, 2005) The combination tested in this work derived from previous works that indicated that the addition of biosurfactants and nutrients at a certain concentrations enhanced degradation (Banat, et al., 2011). Even though the production of DABs was successful, their effectiveness was lower than expected.

The results of the tests of RDABs and SDABs on crude oil, represented in Figure 6 below clearly corroborate that the lyophilised bacteria, even when encapsulated, have little to no effect on this contaminant.

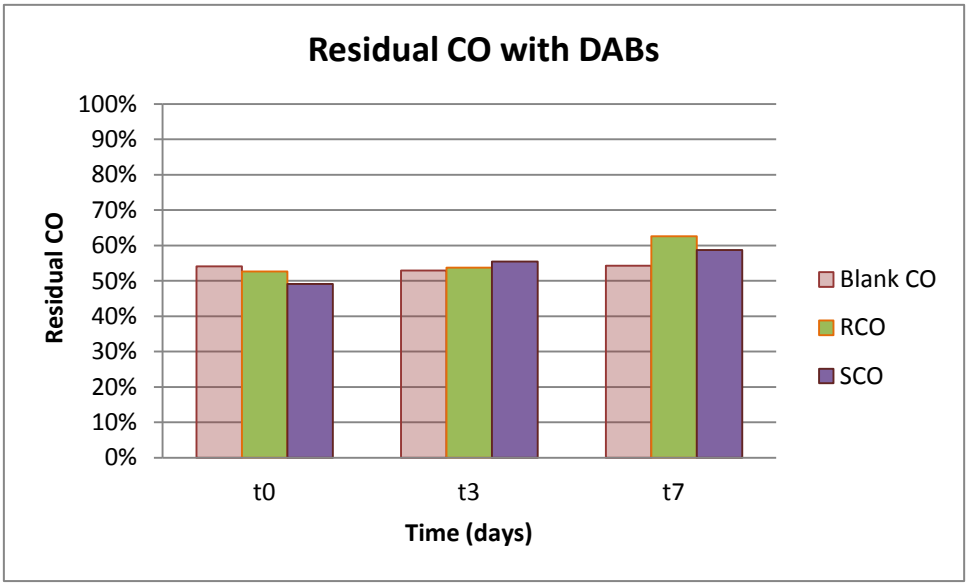


Figure 6: Residual CO with RDABs and SDABs

In the case of n-dodecane, as displayed in Figure 7 below, the RDABs worked well. At time zero the residual C_{12} was similar to the blanks but decreased progressively with time to a maximum result at 7 days of less than a 40%. On the other hand, although SDABs achieved some degradation, the results were not as good. The little degradation achieved does not increase with time; therefore one can assume rhamnolipid provides a better emulsifying effect than sophorolipid.

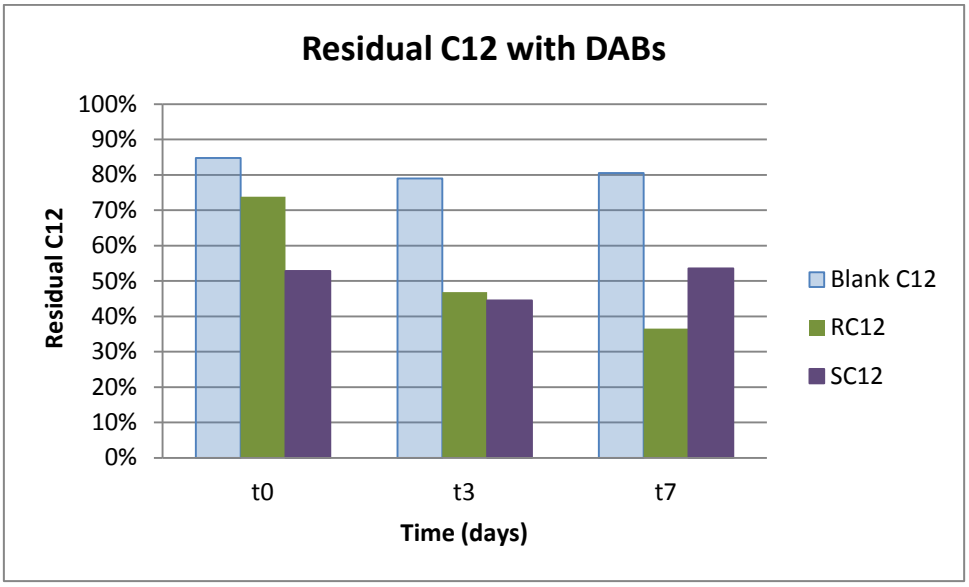


Figure 7: Residual C12 with RDABs and SDABs

The possible amount of contaminant that could have remained trapped in the DABs was also taken into account in these results. The biomass was later extracted and added to the result of the first extraction. However, the value proved to be very low and was not able to influence the overall removal results.

The complete table with the values for each analysis can be found in 4Appendix B.

The fact that the results are lower compared to those achieved with free and lyophilised cells means the immobilisation method is not working as well as it should.

This may have several causes but the most evident ones are the ones that can be conjectured from the urea release and vitality tests, discussed in the two following sections.

3.3 Urea Release

Urea, one of the two nutrients encapsulated in the DABs, is important not only for the optimisation of the cell growth but also acts as an indicator of how well the encapsulation process and the later release are working.

The results of the analysis for the urea release in the DABs are displayed in Figure 8. The blank column represents the maximum urea release in solubilised DABs containing only urea, which was of 0.006g/l. All the DABs were produced at an initial urea concentration of 1g/l, which means that in the case of UreaDABs a great part of the urea content is lost during the formation process of the DABs.

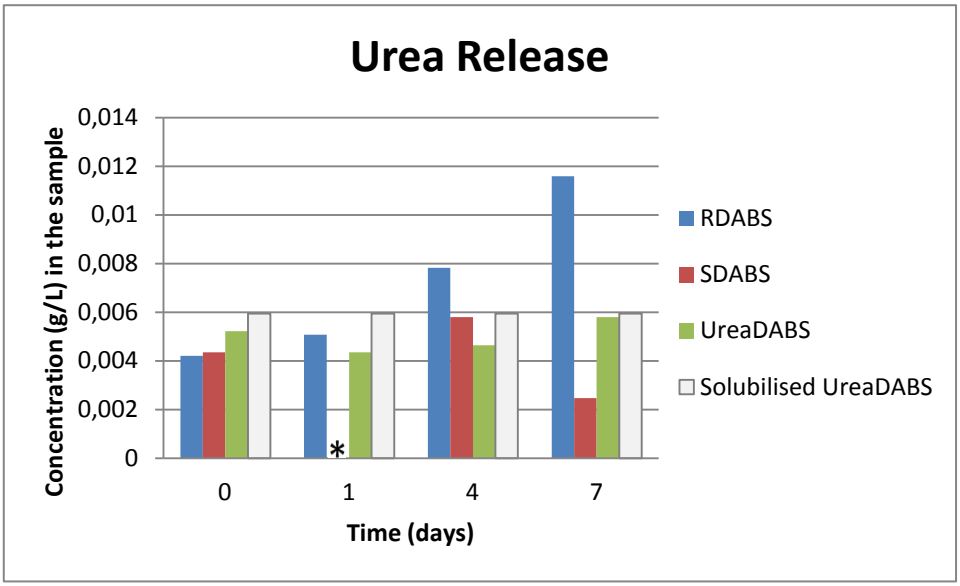


Figure 8: Urea release results for RDABs, SDABs and UreaDABs

*data not available

RDABs seem to have retained more urea during the formation process, since the blue column in Figure 8 shows an increment in release with time, to a maximum value of 0.012 g/L at day 7, higher than the maximum content released in the DABs containing only Urea. Still a large amount of the urea is lost during the immobilization process.

In the case of SDABs, the quantity of urea encapsulated is lower, and so is the quantity that is released with time. The results for this cocktail are not consistent with the increase of urea release with time, so the repetition of this analysis is recommended.

To summarise, during the encapsulation process most of the urea is lost, so the effect that the nitrogen can have as a nutrient becomes limited.

From the comparison of the results obtained from the UreaDABs to the Solubilised ones, one can see that by the incubation time of 7 days almost all of the urea is released.

The complete set of results, together with a copy of the description and methodology provided by the supplier of the equipment used can be found in 4Appendix C.

3.4 Vitality

Vitality of the cells was monitored using flow cytometry. The three graphs displayed in Figure 9: Graphs that indicate the reliability of the results obtained in the flow cytometryFigure 9 below indicate that the flow cytometry is a valid analysis for the vitality of the lyophilised bacteria. The first and third graph show that from all the bacteria being detected, a 99% is coloured by the Sybr green colorant (total count) meaning that the bacteria are detected with a high efficiency. The clear peak in the second graph indicates that the strain of *Marinobacterium hydrocarbonoclasticus* is pure, and that there is no presence of other strains.

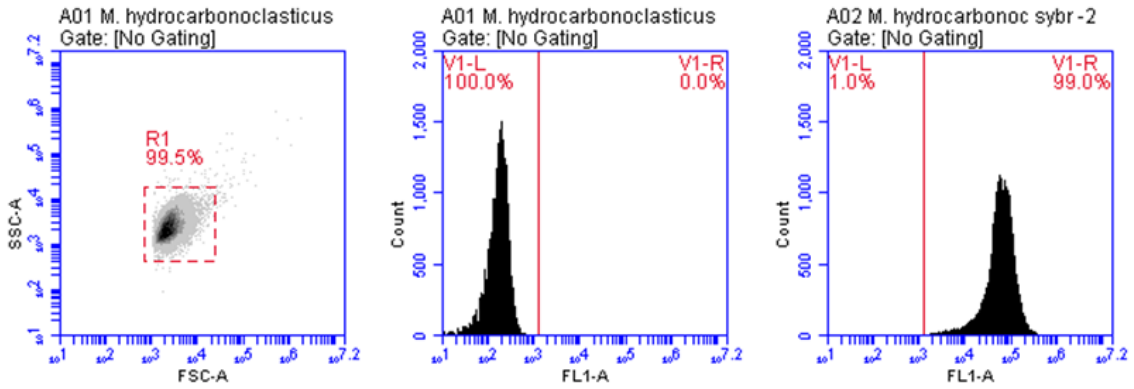


Figure 9: Graphs that indicate the reliability of the results obtained in the flow cytometry

This information gives reliability to the results obtained; therefore flow cytometry is a good system to monitor cell release for further experiments.

Three different sets of samples were analysed:

Table 3 shows the results obtained from the analysis of samples containing lyophilised cells, not encapsulated, but in an equivalent concentration to the one that was calculated to be in 200 mg of DABs.

The unit used for this process is of events per mL (ev/mL), an event meaning the detection of a cell:

Table 4: Flow cytometry results for lyophilised cells (equivalent to DABs)

	Total count (ev/mL) (SYBR Green colorant)	Dead/membrane injured cells (ev/mL) (Propidium Iodide colorant)
Lyophilised eq. to RDABs	$9x10^7$	$9x10^7$
Lyophilised eq. to SDABs	$7x10^7$	$7x10^7$

Clearly, all the cells present were either dead or damaged.

The second set of samples analysed consisted of 200 mg of each type of DAB in 20 mL of sea water (consistent with the degradation tests), to monitor both vitality and cell release in the sea water. The results can be found in table 2 below:

Table 5: Flow cytometry results for DABs

	Total count (ev/mL) (SYBR Green colorant)	Dead/membrane injured cells (ev/mL) (Propidium Iodide colorant)
RDABs T0	$2x10^5$	$3x10^4$
SDABs T0	$6x10^4$	Not detected
RDABs T24h	$4x10^6$	$3x10^5$
SDABs T24h	$3x10^6$	$1x10^5$
RDABs T48h	$6x10^6$	$2x10^5$
SDABs T48h	$5x10^6$	$6x10^5$

The total count increases with time, which means that the cells are actually being released from the DABs, but the result after 48h is one order of magnitude below the cellular content that should be in the sample. A part from this, a large quantity of the released cells still appears as dead or damaged.

The complete report for both sets of samples can be found in 4Appendix E.

Since the results obtained displayed such low levels of vitality, a third sample was prepared, and it was used to be analysed both by flow cytometry and by cellular count by plating. In this way the results from both techniques could be comparable.

Table 6 below shows the results for this sample containing 10,1g of lyophilised cells:

Table 6: Flow cytometry results of lyophilised cells

	Total count (ev/mL) (SYBR Green colorant)	Dead/membrane injured cells (ev/mL) (Propidium Iodide colorant)
Lyophilised cells	$1.4x10^8$	$1.2x10^8$

The same sample used to obtain this result was plated on to the three different growth media, the results obtained for the different dissolutions reported in Table 7 below:

Table 7: Cellular count results by plating

Dissolution	CYSP (CFU)	Marine Broth (CFU)	Onr7al (CFU)
Mother solution	431	397	359
1:10	39	37	20
1:100	3	0	0
1:1000	0	0	0
1:10000	0	0	0
1:100000	0	0	0
1:1000000	0	0	0

Using the Colony Forming Unit (CFU) one can calculate that there are $3.2*10^3$ living cells in the sample, also a very low result that corroborates the results obtained from the flow cytometry analysis.

The vitality tests that were done confirmed that the most probable cause for the lyophilised cells not working well, both when free and immobilised, was that the results indicated that most of the cells were dead. This is probably due to the lyophilisation process damaging the bacterial membrane. When plated, some still grow, meaning that probably the damage to the membrane is not fatal.

As reported in other studies, there where up to a 7% of the cells that took up Propidium Iodide and later exhibited an ability to repair. (Davey & Hexley, 2011).

3.5 Further work

The low vitality rate of the cells seems to be one of the most critical drawbacks. Therefore, further work has to be done to improve vitality while lyophilising, increasing the amount of bacteria encapsulated in the DABs or adding some growth media to enable them to grow once released. The ideal scenario would be to immobilise the cells right after the fermentation process, which would improve drastically the cell content and the vitality, but it would also slow dramatically the response time to a spill, making it not a viable option for application.

The difference between the concentration of 2g/l of cells and the double one is slightly perceptible, but more replication would be recommendable to see if it is really non critical and whether more incubation time is helpful.

More analysis with CO are also highly recommended, to find a solution to the complexity of its analysis.

4 CONCLUSIONS

The effectiveness of the hydrocarbon degrading bacteria isolated was verified, *Marinobacter hydrocarbonoclasticus was* selected as the most effective one and lyophilised to use in the other experiments.

Although on C_{12} the degradation tests continued to present promising results, on CO the process did not work, probably because of its complex composition. Further tests with more incubation time are recommended.

The analytical error during the sample extraction needs to be decreased, in both contaminants, but especially on CO where the recovery rate on blank samples was only of around a 50%

The immobilization process was successful, with better results with the DABs containing rhamnolipid biosurfactant, but there was only degradation on C_{12}.

A possible cause for the low effectiveness of DABs can be derived from the urea release analysis and the vitality tests, which showed that a relevant fraction of the urea and cellular content was lost during the immobilisation process.

The vitality tests also revealed that most cells die during the lyophilisation process, so new protocols should be developed to increase survival rate.

Additional cocktails for immobilization should also be tested to improve both encapsulation and degradation rates.

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APPENDICES

Appendix A Degradation tests of single and combined

Table A-1: Results for residual n-dodecane in single and combined strains

Ref Number	Sample	Area	Final weight of C12 (g)	Initial weight of C12 (g)	Residual C12	Average A&B
1	ANU5 t0 A	5270973	0,1055	0,1445	72,98%	t0h
2	ANU5 t0 B	4612408	0,1026	0,1464	70,07%	71,53%
3	ANU5 t3h A	4529371	0,0906	0,1471	61,61%	t3h
4	ANU5 t3h B	4507933	0,1002	0,1463	68,50%	65,06%
5	ANU5 t3g A	4124435	0,0825	0,1472	56,07%	t3d
6	ANU5 t3g B	1977160	0,0440	0,1425	30,88%	43,47%
7	ANU5 t7g A	3079434	0,0616	0,1463	42,15%	t7d
8	ANU5 t7g B	2085813	0,0464	0,1466	31,67%	36,91%
9	293 t0 A	5030651	0,1007	0,1474	68,30%	t0h
10	293 t0 B	4466466	0,0993	0,1463	67,87%	68,09%
11	293 t3h A	4872790	0,0975	0,1470	66,34%	t3h
12	293 t3h B	4616839	0,1027	0,1511	67,93%	67,13%
13	293 t3g A	4638775	0,0928	0,1424	65,21%	t3g
14	293 t3g B	3866140	0,0860	0,1462	58,81%	62,01%
15	293 t7g A	2530504	0,0507	0,1467	34,55%	t7d
16	293 t7g B	3484872	0,0775	0,1468	52,79%	43,67%
17	339 t0 A	5155800	0,1032	0,1472	70,09%	t0h
18	339 t0 B	4734883	0,1053	0,1515	69,51%	69,80%
19	339 t3h A	4958720	0,0992	0,1416	70,06%	t3h
20	339 t3h B	4305334	0,0957	0,1464	65,38%	67,72%
21	339 t3g A	3618132	0,0724	0,1462	49,53%	t3d
22	339 t3g B	4275373	0,0951	0,1465	64,88%	57,20%
23	339 t7g A	3358624	0,0672	0,1460	46,05%	t7d
24	339 t7g B	3587382	0,0798	0,1470	54,28%	50,16%
25	ANU5+293 t0 A	4873785	0,0975	0,1413	69,01%	t0h
26	ANU5+293 t0 B	4620089	0,1027	0,1499	68,55%	68,78%
27	ANU5+293 t3h A	4698700	0,0940	0,1365	68,87%	t3h
28	ANU5+293 t3h B	3694513	0,0822	0,1241	66,21%	67,54%
29	ANU5+293 t3g A	4133259	0,0827	0,1447	57,18%	t3d
30	ANU5+293 t3g B	2991344	0,0665	0,1429	46,58%	51,88%
31	ANU5+293 t7g A	3648339	0,0730	0,1434	50,93%	t7d
32	ANU5+293 t7g B	2866318	0,0638	0,1433	44,49%	47,71%
33	ANU5+339 t0 A	4937725	0,0988	0,1490	66,32%	t0h
34	ANU5+339 t0 B	4502953	0,1001	0,1475	67,89%	67,10%
35	ANU5+339 t3h A	4693447	0,0939	0,1453	64,64%	t3h
36	ANU5+339 t3g B	928137	0,0207	0,0730	28,35%	46,49%
37	ANU5+339 t7g A	4126010	0,0826	0,1445	57,16%	t3d
38	ANU5+339 t7g B	2894296	0,0644	0,1478	43,57%	50,37%
39	ANU5+339 t3g A	2815539	0,0564	0,1248	45,17%	t7d
40	ANU5+339 t3h B	4334388	0,0964	0,1183	81,50%	63,33%
41	293+339 t0 A	4408979	0,0882	0,1079	81,75%	t0h
42	293+339 t0 B	4979619	0,1107	0,1408	78,64%	80,20%
43	293+339 t3h A	4881966	0,0977	0,1463	66,78%	t3h
44	293+339 t3h B	4705663	0,1046	0,1501	69,71%	68,25%
45	293+339 t3g A	4240005	0,0849	0,1513	56,10%	t3d
46	293+339 t3g B	3876380	0,0862	0,1440	59,88%	57,99%
47	293+339 t7g A	3683144	0,0737	0,1463	50,39%	t7d
48	293+339 t7g B	3284300	0,0731	0,1464	49,89%	50,14%
49	TRP t0 A	5561259	0,1113	0,1436	77,48%	t0h
50	TRP t0 B	5046741	0,1122	0,1488	75,40%	76,44%
51	TRP t3h A	5202792	0,1041	0,1458	71,43%	t3h
52	TRP t3h B	4590545	0,1021	0,1458	70,01%	70,72%
53	TRP t3g A	3191315	0,0639	0,1460	43,76%	t3d
54	TRP t3g B	2563046	0,0570	0,1367	41,72%	42,74%
55	TRP t7g A	2707710	0,0542	0,1424	38,08%	t7d
56	TRP t7g B	3599926	0,0801	0,1467	54,56%	46,32%

A.1 Results for the blanks

Table A-2 Residual C_{12} results for the blanks

	Sample	Area	Final weight of C12 (g)	Initial weight of C12 (g)	Residual C12	Average A&B&C
C12 t0	CN t0 A	554442	0,1115	0,1453	76,73%	79,02%
	CN t0 B	560575	0,1127	0,1550	72,72%	77,92%
	CN t0 C	651825	0,1310	0,1495	87,60%	73,13%
C12 t17h	CN t17h A	528200	0,1062	0,1456	72,97%	52,00%
	CN t17h B	542638	0,1091	0,1395	78,23%	
	CN t17h C	606246	0,1218	0,1476	82,55%	
C12 t3g	CN t3 A	572566	0,1151	0,1476	77,99%	
	CN t3 B	510601	0,1027	0,1425	72,06%	
	CN t3 C	503295	0,1013	0,1461	69,33%	
C12 t7g	CN t7 A	353693	0,0713	0,1452	49,13%	
	CN t7 B	322969	0,0652	0,1462	44,58%	
	CN t7 C	452437	0,0911	0,1462	62,30%	

Appendix B Degradation tests with lyophilised ANU5 and DABs

B.1 Blanks

Table B-1 Results for the blanks on C_{12} and CO

	Sample	Area	Final weight of C12 (g)	Initial weight of C12 (g)	Residual C12	Average residual C12
C12 t0	C12 to 1	902484	0,1811	0,2019	89,70%	84,80%
	C12 to 2	813439	0,1633	0,1997	81,77%	
	C12 to 3	815654	0,1637	0,1974	82,94%	
C12 t3g	C12 t3g 1	809906	0,1626	0,1997	81,41%	80,82%
	C12 t3g 2	802389	0,1611	0,1988	81,03%	
	C12 t3g 3	786474	0,1579	0,1973	80,03%	
C12 t7g	C12 t7g 1	846648	0,1699	0,2004	84,80%	80,49%
	C12 t7g 2	807230	0,1620	0,1987	81,55%	
	C12 t7g 3	745991	0,1498	0,1994	75,12%	
	Sample		Final weight of CO (g)	Initial weight of CO (g)	Residual C12	Average residual CO
CO t0	CO to 1		0,0424	0,0831	51,02%	54,11%
	CO to 2		0,0368	0,0846	43,50%	
	CO to 3		0,0649	0,0957	67,82%	
CO t3g	CO t3 1		0,0489	0,0948	51,58%	52,87%
	CO t3 2		0,0436	0,0852	51,17%	
	CO t3 3		0,0524	0,0938	55,86%	
CO t7g	CO t7 1		0,0405	0,0772	52,46%	54,31%
	CO t7 2		0,0497	0,0860	57,79%	
	CO t7 3		0,0480	0,0911	52,69%	

B.2 Lyophilised ANU5

Table B-2 Results for the degradation tests on C_{12}

Sample	Sea Water (ml)	Cells (g)	C_{12} 1% (g)	Area	Weight	Residual C12
1 C12 t0	20	0,0409	0,2131	5448111	0,10902222	51,16%
1 C12 t3	20	0,0411	0,2086	3461165	0,0692833	33,21%
1 C12 t7	20	0,0417	0,2112	3083130	0,0617226	29,22%
3 C12 t0	20	0,0815	0,2162	620051	0,1246102	57,64%
3 C12 t3	20	0,0827	0,2132	3886994	0,07779988	36,49%
3 C12 t7	20	0,0807	0,2141	1062748	0,02131496	9,96%

Table B-3 Results for the degradation tests on CO

Sample	Sea Water (ml)	Cells (g)	Crude Oil 0.5% (g)	Initial Weight (g)	Total Weight (g)	Weight (g)	Residual CO
2 CO t0	20	0,0419	0,0872	23,7574	23,8187	0,0613	70,30%
2 CO t3	20	0,0426	0,0847	22,6337	22,6787	0,045	53,13%
2 CO t7	20	0,0428	0,0857	26,8318	26,8748	0,043	50,18%
4 CO t0	20	0,0803	0,0834	21,513	21,5505	0,0375	44,96%
4 CO t3	20	0,0816	0,0839	23,046	23,0808	0,0348	41,48%
4 CO t7	20	0,0811	0,0895	28,7046	28,7526	0,048	53,63%

B.3 Encapsulated ANU5 in DABs

Table B-4 Results for the degradation tests with DABs on C_{12}

C12	SW (ml)	C12 (g)	DAB's (g)	Area	Dilution	C12 (g)	weight from solved DAB's	% residuo
RC12t0	20	0,2047	0,2062	752682	1:10000	0,1511364	0	73,83%
RC12t3	20	0,2035	0,2057	469662	1:10000	0,0945324	0,00075	46,82%
RC12t7	20	0,2122	0,2082	375432	1:10000	0,0756864	0,0018	36,52%
SC12t0	20	0,2065	0,2065	542583	1:10000	0,1091166	0	52,84%
SC12t3	20	0,2117	0,2072	467741	1:10000	0,0941482	0,000006	44,48%
SC12t7	20	0,2083	0,202	554936	1:10000	0,1115872	0,000008	53,57%

Table B-5 Results for the degradation tests with DABs on CO

CO	SW (ml)	CO (g)	DAB's (g)	itial Weig	Final Weight	CO (g)	weight from solved DAB's	% residuo
RCOt0	20	0,0889	0,208	23,5165	23,5633	0,0468	0	52,64%
RCOt3	20	0,0897	0,2079	28,7356	28,7838	0,0482	0	53,73%
RCOt7	20	0,0885	0,2047	22,2442	22,2987	0,0545	0,0009	62,60%
SCOt0	20	0,0871	0,2075	26,6424	26,6852	0,0428	0	49,14%
SCOt3	20	0,091	0,2045	17,3079	17,3579	0,05	0,0005	55,49%
SCOt7	20	0,0905	0,2062	23,6104	23,6593	0,0489	0,0042	58,67%

Appendix C Urea release tests

C.1 UV method for the determination of urea

Urea/Ammonia

UV-method

for the determination of urea and ammonia in foodstuffs and other materials and for the determination of nitrogen after Kjeldahl-digestion (see pt. 12.2)

Cat. No. 10 542 946 035

Test-Combination for approx. 25 determinations each

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

For *in vitro* use only

Store at 2-8° C

For recommendations for methods and standardized procedures see ref. (2)

Principle (Ref. 1)

Urea is hydrolyzed to ammonia and carbon dioxide in the presence of the enzyme urease (1).

$$(1)\ \text{Urea} + H_2O \xrightarrow{\text{urease}} 2\,NH_3 + CO_2$$

In the presence of glutamate dehydrogenase (GlDH) and reduced nicotinamide-adenine dinucleotide (NADH), ammonia reacts with 2-oxoglutarate to L-glutamate, whereby NADH is oxidized (2).

$$(2)\ 2\text{-Oxoglutarate} + NADH + NH_4^+ \xrightarrow{GlDH} \text{L-glutamate} + NAD^+ + H_2O$$

The amount of NADH oxidized in the above reaction is stoichiometric to the amount of ammonia or with half the amount of urea, respectively. NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 60 ml solution, consisting of: triethanolamine buffer, pH approx. 8.0; 2-oxoglutarate, approx. 220 mg
2. Bottle 2 with approx. 50 tablets; each tablet contains: NADH, approx. 0.4 mg
3. Bottle 3 with approx. 0.7 ml urease solution, approx. 80 U
4. Bottle 4 with approx. 1.2 ml glutamate dehydrogenase solution, approx. 1000 U

Preparation of solutions

1. Use contents of bottle 1 undiluted.
2. Dissolve **one tablet** of bottle 2 with **one ml** solution of bottle 1 in a beaker or in a reagent tube for each assay (blank and samples) depending on the number of determinations. Use forceps for taking the tablets out of bottle 2. This results in reaction mixture 2*.
3. Use contents of bottle 3 undiluted.
4. Use contents of bottle 4 undiluted.

Stability of reagents

Solution 1 is stable at 2-8°C (see pack label).
Bring solution 1 to 20-25°C before use.
Tablets 2 are stable at 2-8° C (see pack label).
Reaction mixture 2 is stable for 3 days at 2-8°C.
Bring reaction mixture 2 to 20-25°C before use.
The contents of bottle 3 and 4 are stable at 2-8°C (see pack label).

Procedure

Wave length[1]: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.040 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 0.3-14 µg urea/assay[3] or 0.2-8 µg ammonia/assay[3] (in 0.100-2.000 ml sample volume)

Pipette into cuvettes	Blank urea	Urea sample	Blank ammonia	Ammonia sample
reaction mixture 2*	1.000 ml	1.000 ml	1.000 ml	1.000 ml
sample solution**	-	0.100 ml	-	0.100 ml
solution 3	0.020 ml	0.020 ml	-	-
redist. water	2.000 ml	1.900 ml	2.020 ml	1.920 ml
Mix***, and read absorbances of the solutions (A_1) after approx. 5 min at 20-25°C. Start reaction by addition of:				
solution 4	0.020 ml	0.020 ml	0.020 ml	0.020 ml
Mix***, wait for completion of the reaction (approx. 20 min) and read absorbances of the solutions (A_2). If the reaction has not stopped after 20 min, read absorbances in 2 min intervals until the absorbances decrease constantly over 2 min.				

If the absorbance A_2 decreases constantly, extrapolate the absorbance to the time of the addition of solution 4 (GlDH).

Determine the absorbance differences (A_1-A_2) for both, blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample.

$$\Delta A = (A_1\text{-}A_2)_{sample} - (A_1\text{-}A_2)_{blank}$$

This results in $\Delta A_{\text{urea + ammonia}}$ (from urea sample) and $\Delta A_{ammonia}$ (from ammonia sample).

The difference of these values results in ΔA_{urea}.

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt. 4).

If the absorbance differences of the samples (ΔA_{sample}) are higher than 1.000 (measured at 340 nm or Hg 334 nm respectively) or 0.500 (measured at 365 nm), the concentration of urea (or ammonia) in the sample solution is too high. The sample is to be diluted according to the dilution table in that case.

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A\ [g/l]$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = extinction coefficient of NADH at:
340 nm = 6.3 [l × mmol^{-1} × cm^{-1}]
Hg 365 nm = 3.4 [l × mmol^{-1} × cm^{-1}]
Hg 334 nm = 6.18 [l × mmol^{-1} × cm^{-1}]

It follows for urea:

$$c = \frac{3.040 \times 60.06}{\varepsilon \times 1.00 \times 0.100 \times 2 \times 1000} \times \Delta A_{urea} = \frac{0.9129}{\varepsilon} \times \Delta A_{urea} [\text{g urea/l sample solution}]$$

for ammonia:

$$c = \frac{3.040 \times 17.03}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{ammonia} = \frac{0.5177}{\varepsilon} \times \Delta A_{ammonia} [\text{g ammonia/l sample solution}]$$

If the sample has been diluted on preparation, the result must be multiplied by the dilution factor F.

1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 See instructions for performance of assay

* For simplification of the assay performance it is also possible to pipette directly 1.000 ml of solution 1 into the cuvette and add 1 tablet from bottle 2. After dissolution of the tablet with the aid of a spatula continue working as described in the procedure. The difference in volume of approx. 1% (increase of volume by 1 tablet per 3.040 ml assay volume) has to be taken into account in the calculation by multiplication of the result with 1.01.

** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

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When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{Content}_{\text{urea}} = \frac{c_{\text{urea}}\ [\text{g/l sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 100\ [\text{g/100 g}]$$

$$\text{Content}_{\text{ammonia}} = \frac{c_{\text{ammonia}}\ [\text{g/l sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 100\ [\text{g/100 g}]$$

1. Instructions for performance of assay

The amount of urea (ammonia) present in the assay has to be between 0.3 µg and 14 µg (0.2 µg and 8 µg). In order to get a sufficient absorbance difference, the sample solution is diluted to yield an urea (ammonia) concentration between 0.02 and 0.14 g/l (0.01 and 0.08 g/l).

Dilution table

Estimated amount of urea (ammonia) per liter	Dilution with water	Dilution factor F
< 0.14 g (< 0.08 g)	-	1
0.14–1.4 g (0.08–0.8 g)	1 + 9	10
1.4–14 g (0.8–8.0 g)	1 + 99	100

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml. The volume of water added must then be reduced to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

2. Technical information

2.1 Use only freshly distilled water for the assay.

2.2 Work in an atmosphere free from ammonia (ban smoking in the laboratory).

3. Specificity (Ref. 1)

The method is specific for urea and ammonia.

In the analysis of commercial urea and ammonium sulfate results of approx. 100% have to be expected.

4. Sensitivity and detection limit (Ref. 1.4)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 2.000 ml and measurement at 340 of an ammonia concentration of 0.02 mg/l sample solution, resp. of an urea concentration of 0.04 mg/l (if v = 0.100 ml, this corresponds to 0.4 mg ammonia/l, resp. 0.8 mg urea/l sample solution).

The detection limit of 0.08 mg ammonia/l, resp. 0.15 mg urea/l is derived from the absorbance difference of 0.020 (as measured at 340 nm) and a maximum sample volume v = 2.000 ml.

5. Linearity

Linearity of the determination exists from approx. 0.2 µg ammonia/assay (0.08 mg ammonia/l sample solution; sample volume v = 2.000 ml) to 8 µg ammonia/assay (0.08 g ammonia/l sample solution; sample volume v = 0.100 ml), resp. from 0.3 µg urea/assay (0.15 mg urea/l sample solution; sample volume v = 2.000 ml) to 14 µg urea/ assay (0.14 g urea/l sample solution; sample volume v = 0.100 ml).

6. Precision

Ammonia:

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to an ammonia concentration of approx. 0.4–1 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.004–0.01 g/100 g can be expected.)

The following data for the determination of ammonia have been published in the literature:

CV = 1.6 %	(plasma)	(Ref. 1.2)
CV = 0.88–1.16 %	(ammonium chloride solutions)	(Ref. 1.4)
CV = 0.34 %	(ammonium chloride solutions)	
CV = 0.36–0.96 %	(meat samples)	(Ref. 3.2)

Urea:

In a double determination using one sample solution, a difference of 0.005 to 0.015 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to an urea concentration of approx. 0.7–2 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.007 - 0.02 g/100 g can be expected.)

The following data for the determination of urea have been published in the literature:

CV = 2.7 %	(serum)	(Ref. 1.1)
CV = 3 %	(serum)	(Ref. 1.3)

Analysis of swimming-pool water: (Ref. 3.7)

x = 0.611 mg/l	r = 0.1854 mg/l	$s_{(r)} = \pm 0.066$ mg/l
	R = 0.2145 mg/l	$s_{(R)} = \pm 0.076$ mg/l
x = 2.323 mg/l	r = 0.1247 mg/l	$s_{(r)} = \pm 0.044$ mg/l
	R = 0.1883 mg/l	$s_{(R)} = \pm 0.067$ mg/l
x = 5.749 mg/l	r = 0.0707 mg/l	$s_{(r)} = \pm 0.025$ mg/l
	R = 0.1707 mg/l	$s_{(R)} = \pm 0.060$ mg/l

7. Interference/sources of error

During protein precipitation with perchloric acid which is to be carried out in foodstuffs, protein fragments are occasionally obtained. These protein fragments are kept in solution and may gradually form ammonia in alkaline buffer systems leading to creep reactions. This formation of ammonia is very low and can be differentiated and calculated from the ammonia content of the sample by extrapolation of the absorbance A_2 to the time of addition of solution 4 (GIDH).

The common ingredients of foodstuffs do not interfere with the assay of urea and ammonia. Only high concentrations of tannins in fruit juices may cause an inhibiton of the GIDH reaction. Fruit juices should therefore always be treated with PVPP.

As high concentrations of heavy metals cause turbidity, they make a reliable determination of ammonia difficult. In most cases high concentrations of metal ions can be removed as hydroxides by alkalization of the sample solution (pH > 7.5).

Sodium thiosulfate, occasionally added to samples of swimming-pool water, does not interfere with the assay up to 1 mg per assay.

8. Recognizing interference during the assay procedure

8.1 If the conversion of urea and ammonia has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

8.2 On completion of the reaction, the determination can be restarted by adding urea and/or ammonium chloride or ammonium sulfate (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard

The reagents used in the determination of urea and ammonia are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

10. General information on sample preparation

In carrying out the assay:
Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml;
Filter **turbid solutions**;
Degas **samples containing carbon dioxide** (e.g. by filtration);
Adjust **acid samples** to pH 7-8 by adding sodium or potassium hydroxide solution;
Adjust **acid and weakly colored samples** to approx pH 7-8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;
Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with polyvinylpolypyrrolidone (PVPP) - (e.g. 2.5-5 g/100 ml);
Crush or homogenize **solid or semi-solid samples,** extract with water or dissolve in water and filter if necessary;
Deproteinize **samples containing protein** with perchloric acid or with trichloroacetic acid;
Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter;
Break up **emulsions** with trichloroacetic acid.

Important note

The Carrez-clarification should not be used in the sample preparation for urea / ammonia determination due to a too low recovery rate (adsorption of urea/ammonia).

11. Application examples

Determination of ammonia in fruit juices
Add 0.5-1.0 g wet polyvinylpolypyrrolidone (PVPP) to 10 ml fruit juice (clear, turbid or colored juices) - when the sample volume is increased, neutralize, if necessary, and fill up to 20 ml with water - in a beaker and stir for 1 min (magnetic stirrer). Filter sample solution immediately and use it for the assay. In the assay, only "blank ammonia" and "sample ammonia" are to be measured.

Determination of urea and ammonia in water (swimming-pool water)
Dilute sample solution according to the dilution table or use up to v = 2.000 ml sample volume for the assay.

Determination of urea in milk
Mix 1 ml milk with 4 ml trichloroacetic acid (0.3 M). After approx. 5 min centrifuge for separation of the precipitate (for 3 min, ca. 4000 rpm). Use 0.100 ml of the supernatant clear solution for the assay.

Determination of ammonia in milk
Mix 1 ml milk with 4 ml trichloroacetic acid (0.3 M). After approx. 5 min centrifuge for separation of the precipitate. Decant the supernatant and neutralize with KOH (10 M) (dilution factor can be neglected due to the high concentration of KOH), filter and use 1.000-2.000 ml sample solution for the assay.
In the assay, only "blank ammonia" and "sample ammonia" are to be measured.

Determination of ammonia in bakery products
Accurately weigh approx. 10 g of the minced sample into a homogenizer beaker, add approx. 20 ml perchloric acid (1 M) and homogenize for approx. 2 min. Proceed as stated under "meat and meat products". Use at most 1.000 ml for the assay.
In the assay, only "blank ammonia" and "sample ammonia" are to be measured.

Determination of urea and ammonia in meat and meat products
Accurately weigh approx. 5 g of the homogenized sample (from a sample of 100 g, that has been ground and homogeneously mixed in a mixer) into a homogenizer beaker, add approx. 20 ml perchloric acid (1 M) and homogenize for approx. 2 min. Transfer the contents quantitatively with approx. 40 ml water into a beaker. Adjust to pH 7.0 (< 7.5) first with potassium hydroxide (5 M) and then exactly with potassium hydroxide (2 M). Transfer the contents quantitatively with water into a 100 ml volumetric flask, fill up to the mark with water, whereby it must be taken care that the fatty layer is above the mark and the aqueous layer is at the mark.

For separation of fat and for precipitation of the potassium perchlorate refrigerate for 20 min. Afterwards filter. Discard the first few ml. Use the clear, possibly slightly turbid solution for the assay.

Calculate of the amount of urea and ammonia according to the aforementioned calculation formula, whereby it must be multiplied with the volume displacement factor K = 0.98.

12. Further applications

The method may also be used in the examination of fertilizers, pharmaceuticals, cosmetics, paper (Ref. 2.1) and in research when analyzing biological samples. For details of sampling, treatment and stability of the sample see Ref. 1.1-1.4.

Examples:

12.1 Determination of urea and ammonia in fertilizers

Grind approx. 10 g of the sample and mix thoroughly. Accurately weigh approx. 100 mg of the homogeneous material into a 100 ml beaker and add approx. 50 to 60 ml water. Adjust to pH 7-8 with diluted hydrochloric acid (1 M) or in the case of acidic fertilizer with diluted sodium hydroxide (1 M). Warm on a heatable magnetic stirrer for approx. 10 min to 60-70°C. Allow to cool, transfer quantitatively into a 100 ml volumetric flask and fill up to the mark with water. Mix the solution and filter, if necessary.
Use 0.100 ml of the clear solution diluted, if necessary, for the assay.

12.2 Determination of nitrogen after Kjeldahl-digestion

The determination of total nitrogen can be obtained via the ammonia determination in a sample mineralized according to the Kjeldahl-method. Normally, the samples are to be incinerated wet (sulfuric acid). The ammonia, formed from nitrogen, is determined according to the procedure as follows.

Accurately weigh approx. 2 g of the ground and homogenized sample into a 100 ml Kjeldahl-flask, add 20 ml sulfuric acid (specific gravity = 1.84 g/ml) and approx. 30 mg catalyst mixture (e.g., acc. to Wieninger) or one Kjeldahl tablet, heat for approx. 2-3 h until the sample is disintegrated (yellowish or blue-greenish solution). Allow the sample to cool and carefully (protective glasses) transfer quantitatively into a beaker filled with 600 ml ice-cold water, while stirring all the time (magnetic stirrer, icebath). Neutralize with approx. 60 ml KOH (10 M) (pH 6-8). Transfer the neutralized solution quantitatively into a 1 l volumetric flask, fill up to the mark with water and mix. If necessary, filter the mixture (sometimes necessary after disintegration with Kjeldahl tablets); discard the first few ml. Use the solution diluted, if necessary, for the assay.

Calculation:
Nitrogen content of the sample (in %)

$$= \frac{\Delta A \times V \times MW \times 100}{\varepsilon \times d \times v \times 1000 \times \text{amount weighed [g]}} =$$

$$= \frac{\Delta A \times 3.04 \times 14.01 \times 100}{\varepsilon \times 1.00 \times 0.100 \times 1000 \times \text{amount weighed [g]}}$$

12.3 Determination of urea and ammonia in fermentation samples and cell culture media

Place the sample (after centrifugation, if necessary) in a waterbath at 80°C for 15 min to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization can be carried out with perchloric acid. See the above-mentioned examples.

Homogenize gelatinous agar media with water and treat further as described.

C.2 Urea Release results

Table C-1: Urea release test results

		A1.1	A2.1	A2.2	A2.3	Increment	C(g/L) sample solution
t0	R-DABS	0,013	-0,016			0,029	0,00420
	S-DABS	0,013	-0,017			0,03	0,00435
	DABS-U	0,02	-0,016			0,036	0,00522

	(27h)	A1.1	A2.1	A2.2	A2.3	Increment	C(g/L) sample solution
t1	R-DABS	-0,096	-0,13	-0,128	-0,131	0,035	0,00507
	S-DABS	-0,092	-0,217	-0,221	-0,224	0,132	0,00000
	DABS-U	-0,1	-0,13	-0,125	-0,13	0,03	0,00435

	(96h)	A1.1	A2.2	A2.3	A2.3	Increment	C(g/L) sample solution
t4	R-DABS	0,01	-0,044			0,054	0,00782
	S-DABS	0,01	-0,03			0,04	0,00580
	DABS-U	0,012	-0,02			0,032	0,00464

		A1.1	A2.1	A2.2	A2.3	Increment	C(g/L) sample solution
t7	R-DABS	0,003	-0,077	-0,073	-0,077	0,08	0,01159
	S-DABS	0,063	0,046	0,051	0,046	0,017	0,00246
	DABS-U	0,034	-0,006	-0,003	-0,004	0,04	0,00580

		A1.1	A2.1	A2.2	A2.3	Increment	C(g/L) sample solution
solub. DABs	DABS-U 0,2M	0,013	-0,023			0,036	0,00522
	DABS-U 0,5M	0,054	0,013			0,041	0,00594
	STANDARD 0,1gr/L	-0,025	-0,68			0,655	0,09491

	pH t0	pH t4g
R-DABS	7,04	6,39
S-DABS	7,08	6,73
DABS-U	7,64	7,89

Appendix D Growth media preparation

In the vitality experiments, the sample was plated into 3 different growth media. They were all brought to volume with redistilled water and autoclaved:

D.1 CYSP medium

Casein hydrolysate	15 g/L
Yeast	5 g/L
Soytone	3 g/L
Peptone	2 g/L
NaCl	19.4 g/L
$MgCl_2$	8.8 g/L
Na_2SO_4	3.24 g/L
$CaCl_2$	1.8 g/L
$MgSO_4$	15 mg/L
$FeCl_3$	115 mg/L
$MnCl_2$	20 mg /L
Pyruvate	10 mg/mL

D.2 Marine Broth medium

Marine broth 2216	40.2 g/L
Bacto-Agar	15 g/L

D.3 ONR7a medium

Solution 1, to a final volume of 600 mL:

NaCl	22.79 g/L
Na_2SO_4	3.98 g/L
KCl	0.72 g/L
NaBr	0.083 g/L
$NaHCO_3$	0.031 g/L
H_3BO_3	0.027 g/L
NaF	0.0026 g/L
NH_4Cl	0.27 g/L
Na_2HPO_4	0.089 g/L
TAPSO	1.3 g/L

Bacto-agar	15 g/L

Solution 2, to a final volume of 300 mL:

$MgCl_2$	11.18 g/L
$CaCl_2$	1.46 g/L
$SrCl_2$	0.024 g/L

Solution 3, to a final volume of 100 mL:

$FeCl_2$	0.002 g/L

The 3 solutions were autoclaved separately, cooled and mixed. Sodium acetate at a 1% was added as a carbon source.

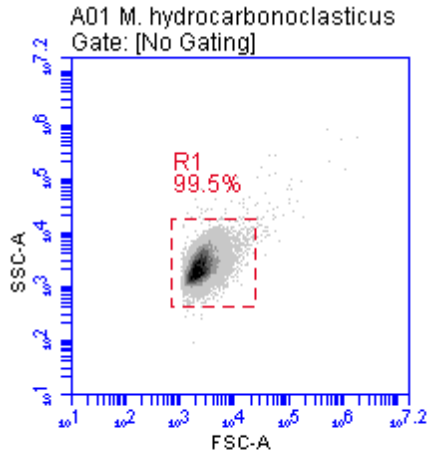
Appendix E Flow cytometry report

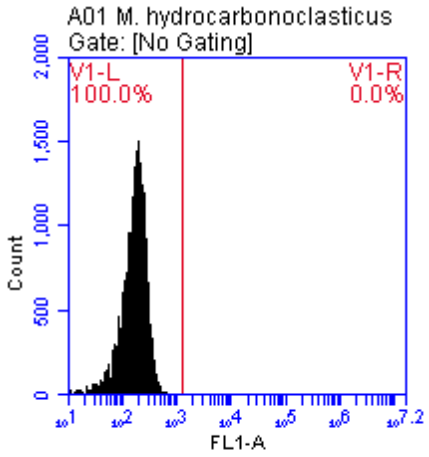
Monitoring of ANU5 cells release in sea water by flow cytometry

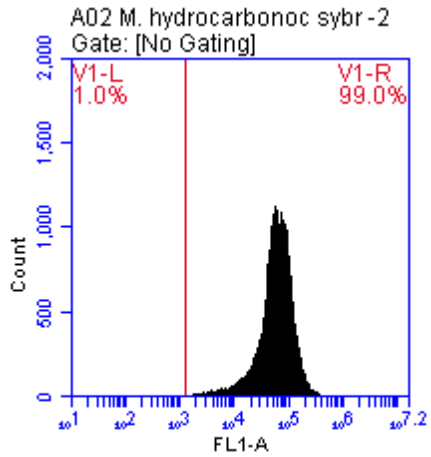
Total cell count with SYBR Green (37C for 10')

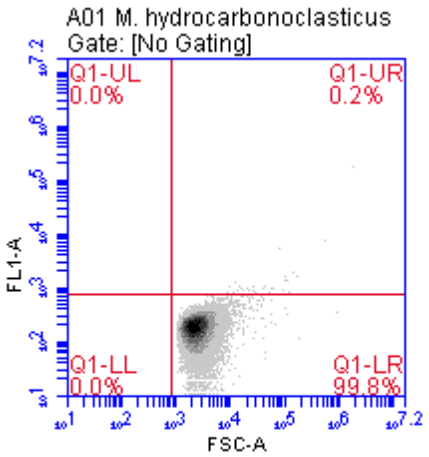
Dead cells or with injured membrane: Propidium Iodide (PI, 5 µg/ml, 37C for 10')

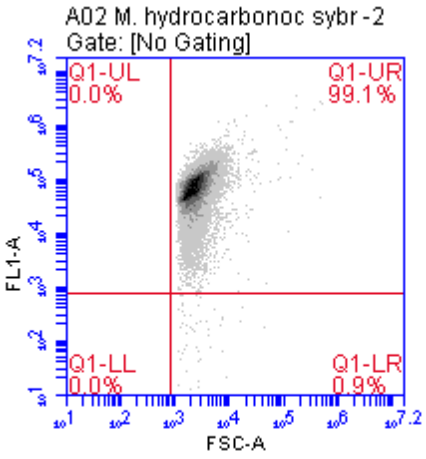
Marinobacterium hydrocarbonoclasticus







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Q1-UR
99.1%
Q1-LL
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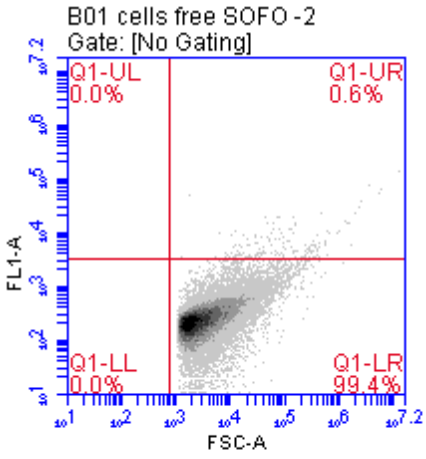
Q1-LR
0.9%
FL1-A
FSC-A


B01 cells free SOFO -2
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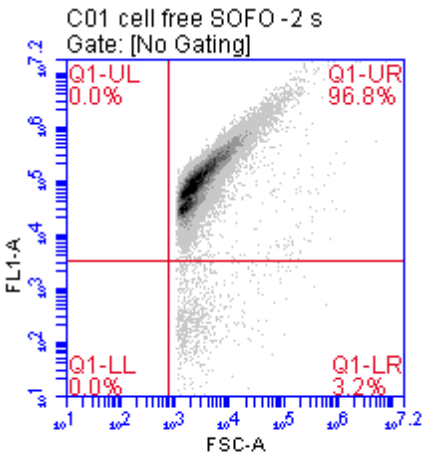
99.4%
FL1-A
FSC-A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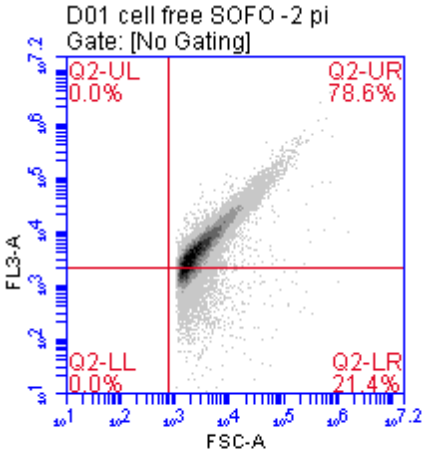
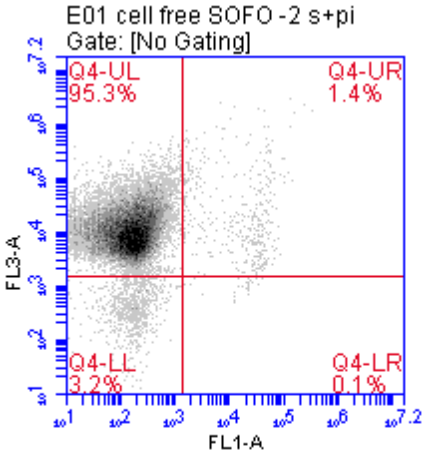
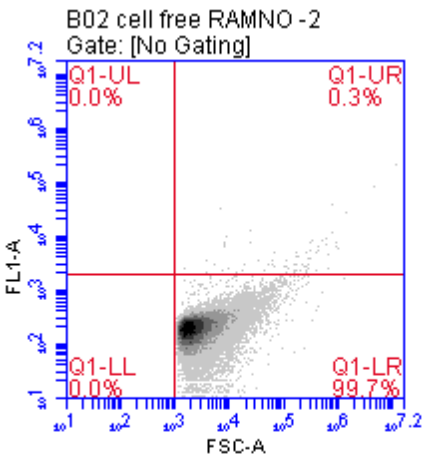
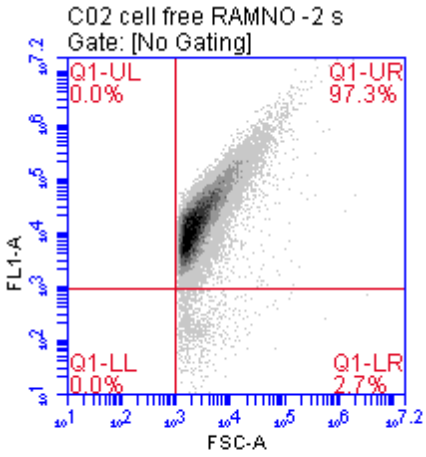
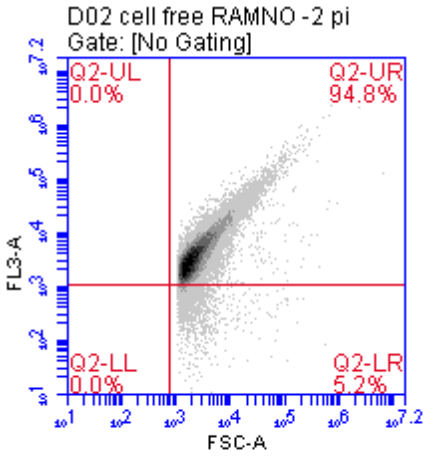
Gate: [No Gating]
Q1-UL
0.0%
Q1-UR
96.8%
Q1-LL
0.0%
Q1-LR
3.2%
FL1-A
FSC-A

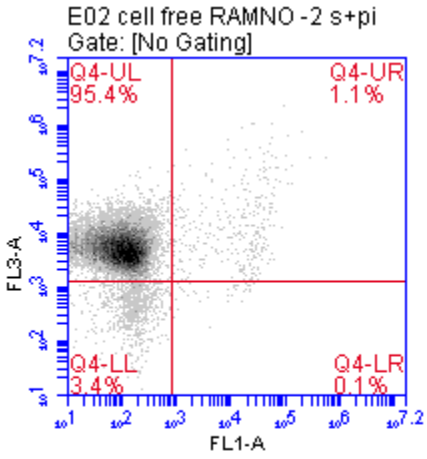

D01 cell free SOFO -2 pi
Gate: [No Gating]
Q2-UL
0.0%
Q2-UR
78.6%
Q2-LL
0.0%
Q2-LR
21.4%
FL3-A
FSC-A

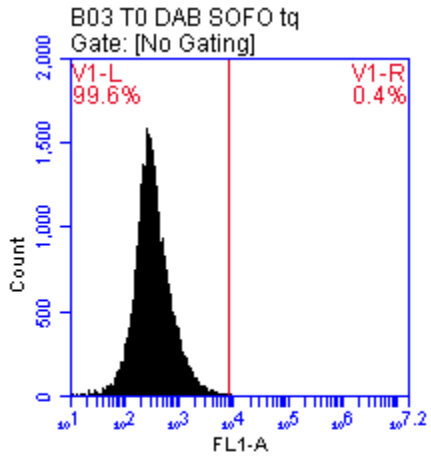

E01 cell free SOFO -2 s+pi
Gate: [No Gating]
Q4-UL
95.3%
Q4-UR
1.4%
Q4-LL
3.2%
Q4-LR
0.1%
FL3-A
FL1-A

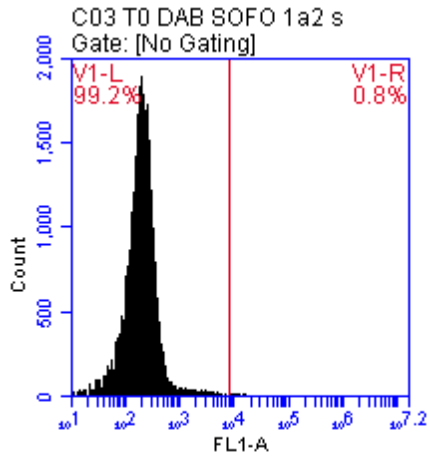

B02 cell free RAMNO -2
Gate: [No Gating]
Q1-UL
0.0%
Q1-UR
0.3%
Q1-LL
0.0%
Q1-LR
99.7%
FL1-A
FSC-A

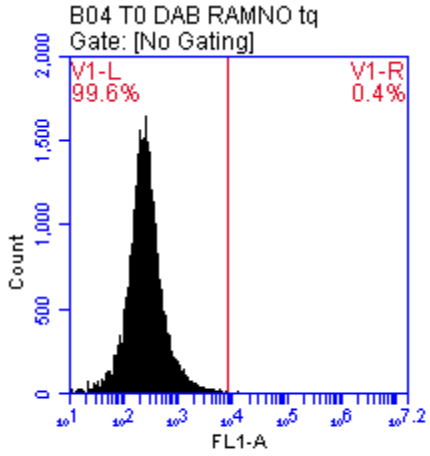

C02 cell free RAMNO -2 s
Gate: [No Gating]
Q1-UL
0.0%
Q1-UR
97.3%
Q1-LL
0.0%
Q1-LR
2.7%
FL1-A
FSC-A

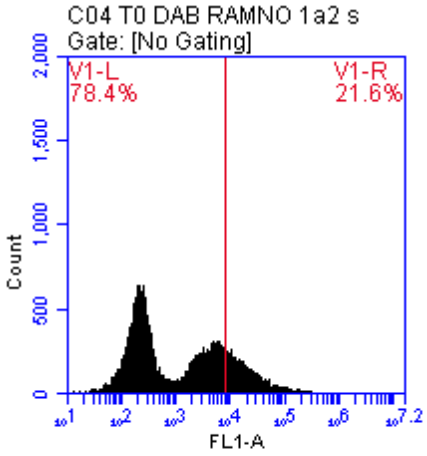

D02 cell free RAMNO -2 pi
Gate: [No Gating]
Q2-UL
0.0%
Q2-UR
94.8%
Q2-LL
0.0%
Q2-LR
5.2%
FL3-A
FSC-A

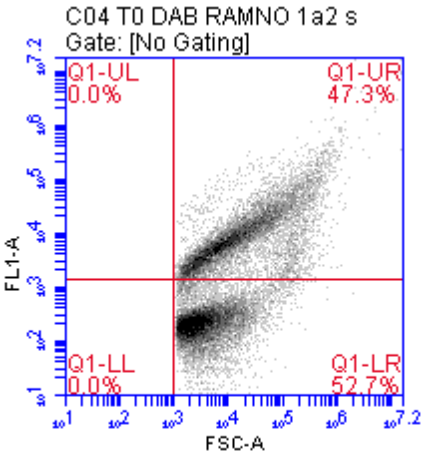

E02 cell free RAMNO -2 s+pi
Gate: [No Gating]
Q4-UL
95.4%
Q4-UR
1.1%
Q4-LL
3.4%
Q4-LR
0.1%
FL3-A
FL1-A


B03 T0 DAB SOFO tq
Gate: [No Gating]
V1-L
99.6%
V1-R
0.4%
Count
FL1-A


C03 T0 DAB SOFO 1a2 s
Gate: [No Gating]
V1-L
99.2%
V1-R
0.8%
Count
FL1-A







	Total count (SYBR Green) (ev/ml)	Dead or membrane injured cells (PI) (ev/ml)
Free cells SOFO	$7x10^7$	$7x10^7$ (treatment?)
Free cells RAMNO	$9x10^7$	$9x10^7$
T0 DAB SOFO	$6x10^4$	nd
T0 DAB RAMNO	$2x10^5$	$3x10^4$

Comments:

Marinobacterium hydrocarbonoclasticus cells could be stained with high efficiency (≥ 99%, see A01 and A02) even in sea water (C01, D01, E01 in sea water + SOFO; C02, D02, E02 in sea water + RAMNO). The flow cytometric approach could be useful for the monitoring of cells release.

The cells used for the assembling of are stained with PI, dead or just membrane injured cells? Reversible condition?

Flow cyto + viable count

<u>After 24 h incubation</u>

	Total count (SYBR Green) (ev/ml)	Dead or membrane injured cells (PI) (ev/ml)
T0 DAB SOFO	$3x10^6$	$1x10^5$
T0 DAB RAMNO	$4x10^6$	$3x10^5$

Comments:

At T0 Higher release of cells in presence of RAMNO than SOFO (? Ramno affects the alginate beads assembling?), but after 24 h the cells release seems the same in the 2 conditions.

<u>After 48 h incubation</u>

	Total count (SYBR Green) (ev/ml)	Dead or membrane injured cells (PI) (ev/ml)
T0 DAB SOFO	$5x10^6$	$6x10^5$
T0 DAB RAMNO	$6x10^6$	$2x10^5$