

## **Optimization of a peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) method for the detection of bacteria and disclosure of a formamide effect**

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### **Abstract**

Despite the fact that fluorescence *in situ* hybridization (FISH) is a well-established technique to identify microorganisms, there is a lack of understanding concerning the interaction of the different factors affecting the obtained fluorescence. In here, we used flow cytometry to study the influence of three essential factors in hybridization – temperature, time and formamide concentration – in an effort to optimize the performance of a Peptide Nucleic Acid (PNA) probe targeting bacteria (EUB338). The PNA-FISH optimization was performed with bacteria representing different families employing response surface methodology. Surprisingly, the optimum concentration of formamide varied according to the bacterium tested. While hybridization on the bacteria possessing the thickest peptidoglycan was more successful at nearly 50% (v/v) formamide, hybridization on all other microorganisms appeared to improve with much lower formamide concentrations. Gram staining and transmission electron microscopy allowed us to confirm that the overall effect of formamide concentration on the fluorescence intensity is a balance between a harmful effect on the bacterial cell envelope, affecting cellular integrity, and the beneficial denaturant effect in the hybridization process. We also conclude that microorganisms belonging to different families will require different hybridization parameters for the same FISH probe, meaning that an optimum universal PNA-FISH procedure is non-existent for these situations.

## 1. Introduction

Fluorescent *in situ* hybridization (FISH) in combination with fluorescence measurement by flow cytometry (flow-FISH) is a well- established technique to detect different bacteria in environmental, clinical or food samples (Cerqueira et al., 2008). Its application ranges from multiplex experiments, frequent in microbial ecology (Almeida et al., 2011; Amann et al., 1990a), to single based organism experiments, particularly useful in clinical diagnosis (Barken et al., 2007; Fontenete et al., 2013; Guimaraes et al., 2007). FISH is based on the hybridization of an oligonucleotide probe to a particular bacterial sequence, typically part of the 16S rRNA, obeying to Watson-Crick hydrogen-bonding (Cerqueira et al., 2008).

There is a considerable variability between FISH procedures described in the literature. The implementation of a FISH methodology usually requires an initial optimization to adjust the hybridization stringency (Herzer and Englert, 2001) which is currently performed as a trial and error procedure. In order to better understand the hybridization efficiency of nucleic acids in bacteria, a systematic approach is lacking.

The efficiency of the FISH hybridization is affected by a wide variety of variables and their interplay (Bouvier and DelGiorgio, 2003). Hybridization time and temperature are crucial variables on the hybridization outcome and formamide is commonly used with the purpose of lowering the temperature at which the hybridization is performed (Blake and Delcourt, 1996; Yilmaz et al., 2006). The hybridization temperature is related to the probe affinity to the target and can be estimated by the Gibbs free energy change associated to the hybridization reaction (Yilmaz and Noguera, 2004). Hybridization time has been associated with the kinetics of the process, which comprises the penetration of the probe through the cell envelope, the binding of the probe to the complementary sequence and the unfolding of secondary and tertiary rRNA structures, as well as of eventual folded portions of the probe (Herzer and Englert, 2001; Yilmaz and Noguera, 2004). Formamide (FA) is a denaturant agent that reduces the thermal stability of the double-stranded polynucleotides, increases the accessibility to the rRNA target and competes for hydrogen bonding, which allows for the hybridization to be performed at lower temperatures (Yilmaz et al., 2006). Therefore, it has been assumed that the FA concentration needed, together with the hybridization temperature and time, regulates the process stringency and depends only on the sequence target and the probe structure (Berndt et al., 1996).

During the last decades, different types of probes have emerged and PNA (Peptide Nucleic Acid) was shown to be a DNA mimic with recognized superior hybridization features, demonstrating increased resistance to nucleases or proteases and higher specificity (Guimarães et al., 2007; Worden et al., 2000). Since PNA has an uncharged backbone, it presents reduced electrostatic repulsion

hindering hybridization and consequent independence of salt concentration, which favours the destabilization of rRNA secondary structures (Almeida et al., 2011; Stender et al., 2002).

Universal bacterial probes, such as EUB338 (Amann et al., 1990b), are commonly used in studies for the detection of different bacteria and as a control for the use of probes specifically designed for one bacterium (Fuchs et al., 1998; Perry-O'Keefe et al., 2001; Suzuki et al., 2005). Therefore, an universal probe is a useful model probe to understand the interplay of FISH variables in the optimal detection of different bacteria, with no influence of the target and probe sequence. This work aimed at optimizing the use of a PNA EUB338 probe for bacteria quantification by PNA flow-FISH, focusing on hybridization temperature, time and formamide concentration. The effect of these variables and their interplay on the fluorescence intensity was studied for different bacteria, through response surface methodology.

## **2. Materials and methods**

*2.1. Bacterial strains and fixation/permeabilization* *Escherichia coli* CECT 434, a *Bacillus cereus* strain isolated from a disinfectant solution and identified by 16S rRNA gene sequencing (Simões et al., 2007b), *Pseudomonas fluorescens* ATCC 13525, *Listeria innocua* CECT 910 and *Staphylococcus epidermidis* RP61A were grown overnight on tryptic soy agar (TSA) (3% (w/v) tryptic soy broth and 1.5% agar) (Merck, Darmstadt, Germany). *L. innocua* and *S. epidermidis* were grown at 37 °C, whereas the remaining bacteria were grown at 30 °C. Cells in the exponential growth phase were harvested from plates, suspended in sterile water and homogenized by vortexing in an approximate concentration of 10<sup>8</sup> to 10<sup>9</sup> cells/mL. Bacteria were then fixed/permeabilized according to Perry-O'Keefe et al. (2001) with minor changes. The cell suspension was pelleted by centrifugation at 10,000 x *g* for 5 min, resuspended in 400 µL of 4% (w/v) paraformaldehyde (Acros Organics, New Jersey, USA) and incubated for 1 h at room temperature.

After centrifugation, the pellet was resuspended in 500 µL of 50% (v/v) ethanol, and incubated at –20 °C until used.

### *2.2. PNA fluorescence in situ hybridization (PNA-FISH)*

An universal PNA EUB338 probe (5'-TGCCTCCCGTAGGA-3') which recognizes a conserved region of the 16S rRNA in the domain *Bacteria*, based on Amann et al. (1990a), was used as a model probe to identify all the bacteria in study. The probe was synthesized and labelled at the N terminus with AlexaFluor488 via a double double 8-amino-3,6-dioxaoctanoic acid (AEAA) linker (Panagene, Daejeon, South Korea).

For the hybridization, 100 µL of the fixed-cell aliquot was pelleted by centrifugation (10,000 x *g* for 5 min) and resuspended in 100 µL of hybridization

solution containing 200 nM PNA probe, 50 mM Tris-HCl (pH 7.5) (Fisher Scientific, New Jersey, USA), 10% (w/v) dextran sulfate (Fisher Scientific), 0.1% (v/v) Triton X-100 (Panreac, Barcelona, Spain) and different formamide concentrations (Acros Organics). Negative controls were resuspended in hybridization solution with no probe. Samples were then incubated during the different times and temperatures under study. After hybridization, cells were centrifuged (10,000x *g* for 5 min), resuspended in 500  $\mu$ L of washing solution (containing 5 mM Tris base, pH 10, (Fisher Scientific), 15 mM NaCl (Panreac) and 1% (v/v) Triton X-100), and incubated for 30 min at the same temperature used in the hybridization step. After centrifugation (10,000x *g* for 5 min), the pellet was resuspended in 700  $\mu$ L of sterile water. Each experiment was performed in triplicate.

### 2.3. Flow cytometry analysis

The fluorescence intensity of hybridized samples and negative controls was quantified by an Epics XL flow cytometer (Beckman Coulter, Hialeah, Florida, USA) equipped with a 488-nm argon ion laser. Forward angle light scatter (FS), side angle light scatter (SS), and green fluorescence (FL1) were detected at logarithmic scale. A minimum of 20,000 events falling into the bacterial gate defined on the FS-SS plot were acquired per sample. Data were analysed with the Expo32 software (Beckman Coulter), and the average fluorescence intensity was determined for each triplicate experiment.

### 2.4. Response surface methodology (RSM)

Response surface methodology (RSM) is a modelling technique that has become popular in recent years for optimization studies (Baş and Boyacı, 2007). RSM evaluates the main and interaction effects of independent variables on a process (Myers and Montgomery, 1995). RSM was employed to study the influence of the hybridization temperature, time and the concentration of formamide (in the hybridization solution) on the optimization of PNA EUB338 bacteria quantification. The average fluorescence intensity obtained after PNA-FISH was used as the dependent variable.

Central composite designs (CCD) were set up for *E. coli*, *B. cereus*, *P. fluorescens*, *L. innocua* and *S. epidermidis*, using the statistical software Design Expert<sup>®</sup> 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA) to estimate the coefficients of the model. The range and levels of all variables were defined according to our previous experience and bibliography (Almeida et al., 2010; Guimarães et al., 2007; Jansen et al., 2000; Lefmann et al., 2006). Each of the three variables assumed five different experimental values which were coded for statistical purposes. The codes used are related to the type of points composing the design. Each CCD

included  $2^3$  factorial points (coded at  $\pm 1$ ), 6 axial points (coded as  $\pm \alpha$ ) that represent extreme values used for the estimation of the model curvature, and 6 centre points (all factors at coded level 0) repeated to take into account the experimental error (Myers and Montgomery, 1995; Silva et al., 2011). Therefore, each design matrix consisted of 20 PNA-FISH experiments. After performing these experiments and quantifying the fluorescence by flow cytometry, the average intensity values obtained were introduced in the software to fit a quadratic model. Each obtained model was analysed using analysis of variance (ANOVA). The interaction of the three independent variables and their effect on the fluorescence intensity was inspected by constructing the response surface and contour plots. In order to obtain the optimum conditions within the experimental range that maximized the fluorescence intensity, the optimization function of the Design Expert software was used. A confirmation experiment of the predicted optimum point was performed for each bacterium model, in triplicate.

These procedures were applied in an iterative process. Firstly, the same design was applied to different bacteria. Nevertheless, every time a bacterium could not be modelled with a significant model or the obtained model did not present an optimum area the CCD was redesigned, changing the levels of the variables. This strategy is illustrated in Fig. 1.

Following the referred iterative process, *E. coli*, *P. fluorescens*, *L. innocua* and *S. epidermidis* were finally modelled using the same experimental range, whereas a different one was applied for *B. cereus*, as shown in Table 1.

### 2.5. Gram staining

*B. cereus* and *L. innocua* were gram stained after subjecting them to the PNA-FISH procedure with hybridization solutions containing no probe and the following formamide (FA) concentrations: 0%, 10%, 50% and 90%. The “hybridization” step was performed at 57 °C, during 100 min. These temperature and time were chosen so that a potential effect of different formamide concentrations could be observed (considering the response surface plots obtained for each bacterium). *E. coli* was used as a control. Moreover, a negative control was performed for each bacterium using bacteria only subjected to the fixation/permeabilization step (fixed). *S. epidermidis* was also tested as a confirmation experiment.

Briefly, after applying the FISH procedure with different formamide concentrations, 20  $\mu$ L of the final suspension were smeared in a microscope slide and an ordinary gram staining protocol was followed. This gram protocol is based on three main sequential steps: addition of crystal violet followed by iodine that acts as a mordant, addition of a decolourant solution (80% alcohol/20% acetone, in the case) and finally the addition of safranin (Bartholomew and Mittwer, 1952).

The experiments were performed in triplicate. The stained smears were observed in a LEICA DMLB2 optical microscope, using LAS V4.2 (LEICA), and 15 photos of each sample were taken. The number of violet and pink cells present in each image was counted and the mean percentage of the number of pink cells over the total number of cells in each sample was calculated. The means obtained for each condition were analysed in SPSS 14<sup>®</sup> (New York, USA), using the Tukey's test ( $\alpha < 0.05$ ).

### 2.6. Transmission electron microscopy

*E. coli* and *B. cereus* exposed to the same "PNA-FISH" conditions as used in the gram staining were also observed by transmission electron microscopy (TEM). The samples were processed by fixing the pellets with 4% (v/v) paraformaldehyde (Acros Organics) and 2.5% (v/v) glutaraldehyde (Merk, Darmstadt, Germany) in 0.1 M Sodium Cacodylate Buffer, pH 7.4 (Fluka, Buchs, Switzerland). They were subsequently postfixed in osmium tetroxide (OsO<sub>4</sub>) embedded in an epoxy resin (EPON 812) at 60 °C and stained using uranyl acetate and lead citrate. Preparations were observed with a Jeol JEM 1400 (Jeol, Tokyo, Japan) electron microscope.

## 3. Results and discussion

### 3.1. Optimization of PNA EUB338 by RSM

The initial CCD designed was tested both in a gram negative (*E. coli*) and a gram positive (*B. cereus*) bacteria (Table S1, supplemental material). The results showed an exponential tendency but no optimum was obtained in the response surface plots for each bacterium. In fact, they showed a significantly different behaviour from one another, since *E. coli* detection showed the best fluorescence signal at the lowest formamide concentration (13%) and the highest temperature (65 °C), with no benefit from hybridization times longer than 30 min, while *B. cereus* detection showed the best fluorescence signal at the highest formamide concentration (47%) together with the longest hybridization period (90 min).

We redesigned the CCD specifically for *E. coli* and *B. cereus* (Table 1) so that satisfactory models were obtained. These designs were tested with the other bacterial strains and all of them (*L. innocua*, *P. fluorescens* and *S. epidermidis*) showed to be adequately modelled by the design of *E. coli* and not the one of *B. cereus*.

Using these final designs (Table 1), significant quadratic models were obtained for each bacterium (Table S2, supplemental material), with the model *p*-value < 0.05 and a non-significant lack-of-fit (*p*-value > 0.05). The coefficients of determination, *R*<sup>2</sup>, ranged from 0.83 to 0.99, indicating that the models were adequate (Mandenius and Brundin, 2008). The *F*-values obtained in the *F*-test with

95% of confidence also showed that the models were statically significant (Table S3, supplemental material). Moreover, the plot of the predicted versus the actual experimental values obtained showed an evenly split of the data points by the 45° line, indicating that experimental values were in adequate agreement with the ones predicted by the model (Figure S1, supplemental material). Negative controls (performed together with all CCD centre points experiments) showed always an average fluorescence lower than 1.5, while samples with a good experimental signal presented values generally ranging from 10 to 125 a.u.

The three-dimensional response surface plots showing the interaction between the hybridization temperature and time, while keeping the formamide concentration at its optimum value, are presented in Fig. 2. Since the optimum formamide concentration is the same for all bacteria from (b) to (e) of Fig. 2, as discussed next, the respective plots are not shown.

It can be seen in Fig. 2 that an optimum space was obtained for every bacteria, with a more or less extended area. The optimum conditions leading to the maximum fluorescence intensity in each bacterium are presented in Table 2. Overall, the predicted maximum values were in reasonable agreement with the ones obtained in the confirmation experiments, falling within the prediction interval. It is noticeable that the values of fluorescence were higher for *E. coli* and *P. fluorescens* than for the other three bacteria in analysis, which was persistently observed in different experiments, using varying FA concentrations. Different ribosome content, as well as cell envelope permeability, can contribute for these differences (Bouvier and Del Giorgio, 2003; Yilmaz and Noguera, 2004). It has been extensively reported that the *Staphylococcus* genus is hard to identify by FISH (Jansen et al., 2000; Nordentoft et al., 1997). Perry-O'Keefe et al., 2001 has also observed lower fluorescence for the gram positive bacteria *S. epidermidis*, *L. innocua* and *B. subtilis* (Perry-O'Keefe et al., 2001).

As shown in Table 2, the optimum hybridization temperature obtained for the different bacteria is in the range of 54–60 °C, except for *E. coli* that presents a higher temperature (69 °C). This increased temperature, possibly being thermodynamically favourable (Yilmaz and Noguera, 2004) and increasing the rRNA accessibility and the cells' permeability (Tang et al., 2005), explains the lower hybridization time needed to maximize the *E. coli* fluorescence (40 min) compared to the remaining bacteria. Those had an optimum hybridization time near 60 min, except *B. cereus* which required almost twice the time.

Concerning formamide concentration, it is noticeable that except *B. cereus* all the bacteria emitted the highest fluorescence with the lowest value considered in the model prediction (coded 1 value), (Table 1). Actually, the removal of formamide ( $\alpha$  point) led to values of fluorescence near or even higher than the ones obtained with the optimum points. To confirm if a low formamide concentration could also benefit the hybridization with other morphologically related bacteria strains/species, we

also tested the strains *Listeria innocua* CECT 5376, *Staphylococcus epidermidis* CECT 231 and *Pseudomonas fluorescens* isolated N3 and the species *Staphylococcus aureus* CECT 976, *Listeria monocytogens* ATCC 15313 and *Pseudomonas aeruginosa* ATCC 10145 (Table S4, supplemental material). Higher fluorescence was obtained for all these bacteria using 5.5% formamide instead of 49.5% (performing hybridization at the optimum conditions obtained for the respective genus). To assess if low formamide concentration could affect the specificity of the hybridization, we performed hybridizations on the yeast *Saccharomyces cerevisiae* IGC 2608 and the archaeon *Methanobacterium formicium* DSM 1535 at two different conditions (59 °C, for 59 min and 69 °C, for 40 min). Results showed that while a limited level of non-specific hybridization could occur (but limited to less than 10 a.u.), this was not related to the percentage of formamide in the hybridization solution. In short, lowering the concentration of formamide in PNA-FISH experiments may not necessarily affect the specificity of the method. On the other hand, optimum *B. cereus* identification requires a formamide concentration of nearly 50% (49.5%), presenting a remarkably distinct behaviour from the remaining bacteria. That hybridizations against other bacteria may also improve when higher concentrations of formamide are used was confirmed using *B. thuringiensis* CECT 197 (Table S4, supplemental material). Similarly to *B. cereus*, it was observed that *B. thuringiensis* hybridization using 49.5% formamide led to a significantly higher fluorescence than using 5.5%.

It is noticeable that the optimum predicted conditions for the detection of all bacteria, excepting *B. cereus*, are similar (Table 2 and Figure S2, supplemental material). It is evident that, despite using the same probe for the identification of all bacteria and starting by applying the same hybridization conditions, two main types of responses were obtained.

The results show that, differently from what has been generally assumed, the use of formamide in FISH is only beneficial for certain species of bacteria. FA was introduced in classic molecular biology due to its nucleic-acid denaturant effect (Thomas et al., 1993), which rendered it as an useful solvent for example in RNA electrophoresis (Pinder et al., 1974). It is thought that the FA destabilization is caused by its ability to compete with H-bonds between base pairs (Fuchs et al., 2010), together with displacement of weakly bound water molecules (Massey and Krull, 2012). The use of FA has long been reported to enable the reduction of the temperature required for the annealing of probes to their target (Bonner et al., 1967) and has, therefore, been generally used in FISH hybridizations. Conversely, some authors have observed that low FA concentrations, or even its removal, could led to higher fluorescence values (Berndt et al., 1996; Bond and Banfield, 2001; Bonner et al., 1967; Manz et al., 1992; Yilmaz and Noguera, 2004), without affecting the specificity (Thomas et al., 1993). Nevertheless, this increase of the fluorescence has been explained as a reduction of the stringency needed, as an excess of FA may hinder the bonding of a probe, depending on the probe and target structure and the temperature,



time and salt concentration on the hybridization (Bouvier and Del Giorgio, 2003; Manz et al., 1992; Yilmaz and Noguera, 2004). However, this explanation does not justify the fact that different bacteria, with the same growth state and subjected to the same hybridization conditions with the same probe present different responses.

In fact, inspecting the *E. coli* flow cytometry plots obtained in the initial CCD design (Table S1, supplemental material), we noticed that when using 58.6% formamide (within 60 min hybridization at 57 °C) the population appeared very scattered and an additional peak before the main one was noticed in the histogram, differently from the use of 1.4% formamide (in the same conditions) (Figure S3, supplemental material). This led us to conclude that despite the thermodynamic influence, formamide could also have a harmful effect on the cells' integrity, originating sub-populations. Therefore, the optimum formamide concentration may also depend on the structure of the microorganism being analysed. It is clear that formamide effect occurs regardless of the gram type, as the inclusion of *L. innocua* and *S. epidermidis* (gram positive bacteria) in the study showed that these two bacteria react to formamide similarly to *E. coli* and *P. fluorescens* (gram negative). On the other hand, it is known that unlike in gram negative bacteria, there is a wide variability among the cell envelope of gram positive bacteria, namely the thickness of peptidoglycan (Schumann, 2011). Peptidoglycan layer represents a barrier that can improve the mechanical and chemical resistance (Wada et al., 2012). Systematic studies about the structure of peptidoglycan are lacking (Vollmer et al., 2008). However, recent data focusing on species resembling the ones studied here (Vollmer and Seligman, 2010) strongly indicate that *B. cereus* and *B. thuringiensis* possess a much thicker peptidoglycan layer than that of the remaining bacteria (Fig. 5A). Moreover, increased resistance of *B. cereus* and *B. thuringiensis*, as well as other *Bacillus* species, to cell wall degradation by lysozyme has been reported (Severin et al., 2004). *Listeria* and *Staphylococcus* species have also been said to possess a peptidoglycan structure resembling that of *E. coli* (Hayhurst et al., 2008; Pucciarelli et al., 2007). The thicker cell wall of *B. cereus* (and *B. thuringiensis*) may increase its resistance to formamide, whereas the remaining bacteria studied can be harmed by formamide. Interestingly, the application of hot formamide for the isolation of peptidoglycan is reported in the literature, which is in agreement with the proposed explanation (Fuller, 1938; Greenblatt et al., 1978; Perkins, 1965). As the preservation of cells integrity is crucial for a proper hybridization, bacteria with cell envelope damaged by FA would not benefit from its hybridization adjuvant function (Sen and Nielsen, 2007).

The thicker peptidoglycan of *B. cereus* can also explain the fact its optimum hybridization time was much longer than the ones obtained for the remaining bacteria in study (Table 2), as more time will be needed to efficiently penetrate the cells to reach the rRNA.

### 3.2. Gram staining

In order to attest the suggested effect of formamide on the cell wall peptidoglycan the gram positive bacteria *L. innocua* and *B. cereus* were gram stained; *S. epidermidis* was also used for confirmation.

Gram staining is a traditional microbiological technique to distinguish gram positive and negative bacteria, based on the different thickness of the peptidoglycan layer (Yazdankhah et al., 2001). Intact gram positive bacteria stain violet, since their thick peptidoglycan layer acts as a barrier, enabling the retention of the complex crystal violet-iodine (Popescu and Doyle, 1996). On the contrary, the thin peptidoglycan layer of gram negative bacteria allows the leakage of the crystal violet-iodine complex and the following entrapment of the pink dye safranin (Popescu and Doyle, 1996). It is known gram positive bacteria may also stain pink when mechanically or chemically damaged and aged, presenting an uneven cell wall thickness (Gram, 1884; Popescu and Doyle, 1996).

It was observed that the violet stain of the samples previous to hybridization (fixed) changed with the use of different concentrations of formamide, becoming clearer and pink in colour. Fig. 3A shows that *L. innocua* started to lose its violet stain at 10% FA, with nearly 28% of the total cells colouring pink. More than 80% cells stained pink at 50% FA. The further increase to 90% FA resulted in a non-significant increase of the pink cells to 90%. *S. epidermidis* showed a similar behaviour than *L. innocua* (data not shown). On the contrary, looking at *B. cereus* (Fig. 3A) no statistically significant change occurred compared with the fixed control, when using FA concentrations up to 50% (included). An increase in the pink cells to 49% was observed when 90% FA was applied. The minor percentage of pink cells observed in fixed and with no FA samples of both *L. innocua* and *B. cereus* is associated with some staining inherent errors. As expected, *E. coli* maintained its pink stain in all the conditions. Relevant example photos of stained *L. innocua* compared with *B. cereus* are shown in Fig. 3B.

The results obtained in gram staining evidenced that formamide affected the thickness of peptidoglycan layer of *Listeria innocua* (and *S. epidermidis*) when employed in the hybridization solution in concentrations higher than 10%, whereas *B. cereus* resisted to concentration higher than 50%. This outcome shows that there is a relationship between the effect of formamide and the thickness of the peptidoglycan layer.

### 3.3. Transmission electron microscopy

In order to look for further confirmation of the effect of formamide, *B. cereus* and *E. coli* were observed using transmission electron microscopy (TEM). Among the three bacteria subjected to gram staining, these two bacteria were chosen since they possess the thickest and the thinnest peptidoglycan layer, respectively.

Some structural changes were perceived in *E. coli* by TEM with crescent

concentrations of formamide, so they were inspected in a more detailed view (Fig. 4). While in the samples hybridized in 10% FA the outer membrane, periplasmic space (PS) and the inner membrane were clearly distinguished, in the samples subjected to 50% FA bacteria were found possessing a fuzzier definition of these layers. Moreover, this sample showed bacteria with the PS filled with electron-dense material. These changes were more pronounced in the sample subjected to 90% formamide, showing some bacteria with a completely disrupted cell envelope.

Concerning *B. cereus* (Fig. 4) no significant effect of formamide was observed at 50%, looking similar to 10%, whereas at 90% some bacteria start to show a blurry cell wall.

The results obtained through TEM showed that 50% FA can damage *E. coli*, being harmless to *B. cereus*. Although *B. cereus* cell envelope was susceptible to the high concentration of 90% FA, it was less injured than *E. coli*.

TEM analysis led us to confirm that bacteria possessing different peptidoglycan thicknesses have different susceptibility to FA, as also verified in the gram staining results.

#### **4. Conclusions**

In this work we have shown, through the use of an universal PNA EUB338 probe, that there is not a single optimum protocol for the probe that can be used for all of microorganisms, as the optimal FISH conditions vary with the characteristics of the target bacteria. Through the study of the interaction effect of three factors, reported to be essential in controlling the stringency of the hybridization, on fluorescence intensity, it was revealed that the optimum hybridization temperature, time and formamide concentration do not depend only on the target and the probe (besides other reagents that can be added to affect the stringency), but also on the specific structure of the targeted bacteria. In fact, bacteria with increased thickness of peptidoglycan, as *B. cereus*, benefited from increased hybridization time what was not actually surprising, since it is reasonable to consider that the thick layer can retard the probe diffusion. Nevertheless, different responses to formamide concentration were unexpected at start. It was found through gram staining and TEM that formamide may harm bacteria cell envelope, depending on the thickness of the peptidoglycan layer. Bacteria with a thin layer, such as *E. coli* or *L. innocua* can be susceptible to formamide, compromising a proper hybridization. On the contrary, bacteria which morphology is not affected by FA, due to its protective thick peptidoglycan layer, will benefit from the denaturant effect of FA in hybridization and consequent decrease of hybridization temperature. Therefore, the effect of formamide can be formulated as a balance between two different effects, as illustrated in Fig. 5B. Hence, the use of formamide should be adjusted according to the microorganism to be detected, so that it does not harm

the cells and when possible favours the hybridization thermodynamics.

To our knowledge, this is the first time that such an effect of formamide in FISH is suggested. It can be a relevant contribution for a deeper understanding of FISH methods, besides having practical impacts on the future developments of FISH procedures particularly the ones used as a diagnosis tool. It should however be highlighted that this work is limited to a PNA probe of the well-known EUB338 sequence. In the future it would certainly be useful to understand if these results also hold for DNA and other DNA/RNA mimics probes, in order to broaden the conclusions of the present study.

Moreover, a pioneering approach of optimization was established, applying response surface methodology to model the effect of the factors in study on the FISH response, so that not only the influence of each factor was studied but also their interplay. Therefore, this study can be important in boosting the modelling of FISH methodology.

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## **Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jbiotec.2014.06.023>.

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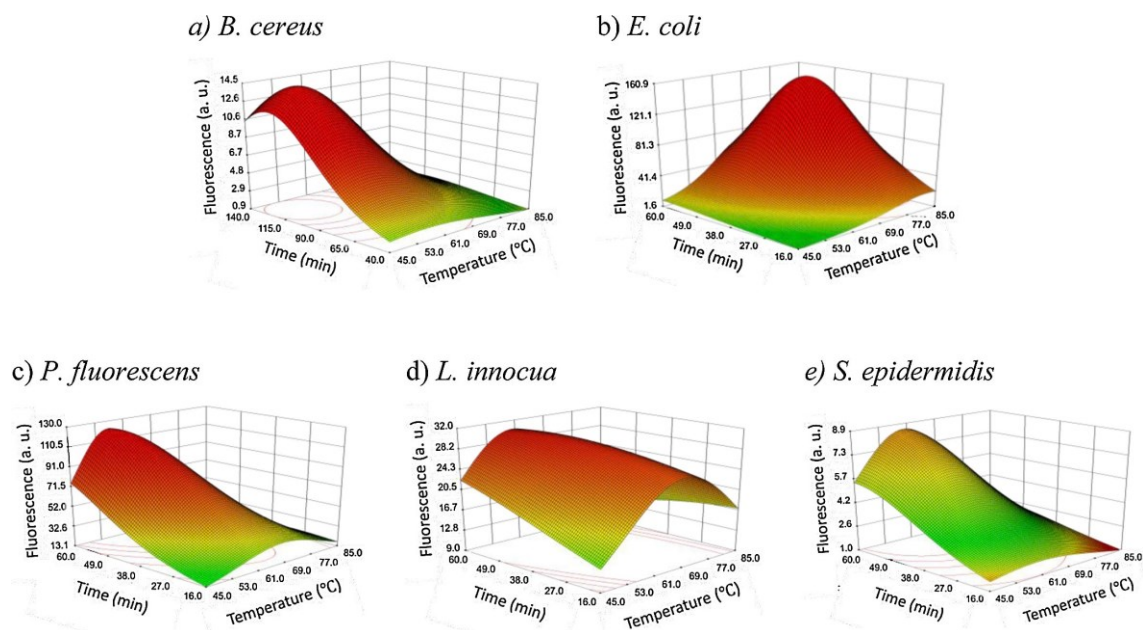
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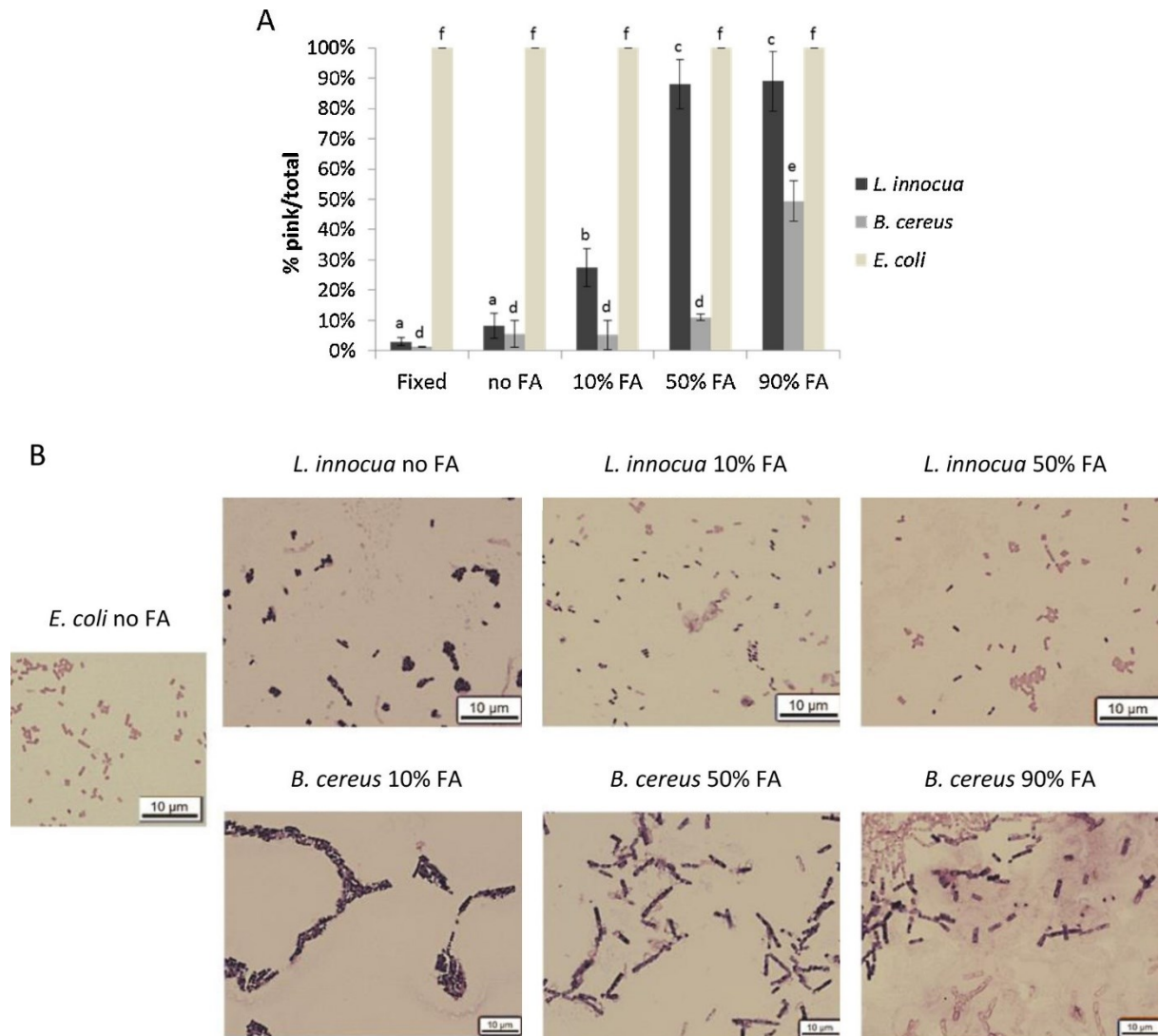
- Bacterium** → **Choose RSM design** → **PNA-FISH** → **Flow cytometry** → **Introduce response and analyse**
- Flow cytometry** output: Events vs Fluorescence (a. u.)
- Introduce response and analyse** output: Table with columns: Run, Rep, Batch, Type, Factors 1-4, Response 1, Response 2
- Significant model?** (Yes/No)
- Yes** → **Surface and contour plots** → **Optimum?** (Yes/No)
- Yes** → **Confirmation experiment**
- Optimum?** (Yes) → **Confirmation experiment**
- Optimum?** (No) → **Choose RSM design**
- Significant model?** (No) → **Choose RSM design**
- Surface and contour plots** output: 3D surface plot of Fluorescence (a. u.) vs Formamide (% v/v) and Temperature (°C).
- Optimum?** (Yes) output: 3D surface plot of Fluorescence (a. u.) vs Time (min) and Temperature (°C).

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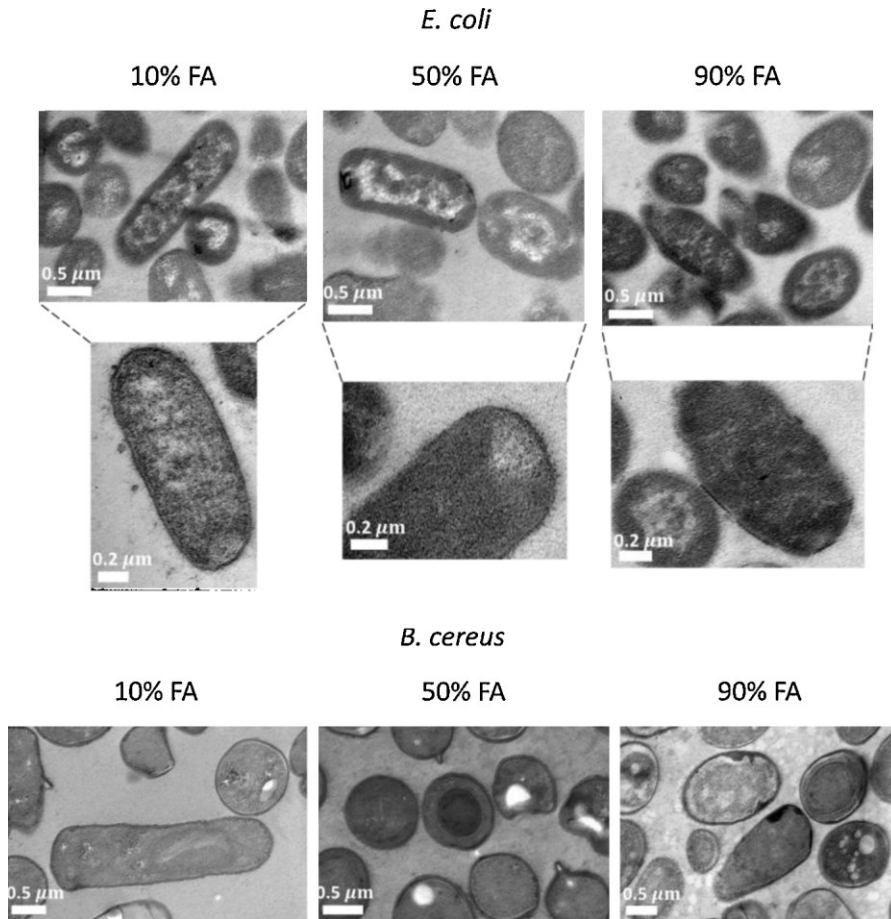




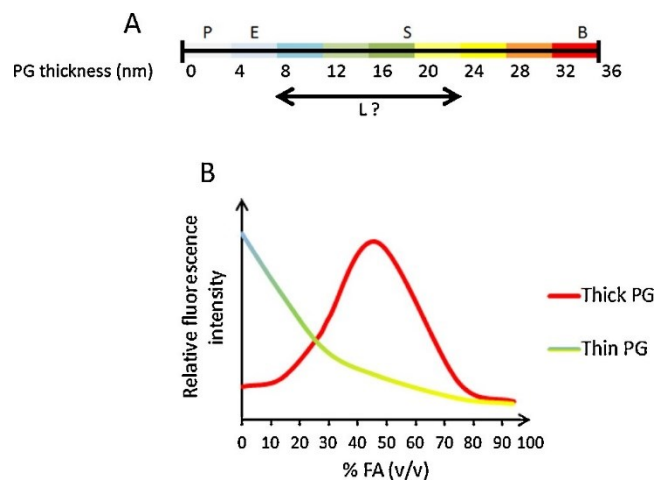
**Fig. 2.** Surface response plots representing the interaction effect of hybridization temperature and time on the fluorescence response of *B. cereus* (a), *E. coli* (b), *P. fluorescens* (c), *L. innocua* (d) and *S. epidermidis* (e). The formamide concentration is kept constant at the optimum value: 49.5% in (a) and 5.5% in (b) to (e). Fluorescence values are presented in arbitrary units (a.u.).



**Fig. 3.** (A) Mean percentage and standard deviation of pink cells over the total cells present in each microscopy image. Bacteria were stained after “PNA-FISH” with no FA, 10%, 50% or 90% FA hybridization solutions. Bacteria subjected only to fixation/permeabilization were used as negative controls (fixed). a-e Means with the same letter indicate there is no significant difference (Tukey’s test:  $\alpha < 0.05$ ) between results. (B) Example photos of *L. innocua* hybridized with no FA, 10% and 50% FA solution in comparison with *B. cereus* hybridized with 10%, 50% and 90% FA solution, as well as a negative control of *E. coli*.



**Fig. 4.** Electron microscopy images after “PNA-FISH” with an hybridization solution containing 10% FA, 50% FA and 90% FA using *E. coli* and *B. cereus*. Scale bars represent 0.5  $\mu\text{m}$  except in the images presenting a more detailed view of *E. coli* where they represent 0.2  $\mu\text{m}$ .



**Fig. 5.** (A) Illustrative comparison of the peptidoglycan (PG) thickness of the bacteria in study, based on the isolated PG of *Pseudomonas aeruginosa* (P), *Escherichia coli* (E), *Staphylococcus aureus* (S) and *Bacillus subtilis* (B), according to Vollmer and Seligman (2010). To our knowledge, apart from qualitative

comparisons (Pucciarelli et al., 2007), no quantitative information is available for *Listeria* (L). Resembling bacteria species were studied, so the relative thicknesses are considered valid. (B) Representation of the effect of the hybridization formamide (FA) concentration on the relative fluorescence intensity obtained when targeting bacteria possessing thick or thin peptidoglycan (PG) layers.

**Table 1**

Experimental levels of variables tested for the different bacteria in study.

Variables		Range and level									
		<i>E. coli</i> , <i>P. fluorescens</i> , <i>L. innocua</i> , <i>S. epidermidis</i>					<i>B. cereus</i>				
		$-\alpha$	-1	0	+1	$+\alpha$	$-\alpha$	-1	0	+1	$+\alpha$
$x_1$	Temperature (°C)	31	45	65	85	99	31	45	65	85	99
$x_2$	Time (min)	1	16	38	60	75	6	40	90	140	174
$x_3$	[Formamide] (% v/v)	0	5.5	13.5	21.5	26.9	0	19	47	75	94

**Table 2**

Optimum temperature, time and formamide concentration predicted through the RSM models for the tested bacteria. The predicted and obtained fluorescence values in those conditions are shown.

Bacteria	Optimum conditions			Predicted fluorescence (a.u.)	Obtained fluorescence (a.u.)
	Temperature (°C)	Time (min)	[Formamide] (% v/v)		
<i>B. cereus</i>	56.1	112.2	49.5	13.3	25.8
<i>E. coli</i>	69.3	39.5	5.5	115.1	101.8
<i>P. fluorescens</i>	54.4	59.6	5.5	121.2	122.6
<i>L. innocua</i>	60.0	56.2	5.5	30.6	30.9
<i>S. epidermidis</i>	59.0	60.0	5.5	8.6	4.4