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Optimization of peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) for the detection of bacteria: The effect of pH, dextran sulfate and probe concentration

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ABSTRACT

Fluorescence *in situ* hybridization (FISH) is a molecular technique widely used for the detection and characterization of microbial populations. FISH is affected by a wide variety of abiotic and biotic variables and the way they interact with each other. This is translated into a wide variability of FISH procedures found in the literature. The aim of this work is to systematically study the effects of pH, dextran sulfate and probe concentration in the FISH protocol, using a general peptide nucleic acid (PNA) probe for the *Eubacteria* domain. For this, response surface methodology was used to optimize these 3 PNA-FISH parameters for Gram-negative (*Escherichia coli* and *Pseudomonas fluorescens*) and Gram-positive species (*Listeria innocua*, *Staphylococcus epidermidis* and *Bacillus cereus*). The obtained results show that a probe concentration higher than 300 nM is favorable for both groups. Interestingly, a clear distinction between the two groups regarding the optimal pH and dextran sulfate concentration was found: a high pH (approx. 10), combined with lower dextran sulfate concentration (approx. 2% [w/v]) for Gram-negative species and near-neutral pH (approx. 8), together with higher dextran sulfate concentrations (approx. 10% [w/v]) for Gram-positive species. This behavior seems to result from an interplay between pH and dextran sulfate and their ability to influence probe concentration and diffusion towards the rRNA target. This study shows that, for an optimum hybridization protocol, dextran sulfate and pH should be adjusted according to the target bacteria.

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1. Introduction

In situ hybridization (ISH) consists of an array of methodologies that ultimately allow the specific detection of nucleic acid sequences in biological samples (Jin and Lloyd, 1997). At the present moment, most ISH techniques use fluorescent dyes as reporter molecules, in a process called Fluorescence *in situ* Hybridization (FISH) (Speicher and Carter, 2005; Trask, 2002). On its original form, FISH consists essentially on hybridizing an oligonucleotide probe to its complementary sequence in previously fixed samples, obeying to the Watson-Crick hydrogen-bonding rules (Cerqueira et al., 2008; Volpi and Bridger, 2008). FISH is widely used in the field of microbiology (Amann and Fuchs, 2008), namely in the identification, quantification and characterization of phylogenetically defined microbial populations in complex environments (Wagner et al., 2003).

Since the first application of FISH to microorganisms by DeLong et al. (1989), diverse FISH-based diagnostic assays have been developed (see review from Volpi and Bridger, 2008). These result from combinations of FISH with other techniques or improvements at

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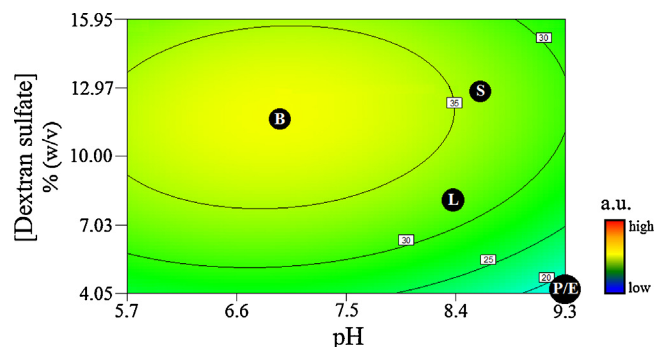

Fig. 1. Contour plot of *B. cereus* showing the effect of pH and DS (500 kDa) concentration on the fluorescence intensity (with probe concentration at the optimum of 300 nM). The fluorescence values (in arbitrary units) of the contour lines are the ones obtained for *B. cereus*. The optimum points predicted by the software for *E. coli*, *P. fluorescens*, *L. innocua*, *B.cereus* and *S. epidermidis* are represented in black circles with its respective initial letter. For *E. coli* and *P. fluorescens* no optimum value was obtained, but the overall behaviour observed indicates that lower DS concentrations and higher pH values should be preferred to redefine the testing concentrations.

the FISH procedure level, such as the use of other molecules, rather than standard DNA or RNA, as probes. A good example of this is the application of peptide nucleic acid (PNA), a nucleic acid mimic with recognized superior hybridization features (Cerqueira et al., 2008; Stender et al., 2000, 1999). PNA is comprised of a neutral polyamide backbone (Nielsen et al., 1991) with an identical chemical configuration to the DNA molecules that allows PNA to hybridize with complementary DNA or RNA sequences (Nielsen, 2001; Shakeel et al., 2006). Its superior hybridization features arise from the lack of electrostatic repulsion between the non-charged polyamide backbone and the charged DNA/RNA phosphodiester backbone. This is translated into an improved thermal stability of the duplex (Nielsen, 2001; Perry-O'Keefe et al., 2001) and allows the hybridization step to be performed under low salt concentrations (Orum et al., 1995), a condition that destabilizes the rRNA secondary structures and results in an improved access to target sequences (Azevedo et al., 2003; Fuchs et al., 1998; Yilmaz et al., 2006). The neutrally-charged PNA also diffuses well through the bacterial membrane (Drobniewski et al., 2000) and its synthetic nature leads to an increased resistance to nucleases and proteases (Demidov et al., 1994; Stender et al., 2002; Wagner et al., 2003).

In spite of PNA-FISH robustness, there is a considerable variability between the procedures described in the literature and its implementation usually requires an initial optimization to adjust the hybridization efficiency (Herzer and Englert, 2001), currently performed as a trial-and-error approach. This is a laborious and time-consuming step that could be greatly shortened if knowledge on how to develop a novel PNA-FISH method was at hand. In fact, variables such as type of fixative used (aldehyde or alcohol-based fixation), hybridization time, temperature, pH, concentration of probe, dextran sulfate (DS) and formamide, among others, are known to affect hybridization efficiency. Santos et al. (2014) recently assessed the effects of formamide, temperature and time on the hybridization efficiency, while successfully establishing an approach for FISH optimization, applying response surface methodology (RSM).

The present work aimed to understand the effect of hybridization pH, DS and probe concentration (and their interplay) on PNA-FISH efficiency for different bacteria. To this end, Response Surface Methodology was used to model the hybridization of an universal *Eubacteria* PNA probe (EUB338) (Amann et al., 1990; Santos et al., 2014), and signal quantification was assessed by flow cytometry.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains selected for this study were *Escherichia coli* CECT 434, *Pseudomonas fluorescens* ATCC 13525, *Listeria innocua* CECT 910, *Staphylococcus epidermidis* RP61A and *Bacillus cereus* isolated from a disinfectant solution and identified by 16S rRNA gene sequencing (Simões et al., 2007). *E. coli* and *L. innocua* were grown on tryptic soy agar (TSA) [3% (w/v) tryptic soy broth and 1.5% (w/v) agar] (Oxoid, Basingstoke, England and Merck, Darmstadt, Germany). *B. cereus*, *P. fluorescens* and *S. epidermidis* were grown in plate count agar (Merck). All cultures were grown overnight at 30 °C and streaked onto fresh plates every 2 or 3 days.

2.2. PNA-FISH method

In order to evaluate the influence of pH, DS and probe concentration in the fluorescent signal outcome, a PNA-FISH protocol similar to the one described by Santos et al. (2014) was implemented, followed by signal quantification using flow cytometry. A universal PNA probe EUB338 (5′-TGCCTCCCGTAGGA-3′), based on the work of Amann et al. (1990), which recognizes a conserved region of the 16S rRNA in the domain *Eubacteria*, was used. The probe was synthesized and labelled at the N terminus with AlexaFluor488 via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker (Panagene, Daejeon, South Korea).

Bacterial cells were harvested from plates and suspended in sterile water to a final concentration of 10^8–10^9 cells mL^{-1}. For sample fixation, the cell suspension was pelleted by centrifugation at 10,000*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 at 10,000*g* for 5 min, the pellet was resuspended in 500 μL of 50% (v/v) ethanol and incubated at −20 °C for at least 30 min. For hybridization, 100 μL of the fixed-cell aliquot were pelleted by centrifugation (10,000*g* for 5 min) and resuspended in 100 μL of hybridization solution. With the exception of the parameters under study, the composition of the hybridization solution was the same as the one reported by Santos et al. (2014), with the optimum formamide concentration obtained on that study. Consequently, formamide (Acros Organics) at 5.5% (v/v) was used for *E. coli*, *P. fluorescens*, *L. innocua* and *S. epidermidis* and at 49.5% (v/v) for *B. cereus*. Regarding the 3 parameters under study, the ranges selected are presented in Table 1. The conditions for assay 1 were selected to cover the values commonly described in the literature (Almeida et al., 2010; Brehm-Stecher et al., 2005; Cerqueira et al., 2011; Oliveira et al., 2001; Perry-O'Keefe et al., 2001; Stender et al., 1999; Zhang et al., 2012) (See further details on hybridization conditions in Table S1 of the Supplemental material). Based on the results obtained in assay 1, new ranges were selected for assay 2, to achieve a suitable model for *E. coli* and *P. fluorescens*. Ranges defined in assay 3 and 4 were used to further evaluate the influence of DS molecular weight (MW) and pH on the signal outcome of Gram-positive bacteria. Different buffers were used at a concentration of 50 mM to control the pH of the hybridization solution, specifically citrate-phosphate (for pH 4–6); Tris-HCl (pH 7–8); Glycine-NaOH (pH 9–10); Sodium bicarbonate-NaOH (pH 11.2 and 11.3) and potassium chloride-NaOH (for pH above 12). Samples were hybridized at 60 °C for 55 min, except for *B. cereus* samples that were incubated for 110 min, based on the optimum conditions found by Santos et al. (2014). As a negative control, all procedures described above were repeated for each condition, but the PNA probe was not added to the hybridization solution. After hybridization, cells were centrifuged (10,000*g* for 5 min), resuspended in 500 μL of washing solution containing 5 mM Tris base (pH 10; Fisher Scientific, New Jersey, USA), 15 mM NaCl

Table 1
Experimental levels for the variables used in the optimization of the PNA-FISH hybridization protocol for *E. coli*, *P. fluorescens*, *L. innocua*, *S. epidermidis* and *B. cereus* species.

Assay	Variables		Range and level				
			−α	−1	0	+1	+α
1[a]	x_1	pH	4.5	5.7	7.5	9.3	10.5
	x_2	[DS 500 kDa] % (w/v)	0.0	4.1	10.0	16.0	20.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
2[b]	x_1	pH	5.9	7.3	9.3	11.3	12.6
	x_2	[DS 500 kDa] % (w/v)	0.0	1.0	2.5	3.9	5.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
3[c]	x_1	pH	4.5	5.7	7.5	9.3	10.5
	x_2	[DS 10 kDa] % (w/v)	0.0	4.1	10.0	16.0	20.0
	x_3	[PNA EUB338] nM	32	100	200	300	368
4[d]	x_1	pH	6.5	7.3	9.3	11.2	12.0
	x_2	[DS 0.5 kDa] % (w/v)	1.9	5.0	12.5	20.0	23.1

[a] Experimental levels set in the optimization protocol for *E. coli*, *P. fluorescens*, *L. innocua*, *S. epidermidis* and *B. cereus*.
[b] Experimental levels set in the optimization protocol for *E. coli* and *P. fluorescens*.
[c] Experimental levels set in the optimization protocol for *L. innocua*, *S. epidermidis* and *B. cereus*.
[d] Experimental levels set in the optimization protocol for *L. innocua*. Probe concentration at 200 nM.

(Panreac, Barcelona, Spain) and 0.1% (v/v) Triton X-100 (Panreac) and incubated for 30 min at 60 °C. After centrifugation (10,000*g* for 5 min), the pellet was resuspended in 700 μL sterile saline solution, 0.9% (w/v) NaCl (Panreac). 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, Florida, USA) equipped with a 488 nm argon ion laser. Forward angle light scatter (FS), side angle light scatter (SS), and green (FL1) fluorescence 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. The data was analysed with the Expo32 software (Beckman Coulter), and the average fluorescence intensity was determined for each triplicate experiment.

2.4. Response surface methodology (RSM)

In order to model the effect of pH, DS and probe concentration in the hybridization of PNA EUB338 probe in bacteria, RSM was employed according to the procedure applied by Santos et al. (2014). 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® 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 previous studies (Table S1 of the Supplemental material) and the results obtained within this study. Each CCD for assays 1, 2 and 3 included 2^3 factorial points (coded at ± 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. For the assay number 4 the CCD included 2^2 factorial points (coded as ± 1), 4 axial points (coded as $\pm\alpha$) and 5 centre points (all factors at coded level 0). Therefore, this design matrix consisted of 13 PNA-FISH experiments.

2.5. Viscometer analysis

Viscosity measurements of DS 500 kDa solutions at pH 6, pH 9 and pH 12 were performed using a Cannon-Fenske viscometer size 100 (Hipex, Portugal). Different buffers were used at a concentration of 50 mM to control de pH of the DS solutions, specifically citrate-phosphate for pH 6, Glycine-NaOH for pH 9 and potassium chloride-NaOH for pH 12. The viscometer was placed in a water bath at a constant temperature of 25 ± 1 °C. The viscosity of DS solutions at different pH was determined by the comparison of the flow time of DS solutions against the flow time of distilled water in triplicate.

2.6. Statistical analysis

In order to find the optimum hybridization conditions for all five species in the study, the average intensity fluorescence values obtained by flow cytometry were introduced in the software Design Expert® 8.0.7.1 to fit a quadratic model and 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. The optimization function of the software was then used to estimate the optimum conditions within the experimental range that maximized the fluorescence intensity. A confirmation experiment of the predicted optimum point was performed for each bacterium in triplicate.

3. Results and discussion

3.1. PNA-FISH optimization in bacteria: pH, DS and probe concentration

In this work, the effect of three parameters (pH, DS and probe concentration) on the hybridization efficiency of PNA-FISH was studied. To model their effect, RSM was applied to the hybridization data obtained from 3 Gram-positive (*L. innocua*, *S. epidermidis* and *B. cereus*) and 2 Gram-negative species (*E. coli* and *P. fluorescens*). This setup was selected in order to include bacteria with different cell wall thicknesses, ranging from thin, *e.g.* Gram-negative *P. fluorescens*, to thick cell walls, *e.g.* Gram-positive *B. cereus* (Santos et al., 2014)Please revise the word Gramäll along the document..

The first range of pH, DS and probe concentrations tested in the CCD were based on the values typically described in the literature for PNA-FISH methods (Table 1, assay 1 and Table S1 of the Supplemental material). After performing the CCD set of experiments, significant quadratic models (p-value <0.05), a non-significant lack of fit (p-value >0.05) and a satisfactory coefficient of determination (R^2) combined with an optimum on the response surface plots were obtained for all three Gram-positive species tested (Fig. 1). However, for the Gram-negative species, an optimum value from the response surface plots was not obtained, although a general tendency for lower DS concentrations and higher pH values was observed (Fig. 1).

In order to obtain a satisfactory model for the Gram-negative species, the range of pH and DS concentration on the CCD were redesigned for higher pH values and lower DS concentrations (Table 1, assay 2), while maintaining the probe concentration level.

Using those designs for Gram-positive (Table 1, assay 1) and Gram-negative species (Table 1, assay 2), significant quadratic models were obtained for all five species tested (Tables S2 and S3 of the Supplemental material). The successful modelling of the three studied parameters (pH, DS and probe concentration) allowed the determination of the optimal conditions for the maximum fluorescence (Fig. 2). Moreover, the confirmatory experiment showed an

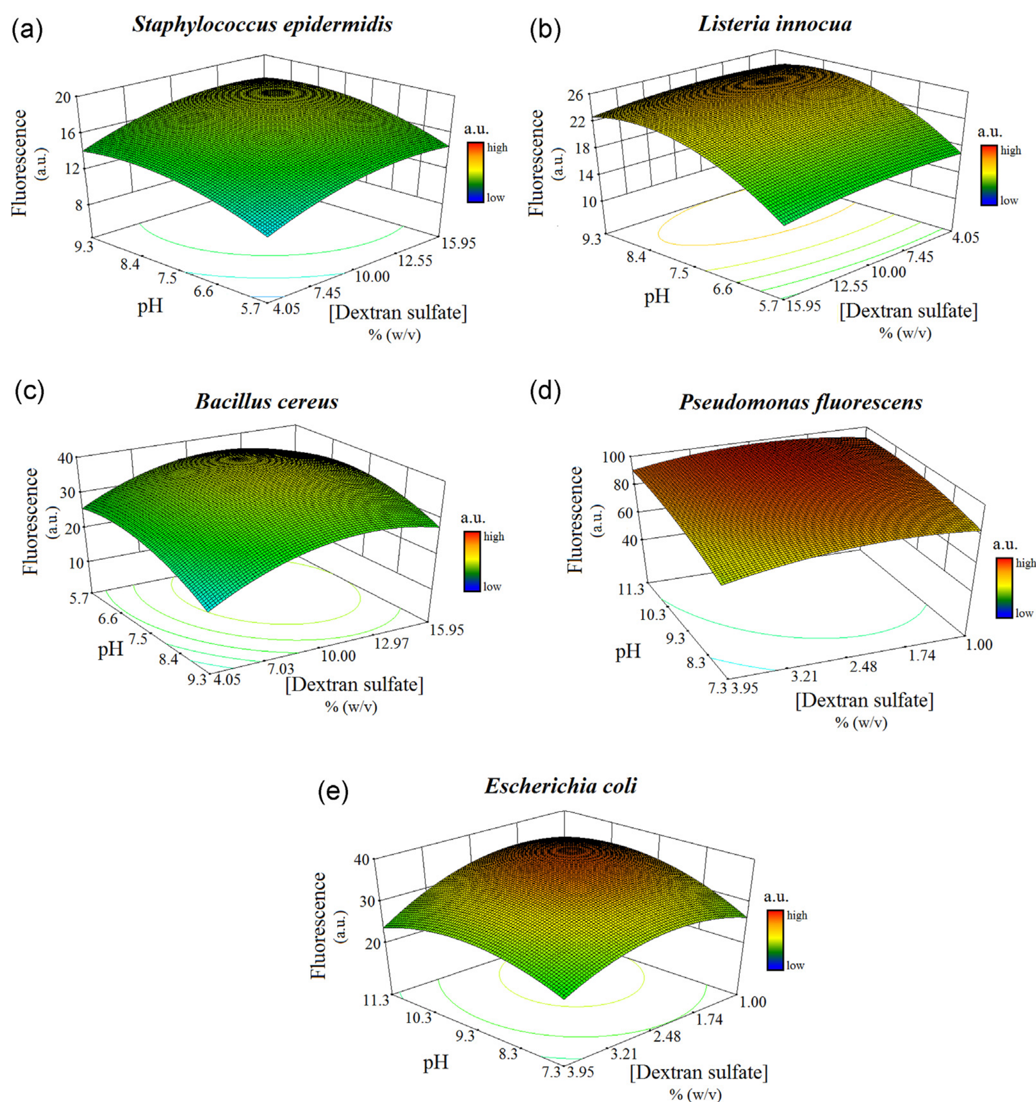

Fig. 2. Surface response plots representing the interaction effect of pH and DS on the fluorescence response of *S. epidermidis*, *L. innocua*, *B. cereus*, *E. coli* and *P. fluorescens*. The optimal PNA EUB338 probe concentration was 300 nM for all 5 strains. Fluorescence values are presented in arbitrary units (a.u.).

Table 2
Optimum hybridization pH, DS and probe concentration predicted through the RSM models for the tested species. The negative control, predicted and obtained fluorescence values in those conditions are shown.

Bacteria	Optimum conditions			Predicted Fluorescence (a. u.)	Obtained Fluorescence (a. u.)	Negative Control (a. u.)
	pH	DS (% w/v)	Probe (nM)			
E. coli	9.87	1.93	300	37.1	37.7 ± 1.5	0.6 ± 0.1
P. fluorescens	10.83	2.32	300	98.2	171.7 ± 8.3	0.9 ± 0.1
L. innocua	8.36	7.94	300	24.9	21.6 ± 0.2	0.6 ± 0.1
B. cereus	6.92	11.70	300	37.9	30.6 ± 1.4	0.5 ± 0.1
S. epidermidis	8.56	12.84	300	18.0	17.4 ± 1.6	0.4 ± 0.1

agreement between experimental and predicted values (Table 2). The average fluorescence for negative controls was equal or lower than 1 a.u. (data not shown), while for positive samples the values ranged from 7 to 150 a.u., depending on the microorganism and the conditions tested (Fig. 2).

Analysing Table 2 we can also notice a difference in terms of fluorescence intensity, with Gram-negative *P. fluorescens* and *E. coli*, exhibiting a higher signal than Gram-positive species, *L. innocua*, *S. epidermidis* and *B. cereus*. This pattern was also reported in the previous optimization performed by Santos et al. (2014). FISH signal is influenced (not only) by accessibility and target content. While accessibility is dependent on the permeability of the cell envelope to the probes, target content is correlated with the growth rate of bacteria (DeLong et al., 1989; Roller et al., 1994; Wallner et al., 1993). With this in mind, the Gram-differences found can be attributed to varying ribosomal content as well as cell envelope permeability. Despite the observed Gram-differences, our results still show that positive results are achieved even using favorable Gram-negative hybridization protocols on Gram-positive and vice

versa, since the outcome signal is still far greater than the respective negative control value.

From Table 2 we can observe as well that the optimal probe concentration for all species was the maximum tested and considered by the model, 300 nM (+1 factor). This was expected, since the probe concentration is a key factor for the nucleaction reaction and the time needed for hybridization (Bruns et al., 2007). The nucleation reaction is the rate-limiting step in the hybridization of nucleic acids, being characterized by the formation of a small number of base pairs that initiate the hybridization, proceeding then as a rapid zippering of the remaining nucleotides (Bruns et al., 2007). If the concentration of hybrid strands in solution is similar, the hybridization follows a second order kinetics, meaning that the higher the concentration of hybrid strands in solution, the higher the annealing rate will be (Bruns et al., 2007). However, as FISH protocols usually use probe concentration in excess relatively to the number of target sequence(s) (Yilmaz and Noguera, 2004) a pseudo-first order kinetics is applied (Bruns et al., 2007), and in this case the hybridization depends only on the concentration of the target. However, the time required to hybridize the probe to the target remains inversely proportional to the probe concentration (Bruns et al., 2007). Other variables such as target accessibility, probe length and complexity have also an impact on the hybridization (Bruns et al., 2007), but these were not of concern since the same probe (PNA EUB338) was used throughout this work.

Interestingly, analyzing the results of the optimal pH and DS concentration (Table 2), it is possible to distinguish 2 different behaviors. A higher pH, approx. 10, combined with lower DS concentration, approx. 2% (w/v), were found to be favorable for Gram-negative species (*E. coli* and *P. fluorescens*), while near-neutral pH, approx. 8, together with higher DS concentrations, approx. 10% (w/v), favored Gram-positive species (*L. innocua*, *S. epidermidis* and *B. cereus*).

The application of DS in the hybridization solution has two main effects in FISH. On the one hand, higher concentrations of DS should be favorable to FISH as they cause an apparent increase in probe concentration (Azevedo, 2005; Cmarko and Koberna, 2007). On the other hand, it is well known that DS increases the viscosity of a solution, hence decreasing molecular diffusion (Kosar and Phillips, 1995; Zustiak et al., 2011). In order to understand why DS affected differently Gram-positive and Gram-negative bacteria, we considered that the access of the probe to the target rRNA occurs in three steps: 1) diffusion on the suspension, 2) diffusion through the cell envelop (including the cell wall) and 3) diffusion in the cytoplasm. For the Gram-positive, the limiting step is possibly 2), considering that they possess a peptidoglycan layer much thicker than Gram-negative bacteria (Roller et al., 1994; Franks et al., 1998) and as such, a higher probe concentration gradient is needed. For the Gram-negative the limiting diffusion step is 1), so the increase in viscosity might be more relevant.

In order to explore this hypothesis of the interplay between viscosity and optimum DS concentration needed for the probe to overcome the thick cell wall of Gram-positive bacteria, we further tested different MW DS (besides the previously used 500 kDa in Table 1, assays 3 and 4), as the viscosity of DS molecules in solution decreases with lower MW DS (Joosse et al., 2007). At 30 °C 10% (w/v) DS of 500 kDa presents a viscosity of ≈35 mPa.s (Demetriades and McClements, 1998), whereas 10% (w/v) DS of 10 kDa presents ≈2 mPa s (Algotsson et al., 2013). So, using lower MW DS we would expect to observe an increase in the optimum DS concentration values, due to the lower viscosity of the hybridization solution obtained. The results presented in Table 3 confirmed the anticipated outcome stated above.

Lastly, the pH of the hybridization solution may also impact FISH in 2 different ways. On one side, it affects the ionization of nucleotides (Blackburn et al., 2006; Vieregg, 2010). In fact, from pH 5–9 all bases are uncharged so hybridization occurs without interference. At higher pH, guanine, uracil and thymine bases become deprotonated (pK_a 9.2–9.7), while at lower pH, adenine and cytosine bases become protonated (pK_a 3.5 and 4.2). This ultimately disfavors pairing, through an increase in electrostatic repulsion at high pHs and destabilization of hydrogen bonding (Blackburn et al., 2006; Vieregg, 2010). On the other side, pH ionizes DS molecules, which affects its viscosity (Katchalsky, 1964). This was actually confirmed by viscosity measurements at 25 ± 1 °C of DS 500 kDa 10% (w/v) solutions at pH 6, pH 9 and pH 12, having respectively 57.20 ± 0.01 mPa s, 60.38 ± 0.02 mPa s and 55.25 ± 0.03 mPa s (Fig. S1 of the Supplemental material).

Table 3
Optimum pH and DS concentration, for 500, 10 and 0.5 kDa MW molecules, in hybridization solution predicted through RSM models for Gram-positive species in study.

Dextran sulfate (MW)	Species					
	S. epidermidis		*L. innocua*		*B. cereus*	
	pH	[DS] (% w/v)	pH	[DS] (% w/v)	pH	[DS] (% w/v)
500 kDa	8.56	12.84	8.36	7.94	6.92	10.70
10 kDa	9.30	15.43	9.14	10.52	8.09	12.16
0.5 kDa	NE	NE	9.76	12.66	NE	NE

NE—Not Evaluated.

Taking into account the viscosity measurements we could argue a limiting effect of increased viscosity with pH allied to a high content in DS. Nonetheless, viscosity readings show a rather small impact on this parameter when compared with the variance in viscosity of DS with different MW. Still, when using lower MW DS (Table 1, assay 3 and 4), that produces a far less viscous hybridization solution than the one using 500 kDa, we observe a higher optimum pH for Gram-positive (Table 3) close to the ones obtained for Gram-negative species with a DS of 500 kDa.

Taking into account the results obtained we were able to reach to an optimized PNA-FISH procedure for bacteria in terms of hybridization pH, DS and probe concentration. These results can be added to previous optimization disclosed by Santos et al. (2014) to greatly improve the efficiency of the hybridization protocols used. In fact, putting all this information together, a more optimized PNA-FISH hybridization procedure can be obtained in accordance to the properties of the target bacteria (Table 4).

It is possible that the optimized conditions of pH, dextran sulfate and probe concentration can be applicable to all protocols using PNA probes. Nonetheless, some minor adjustments to the optimum conditions described here cannot be excluded. We should point out that in order to access the effects of the conditions under study we worked with a simplified hybridization solution, so changes in composition and viscosity or even probe length will impact PNA-FISH outcome. It is also important to notice that the optimization described here is not applicable to DNA, RNA and other nucleic acid mimics probes such as LNA or 2′OMe RNA, as their molecular structure differs markedly from PNA oligonucleotides (Cerqueira et al., 2008).

4. Conclusions

While optimum values/concentrations were obtained for the three parameters under study, an important observation of the present work was how pH and dextran sulfate interplay, affecting the probe gradient and consequently the hybridization efficiency. In Gram-positive bacteria, differently from Gram-negative species, a compromise between pH and DS concentration should be taken into consideration in order to maximize the hybridization efficiency (Fig. 3).

Table 4
Optimized hybridization variables for PNA-FISH in 5 Gram-positive and Gram-negative species, by RSM, obtained in this work and reported in Santos et al. (2014).

	Variable	Time (min)	Temperature (°C)	Formamide (% v/v)	pH	DS (% w/v)	Probe (nM)
Bacteria	*E. coli*	55	60	5.5	10	2	≥300
	P. fluorescens						
	L. innocua				8	10	
	S. epidermidis						
	B. cereus	120		49.5			

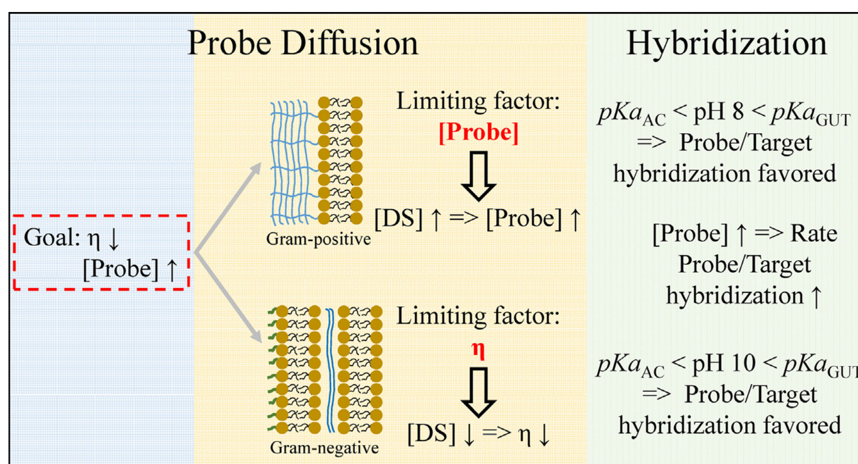

Fig. 3. Schematic illustration showing the influence of pH, DS and probe concentration in PNA-FISH for Gram-positive and Gram-negative species. The identification of the limiting factor for Gram-positive and Gram-negative bacteria regarding probe diffusion inside the cell and the adjustment in terms of [DS] needed in order to maximize it. The η stand for viscosity and the A; C; G; U and T in front of *pKa* stand for the Watson-Crick nucleotide bases.

Bacteria with thick peptidoglycan cell walls are harder to permeabilize (Roller et al., 1994), so a higher probe gradient between the extracellular environment and the cell cytoplasm is necessary to improve probe diffusion through the cell wall. This is accomplished using high concentrations of probe, 300 nM, and DS. The concentration of DS is, however, limited by the viscosity conferred by this molecule to the hybridization solution and in some extent by the pH. If the viscosity is too high, the diffusion of the probe in solution will be the limiting step, if it is too low, the probe gradient driving its diffusion across the cell envelope will be the limiting step for hybridization. So, a balance of DS and pH should always be considered for an efficient hybridization and this work might be used as a guideline according to the bacteria properties. Future work can expand the scope of this optimization to other steps of the FISH procedures, to a broader range of microorganisms, including species from the other two Domains, *Archea* and *Eukarya* and eventually, to a set of different nucleic acid mimic probes.

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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.2016.03.047.

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