



**CPS 2013 RFP
FINAL PROJECT REPORT**

Project Title

Feasibility of using nitric oxide donors to disperse biofilms of industrial significance to strengthen the efficacy of current industrial disinfectants

Project Period

January 1, 2014 – December 31, 2014

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Objectives

Objective 1. To establish which of the NODs is most effective at low concentration in disrupting existing biofilms formed on polypropylene, polystyrene and stainless steel by mixtures of Salmonella enterica pathogenic E. coli and Listeria monocytogenes isolated from recent outbreaks

Objective 2. To test whether hydrogels enriched with NODs associated with quaternary ammonium salts or chlorine dioxide possesses a synergistic effect in promoting biofilm dispersion from plastics and stainless steel

Funding for this project provided by the Center for Produce Safety through:

CPS Campaign for Research

FINAL REPORT

Abstract

Protected by extracellular polymers, microbes within biofilms are significantly more resistant to chlorine and other disinfectants. As a result, several disinfectants fail to completely eradicate biofilms from food contact surface materials. Current research has been instrumental in identify nitric oxide donors and hydrogels as potential additives to disinfectant. Nitric oxide donors are considered very promising molecules as biofilm dispersal agent and hydrogels have recently attracted a lot of interest due to their chemical properties. With this proof-of-concept proposal, we compared the ability of five nitric oxide donors (molsidomine, MAHMA NONOate, diethylamine NONOate, diethylamine NONOate diethylammonium salt, spermine NONOate) to dislodge biofilms formed by non-typhoidal *Salmonella enterica* and pathogenic *Escherichia coli* on plastic and stainless steel surfaces at different temperatures. All five nitric oxide donors induced significant (15-80%) dispersal of 24-hour old biofilms, however, the degree of dispersal and the optimal conditions varied. MAHMA NONOate and molsidomine were strong dispersants of the *Salmonella* biofilms formed on polystyrene. Importantly, molsidomine induced dispersal of up to 50% of the pre-formed *Salmonella* biofilm at 4°C, suggesting that it could be effective even under refrigerated conditions. Biofilms formed by *E. coli* O157:H7 were also significantly dispersed. Nitric oxide donor molecules were highly active within 6 hours of application.

We also measured the additive effect of molsidomine and MAHMA NONOate with the industrial disinfectant SaniDate 12.0. The association SaniDate 12.0 and nitric oxide donor was ~15% more effective in removing the preformed biofilm when compared with SaniDate treatment only. Finally, nitric oxide donors were also tested on well structured biofilms (1-week old). When MAHMA NONOate was dissolved in phosphate saline buffer (PBS), it was able to reduce the biomass of well-established biofilms up to 15% for at least 24 hours of contact time. No significant results were measured when the contact time was decreased to 6 hours. The association of cellulose nanocrystals (CNC) with the nitric oxide donors MAHMA NONOate and molsidomine has shown a synergistic effect in dispersing well-established biofilms: After 2 hours of exposure moderate but significant dispersion was measured. After 6 hours of exposure, the number of cells switching from the biofilm to the planktonic state was up to 0.6 log higher when compared with the not-treated. Because diffusion is the predominant transport process within hydrogels and cell aggregates, we measured the nitric oxide diffusion, at 25°C, of 0.1 μM MAHMA NONOate-CNC. Nitric oxide diffuses up to 500 μm from the source, with a nitric oxide flux decreasing 0.1 $\text{pmol cm}^{-2} \text{sec}^{-1}$ every 100 μm of distance from the capillary opening. With reference on the releasing time, 60% of the nitric oxide is released as gas during the first 23 minutes.

At the end of this proof-of-concept project, we can definitively confirm the effectiveness of nitric oxide donors as biofilm dispersant in association with industrial disinfectants and hydrogels (cellulose nanocrystals) on surfaces of industrial interest. This, in turn, can implement new sustainable cleaning strategies by expanding the tool-kit of pro-active practices for GAPs, HACCP and Cleaning-in-place (CIP) protocols.

Background

In post-harvest handling facilities where produce is washed, processed or otherwise handled using aqueous solutions, biofilms could be particularly problematic. Encased in EPS, human and plant pathogens within biofilms are significantly more resistant to chlorine and other disinfectants^{1,2}. Several disinfectants, antibiotics and messenger molecules have been studied

for their ability to dislodge existing biofilms. Of these, nitric oxide appears to be very promising in industrial applications and it can be released by using nitric oxide donors. The release of nitric oxide occurs with different speeds and has different effectiveness according with the molecules used, the temperature applied and the kind of biofilm treated. Biofilms formed on washing systems in produce production facilities can be recalcitrant reservoirs of human pathogens, which are difficult to control and can potentially originate costly outbreaks³. Pathogens in biofilms are resistant to common disinfectants and contribute to increase of the potential risk of produce contamination posed by the use of sanitization water that may have been potentially contaminated^{1,2}. Several disinfectants, antibiotics and messenger molecules have been studied for their ability to dislodge existing biofilms⁴⁻⁶. Of these, nitric oxide appears to be very promising, and nitric oxide donors are currently used clinically. Nitric oxide is effective as a biofilm dispersant, functioning as a messenger rather than a generic poison⁴⁻⁷. It can be delivered to biofilms by using donor molecules⁸ and the application of nitric oxide donors has the same effect as the direct addition of nitric oxide gas⁹. In bacteria, nitric oxide is a signaling molecule active at very low concentrations and able in dispersing preformed biofilms at nano- or picoMolar concentration, indicating that truly minute quantities of the chemical are sufficient for dislodging much of the biofilm⁴⁻⁷. Nitric oxide could have a universal effect on dispersal of bacteria biofilm including both Gram-positive and Gram-negative bacteria¹⁰. The mechanisms of biofilm dispersion are not clear, but it appears to function in the transition of sessile biofilm organisms to free-swimming bacteria. For example, microarray studies have revealed that genes involved in adherence are downregulated in *P. aeruginosa* upon exposure to nitric oxide donors¹¹.

Over 105 nitric oxide donors have been characterized, and 6 have been currently tested as biofilm dispersal. Among these, the main studied are the sodium nitroprusside,^{4, 7, 12, 13} molsidomine, diethylamine NONOate diethylammonium, NONOate diethylammonium, MAHMA nonoate [studied in this seed project]⁷ and NO-releasing silica nanoparticles¹⁴. From these data, MAHMA NONOate is one of the best candidate in biofilm dispersion: It spontaneously dissociates in a pH/temperature dependent manner, in serum it has a half-life of 1 minute and 3 minutes at 37°C and 22-25°C respectively, at pH 7.4 to liberate 2 moles of NO per mole of parent compound^{8, 15, 16}.

Nitric oxide donors can be associated with other disinfectants obtaining a synergistic effect in terms of biofilm dispersion. For example, a 20-fold increase in the efficiency was observed when nitric oxide was applied together with chlorine in removing multispecies biofilms⁹. We used this foundation to hypothesize that nitric oxide donors can also be applied to hydrogels and other disinfectants in order to obtain a synergistic effect in terms of biofilm dispersal. In this project we used the Sanidade 12.0, one of the most common disinfectant used in industry.

As hydrogels we used cellulose nanocrystals (CNC). Hydrogels have recently attracted a lot of interest due to high strength/stiffness, optical transparency, biocompatibility, biodegradability, and highly porous structural network^{17, 18} and the ability to carries bactericidal molecules¹⁹⁻²¹. Hydrogels are water-swollen, and cross-linked polymeric network produced by the simple reaction of one or more monomers²². The industrial applications of hydrogels are extremely heterogeneous, ranging from agriculture, drug delivery systems, food additives, to biomedical applications and separation of biomolecules^{22, 23}.

Among the hydrogels, cellulose nanocrystals (CNC) is one of the most promising polymer because it is an eco-friendly product, cheap and safe²⁴. CNC has high crystallinity, high water holding capacity and excellent mechanical and thermal properties²⁴. For our scope, CNC has an ionic charged surface appropriated for attaching the biofilm outer extracellular polymeric substances (EPS)^{7, 24, 25}.

Research Methods and Results

Objective 1.

For detailed research methodologies please refer to Marvasi *et al.* 2014⁷.

1. Biofilm dispersal by molsidomine.

Molsidomine releases nitric oxide and forms polar metabolites rapidly; its half-life is 1 to 2 hours in plasma at pH 7.4²⁶. In terms of biofilm dispersal, molsidomine was one of the most potent molecule. It was effective in dislodging biofilms formed by *S. enterica* sv Typhimurium 14028, the cocktail of the six *Salmonella* outbreak strains and *E. coli* O157:H7. Molsidomine was particularly effective on polypropylene (Fig. 1). It was most effective at 22°C, inducing dispersal of ~50% of biofilms formed by *Salmonella* 14028 and the cocktail of six *Salmonella* outbreak strains after incubation for 6 hours (Fig.1, $p < 0.0001$). Up to 75% of biofilm dispersal was observed after a 24-hrs treatment of *E. coli* O157:H7 biofilms with molsidomine (Fig. 1, $p < 0.0001$). Biofilm dispersal by molsidomine was also observed on polystyrene (Table 1). *Salmonella* biofilms preformed in polystyrene wells were dislodged after 24 hours of contact time at room temperature. Molsidomine was able to induce some dispersal even when biofilms were treated with the compound at 4°C. Intriguingly, the strongest dispersal was observed in response to the treatment with 10 picomolar concentrations of molsidomine (Fig. 1). Such potency of the compound at very low concentrations makes it a potentially interesting candidate for commercial applications.

To test whether the decrease in the staining of the attached cells was caused by an increase in the number of planktonic cells, biofilms formed by fluorescent *Salmonella* 14028 pGFP-ON on polypropylene were treated with molsidomine. Fluorescence of the planktonic and attached cells was measured after 0, 3 and 6 hours of exposure to the nitric oxide donor. The increase in total fluorescence of planktonic cells upon treatment of biofilms with molsidomine was statistically significant (Table 1), reflecting an increase in detachment at the tested concentrations. Similarly, molsidomine treatment of the *Salmonella* biofilms formed on stainless steel resulted in ~0.3 log increase in fluorescence of the planktonic cells (data not shown). However experiments with stainless steel were not further performed due to the difficulty to measure the detachment from such material. In our experimental conditions the *Salmonella* biofilm formed on stainless steel were very strong and not always consistent in terms of variability within the replicas.

The ability of the constitutively luminescent *Salmonella* construct driven by a phage promoter to produce light in the presence of molsidomine was used as an indirect assessment of the toxicity of the compounds, and its ability to generally disrupt metabolism or respiration of the

□□ promoter

bacteria. Our result showed that even though modest decrease in luminescence was observed in molsidomine-treated cultures, the reduction of luminescence was not greater than 1-10% of the control ⁷.

2. Dose-dependent biofilm dispersal by diethylamine NONOate diethylammonium.

Diethylamine NONOate diethylammonium represents a class of molecules which spontaneously dissociate in a pH-dependent manner, with a half-life of 16 minutes at 22-25°C, pH 7.4 to liberate 1.5 moles of NO per mole of the parental compound ^{15,27}. Biofilm dispersal in response to diethylamine NONOate diethylammonium was dose-dependent (Fig. 2), however the relationship was inverse: low concentrations of the nitric oxide were associated with higher biofilm dispersion. The strong activity in the picomolar range is similar to what was observed with molsidomine.

Diethylamine NONOate diethylammonium was potent at dispersing biofilms on polypropylene following a 6-hours incubation. A strong dispersal of biofilms formed by *Salmonella* ATCC 14028 on polypropylene was observed (Fig. 2). Biofilms formed by *E. coli* O157:H7 were also dispersed by diethylamine NONOate diethylammonium, but the dispersal was lower when compared with *Salmonella*. The biofilm formed by the cocktail of six *Salmonella* outbreak strains formed on polystyrene was also dispersed (Fig. 2).

3. The effect of diethylamine NONOate sodium salt hydrate, MAHMA NONOate and spermine NONOate on biofilms.

Of the tested compounds, diethylamine NONOate sodium salt was the least effective biofilm dispersant following 24 and 6 hours incubation. The strongest dispersion was observed in biofilms formed on polypropylene by the cocktail of *Salmonella* strains and *Salmonella* Typhimurium ATCC 14028 (Figure 3). Up to 50% of reduction of biofilm was detected at room temperature for *Salmonella* 14028 and the outbreak strains on polystyrene (Fig. 3). On polystyrene, biofilm formation by the cocktail of the *Salmonella* strains was reduced by up to 30% when compared with the control. Diethylamine NONOate was also active in dispersing biofilms formed by *E. coli* O157:H7, even though the dispersion was very limited (~20% when compared with the control) ⁷.

The effectiveness of MAHMA NONOate (which has half-life of minutes) was compared to that of spermine NONOate, which has half-life of several hours. This affects potency and sustainability of the treatment, for MAHMA NONOate showing up to 40 times the potency of spermine NONOate, but the latter showing activity for hours, compared with few minutes for MAHMA NONOate ⁸. MAHMA NONOate is also an optimal dispersant of preformed biofilm of *Pseudomonas aeruginosa* biofilm ²⁸. Therefore, the two molecules were compared within 6 hours of incubation with *Salmonella* and *E. coli* biofilms. As expected MAHMA NONOate was a more effective dispersant on both the materials ⁷. However, spermine NONOate was mainly effective at releasing biofilms formed on polypropylene ⁷.

MAHMA NONOate also appears to be a broadly active molecule in dispersing biofilm formed both on polypropylene and polystyrene (Fig. 4): it released up to 70% of *E. coli* biofilms pre-

formed on polystyrene at room temperature after a 6-hour treatment (Fig. 4). Biofilms formed by *S. enterica* sv Typhimurium ATCC14028 were also dispersed by MAHMA NONOate at room temperature, but compared to spermine NONOate, it was more effective on polystyrene (Fig. 4). On polypropylene, up to 50% of *S. enterica* sv Typhimurium ATCC14028 and the cocktail of six *Salmonella* outbreak strains biofilms were dispersed by MAHMA NONOate when incubated for 24 hours at room temperature (Fig. 4). Biofilms formed by *E. coli* O157:H7 were also effectively dispersed on polypropylene but less when compared with polystyrene (Fig.4).

4. *The effect of NOC-5 on preformed biofilms of Listeria innocua.* As preliminary experiments, *Listeria monocytogenes* was replaced with the not pathogenic *Listeria innocua*. The nitric oxide donor 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) was active in dispersing biofilm formed both on polystyrene at 4°C and 22°C: it released up to 15% of *Listeria* after a 24-hour treatment.

Objective 2.

5. *Additive effect of molsidomine and the donor MAHMA NONOate and the industrial disinfectant SaniDate 12.0.*

Quaternary ammonium salts or chlorine dioxide were replaced with Sanidate 12.0, one of the most used disinfectant in industry. To determine whether there is an additive effect of molsidomine and the donor MAHMA NONOate in association with this disinfectant, biofilms of *S. Typhimurium* ATCC14028 were established on polystyrene surfaces in Colonization Factor Antigen medium for 24 hours. The biofilms were pretreated for 6 hours with 10nanaMolar of Molsidomine or MAHMA NONOate. After the treatment wells were exposed to SaniDate 12.0 (BioSafe System, Hartford, CT, USA) as per manufacturer's recommendations. Treated biofilms were stained with 1% crystal violet, washed and the absorbed crystal violet in the biofilm was dissolved in 33% acetic acid and measured with a spectrophotometer. The association SaniDate 12.0 and nitric oxide donor was ~15% more effective in removing the preformed biofilm when compared with SaniDate treatment only (Figure 5).

Experiments on stainless steel were not performed at this time.

6. *Synergistic effect of the association of nitric oxide donors with cellulose nanocrystals (CNC).*

The effect of MAHMA NONOate as biofilm dispersal was previously assessed in our recent publication in a range of biofilm-forming microorganisms of industrial and/or clinical significance including *Salmonella enterica*⁷. Our previous efforts were focused on studying young biofilms (maximum 24 hours old) and exposure time up to 6 hours. Because concerning on more applicative approaches of the nitric oxide donor technology, we tested the nitric oxide donors on well-structured biofilms (1 week old biofilms) and for a shorter contact time (up to 1 hour of exposure).

As first approach, we exposed a 24-hours old *Salmonella* biofilm for a minimum of 2 hours to different concentrations of MAHMA NONOate dissolved in PBS (10 µM, 10nM and 10pM): no significant results were measured (Fig 6, A). The experiment was replied with the nitric oxide donor molsidomine observing a similar result (Fig.6, B). In further experiments, MAHMA

NONOate or molsidomine were dissolved into cellulose nanocrystals (CNC) hydrogel to 10 μ M (CNC-NONOate or CNC-molsidomine) to test whether the association of CNC was instrumental in increasing the dispersion potential of the nitric oxide donors within the 2 hours.

CNC-nitric oxide preparation. 1 mmol L⁻¹ stock solutions of MAHMA NONOate or molsidomine were prepared in phosphate-buffered saline, pH 7.3 (PBS, Fisher, Waltham, MA, USA). Final solutions to 1 μ M were prepared by dispersing the nitric oxide stock solution in an opportune volume of CNC. The solution was further vortexed for 30 seconds and applied of prewashed biofilm formed into polypropylene plates.

A fluorescent *Salmonella* 14028 pGFP-ON biofilm preformed on polypropylene was treated with 10 μ M NONOate-CNC or 10 μ M molsidomine-CNC. Biofilms dispersion treated with CNC (or control, CNC+PBS) was measured by directly monitoring the increase of fluorescence of planktonic cells of *S. Typhimurium* 14028 pGFP-ON. At the end of the period of exposure, 170 μ L of CNC-nitric oxide solution were transferred into a black 96-wells plate and measured by using Victor-2 multimode plate reader with a 485nm/535nm excitation/emission filter (Perkin Elmer, Waltham, MA, USA). Increasing of fluorescence represent an increase of detached cells. Fluorescence of the planktonic cells was measured after 2 hours and 1 hour of exposure to the nitric oxide donors. The increment of cells switching to the planktonic state was significant after 2 hours of exposure (Fig. 6 C), but not significant after 1 hour of exposure (Fig. 6 D).

To measure the responsiveness of a well-established biofilm such as a 1-week old biofilm, *Salmonella* 14028 biofilms were exposed to different concentrations of MAHMA NONOate dissolved in PBS for 24 and 6 hours. 24 hours of exposure to MAHMA NONOate was able to reduce the biomass up to 15% (Fig. 7, A). 6 hours of exposure was instead not significant (Fig. 7, B). As before, we tested whether the association of CNC was instrumental in increasing the dispersal potential of the nitric oxide donor. MAHMA NONOate and molsidomine were dissolved into CNC at a concentration of 10 μ mol L⁻¹. Interesting total fluorescence of planktonic cells increased significantly in the wells with biofilms treated with both the donors (Fig. 7, C and D) reflecting an increase in cellular detachment at the tested concentrations. The biofilm exposed for 2 hours showed an average of 0.3 log increasing in fluorescence when compared with the control (PBS) (Fig. 7, C). When the same treatment was extended for 6 hours, a difference up to 0.6 log was measured (Fig. 7, D). One-hour exposure was also tested but it was not significant (data not shown).

7. Releasing profile of MAHMA NONOate in BPS and CNC. Co-PI Eric McLamore.

This part of the project has been completed in collaboration with the CoPI Dr. McLamore. Nitric oxide diffusion into CNC-MAHMA NONOate has been studied by using nitric oxide-specific microelectrodes. During operation, nitric oxide is oxidized at the surface of the electrode, generating the nitrosonium cation (NO⁺) and one free electron; free electrons are detected as current (Eq. 1). Electrodes were calibrated by using different concentration of nitric oxide and within ten minutes.



In order to better understand the mass transfer of nitric oxide from the hydrogel, a nitric oxide

microelectrode was positioned at the surface of the hydrogel to measure surface concentration, flux, and release rate of nitric oxide. At 25°C, 0.1 μM the nitric oxide dissociated from the MAHMA NONOate in CNC diffused up to the first 400 μm from the capillary open (Fig. 8, A). When the MAHMA NONOate in CNC was diluted up to 1:10 the detection of the nitric oxide was up strongly reduced and detected up the first 100 μm from the capillary open (Fig. 8 A). At 25°C, 0.1 μM MAHMA NONOate diffused in CNC up to 500 μm from the capillary opening, with a nitric oxide flux decreasing roughly 0.1 $\text{pmol cm}^{-2} \text{sec}^{-1}$ every 100 μm of distance from the capillary opening (Fig 8 B). 1:10 dilution of MAHMA NONOate in CNC significantly reduced the flux and diffusion as expected (Fig. 8 B).

Although the exact amount of nitric oxide liberated within biofilms from nitric oxide donors have not yet been established, we measured the releasing profile of nitric oxide occurring from the CNC hydrogel enriched with MAHMA NONOate on a time scale. MAHMA NONOate was initially dissolved in phosphate-buffered saline (PBS) to a concentration of 500 mM, and then it was dissolved in CNC at a final concentration of 1 μM . A nitric oxide electrode was used to observe the releasing profile (Fig. 9). Our measurements (three replicas) showed that 60% of the nitric oxide is released as gas during the first 23 minutes (~1400 seconds). The electrode did not measure 1:10 dilution of MAHMA NONOate in CNC, as probably the flux was below the electrode sensitivity.

8. The *recA-hydN* genomic region is involved in *Salmonella* biofilm dispersal. In order to better understand how nitric oxide donors induce dispersal of *Salmonella* biofilms, the genome of *S. Typhimurium* 14028 was scanned for the homologs of known genes involved in nitric oxide signaling. A mutant lacking the ~15kB *Salmonella enterica* sv Typhimurium ATCC14028 genomic region spanning 15 genes (*recA-hydN*) was studied (Fig. 10). This region includes putative NO-reductase machinery: *ygaA*, an anaerobic nitric oxide reductase transcriptional regulator; STM2840, an anaerobic nitric oxide reductase flavorubredoxin; *ygbD*, nitric oxide reductase; *hypF*, hydrogenase maturation protein; *hydN*, an electron transport protein HydN. Proteins encoded region can be involved in transferring electron to NO, its detoxification and generation of nitrous oxide. Consistent with the predicted functions of the deleted genes, the Δ *recA-hydN* mutant formed a more robust biofilm on polystyrene compared to the wild type, and did not respond to the 24-hrs treatment with molsidomine (Fig 10).

Outcomes and Accomplishments

At the end of this proof-of-concept project we have produced the following outcomes:

- We increased the current scientific knowledge on the effectiveness of nitric oxide donors as dispersal agents and in association with i) disinfectants and ii) hydrogel cellulose nanocrystals. At the end of this project the following nitric oxide donors have been characterized: molsidomine, MAHMA NONOate, diethylamine NONOate, diethylamine NONOate diethylammonium salt, spermine NONOate. Molsidomine and MAHMA NONOate were identified as the best candidates.

- Our results have been published in one peer-reviewed paper and another paper is in preparation. We expect the publication of the second paper during the first half of 2015. We presented our work in 9 communications at different conferences.
- We attracted the interest of sanitization companies on the nitric oxide technology and established connection for future collaborations.
- Preliminary results from this proof-of-concept project were used as preliminary findings to support full grants.

Summary of Findings and Recommendations

The scientific findings are summarized in five key-points and they are supported by recommendation for their future development.

1. *Dispersal potential.* All the nitric oxide donors tested were effective as biofilm dispersal. All five nitric oxide donors induced significant (15-80%) dispersal of 24-hour old biofilms, however, the degree of dispersal and the optimal conditions varied when dissolved in phosphate saline buffer. Some donors are more efficient than others, for example moldisomine and MAHMA NONOate have shown the strongest activity, possibly due to their chemical structure. In addition, the donors showed different dispersion potentials when used in different conditions (for example different temperatures, contact times, or materials affected the biofilm dispersal). Further research should be focused in characterizing the more effective chemical structures and the best conditions for the treatments.
2. *Association of nitric oxide donors with other molecules.* Our research has demonstrated the synergistic effect of the nitric oxide donors moldisomine and MAHMA NONOate in association with disinfectant and cellulose nanocrystals hydrogel (CNC). This aspect is extremely important in industry: for example the association with CNC has proved to reduce the effective contact time up to 2 hours on well established biofilms (1 week-old *Salmonella* biofilms). The association with molsidomime and MAHMA NONOate with the disinfectant Sanidate12.0 has also shown that the association was ~15% more effective in removing the preformed biofilm when compared with the Sanidate 12.0 treatment only. Further research should focus on the study of the association of donors with other disinfectants, hydrogels and foaming solutions.
3. *Nitric oxide diffusion* (in collaboration with the Co-PI Dr. Eric McLamore). Diffusion is the predominant transport process within hydrogels and cell aggregates. To that end, we measured the nitric oxide diffusion of nitric oxide in MAHMA NONOate-CNC. We characterized the flux, diffusion, and the releasing time; all these data are important in order to define the chemical characteristic of this association and to predict the nitric oxide release profile in different conditions. However, for a better understanding of nitric oxide diffusion we advise that future research should be focused on the measurement of nitric oxide penetration into the biofilm under various donor exposures.
4. *Genetics.* In this project we established that the *recA-hydN* genomic region is involved in *Salmonella* biofilm dispersal. Further genetic studies need to well characterize this region and clarify the metabolic cascade upon nitric oxide perception. Further research should be addressed to determine how physico-chemical properties of surfaces,

temperatures and nitric oxide donors contribute to the regulation of *Salmonella* regulatory and structural determinants during nitric oxide-mediated biofilm dispersal.

5. *Clinical trials*. Nitric oxide donors are not considered as “Generally Recognized As Safe” (GRAS). Even though some donors are used as therapeutic agents for treatment of cardiovascular diseases, the concentrations we are showing as effective in biofilm dispersal are about 10^{-7} less than the therapeutic dose. It is important to recognize that it is almost certain that no GRAS compound or an herbal extract would ever be cost-effective and efficient for disrupting industrial biofilms. Clearly, addressing potential toxicity of nitric oxide donors will be an important consideration before proceeding to scale up.

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- 27 Maragos CM, Morley D, Wink DA *et al*: Complexes of NO with nucleophiles as agents for the controlled biological release of nitric oxide: Vasorelaxant effects. *J Med Chem* 1991; **34**: 3242-3247.
- 28 Barnes RJ, Bandi RR, Wong WS *et al*: Optimal dosing regimen of nitric oxide donor compounds for the reduction of *Pseudomonas aeruginosa* biofilm and isolates from wastewater membranes. *Biofouling* 2013; **29**: 203-212.

APPENDICES

Publications and Presentations (required)

Peer reviewed publication (one in preparation):

1. **Marvasi M**, Chen C, Carrazzana M, Durie I, Teplitski M. 2014 Systematic analysis of the ability of Nitric Oxide donors to dislodge biofilms formed by *Salmonella enterica* and *Escherichia coli* O157:H7. *Applied Microbiology and Biotechnology Express*. DOI:10.1186/s13568-014-0042-y. (see attached pdf “Marvasi et al 2014”)
2. **Marvasi M**, Durie AI, Vanegas D, McLamore E. 2015 *Salmonella enterica* biofilm-mediated dispersal by nitric oxide donors in association with cellulose nanocrystals. In preparation for *FEMS Microbiology Letters*.

Conferences communications:

- 1) Durie I, Teplitski M, Marvasi M. 2014 “Genes involved with Nitric Oxide Biofilm Dispersal” Florida Genetics. October, University of Florida Symposium.
- 2) Durie I, Teplitski M, Marvasi M. 2014 “Genes involved with Nitric Oxide Biofilm Dispersal” UF Soil and Water Science Research Forum. September, Gainesville, University of Florida
- 3) Marvasi M, McLamore ES, Vanegas D, Durie IA, Teplitski M. 2014 Nitric oxide donors as molecules capable of effectively disperse biofilms formed by *Salmonella*, *Listeria*, pathogenic *E. coli* and *Pectobacterium carotovorum*. Southeastern and Florida ASM Branches Meeting. Ponte Vedra, FL, September 2014.
- 4) Marvasi M, Durie IA, Jenkins KT, Teplitski M. 2014 Nitric oxide donors as molecules capable of effectively disperse biofilms formed by *Salmonella*, *Listeria*, pathogenic *E. coli* and *Pectobacterium carotovorum*. Southeastern and Florida ASM Branches Meeting. Ponte Vedra, FL, September 2014.
- 5) M Marvasi. 2014 Feasibility of using nitric oxide donors to disperse biofilms of industrial significance to strengthen the efficacy of current industrial disinfectants. Center For Produce Safety Meeting. 24-25 June. Newport, CA, USA.
- 6) Jenkins TK, Durie I, Teplitski M, Marvasi M 2014 Nitric Oxide (NO) Signaling in *Salmonella*: Genes Involved in Biofilm Dispersion. Florida Association for Food Protection 28-30 May Sand Key in Clearwater Beach, FL.
- 7) Durie I, Marvasi M, Teplitski M 2014 Feasibility of Using Nitric Oxide Donors on Stainless Steel. Florida Association for Food Protection 28-30 May Sand Key in Clearwater Beach, FL.
- 8) Marvasi M, Jenkins K, Durie AI, Teplitski M. 2014. Nitric oxide (NO) donors to disperse biofilms of industrial significance formed by *Salmonella enterica* and *Listeria sp.* ASM General Meeting 17-20 May. Boston.
- 9) Jenkins KT, Teplitski M, Marvasi M. 2014. Nitric Oxide (NO) Signaling in *Salmonella*: Genes Involved in Biofilm Dispersion. University of Florida. Undergraduate Research Symposium, March 27, 2014.

Budget Summary (required)

Funding was sufficient to successfully accomplish the project. Detailed fiscal report will be provided by the University of Florida fiscal office.

Tables and Figures (optional)

Table 1. Fluorescence of the *Salmonella* cells detached by molsidomine treatment. Measurement of *Salmonella* 14028 planktonic cells detached from preformed biofilm during treatment with molsidomine.

Conc.	Log (Fluorescence 485nm/535nm)			P<0.05
	0 hours	3 hours	6 hours	
<i>Biofilms formed on polypropylene</i>				
0	4.335 ± 0.065	n.a.	4.450 ± 0.177	0.5492
10 µM	4.307 ± 0.067	4.846 ± 0.054	4.466 ± 0.080	<.0001*
10 nM	4.275 ± 0.055	4.780 ± 0.050	4.753 ± 0.075	<.0001*
10 pM	4.355 ± 0.064	4.815 ± 0.125	5.002 ± 0.144	0.0024*

*Statistically significant effects (p<0.05) of molsidomine treatment.

The effect of molsidomine on biofilms

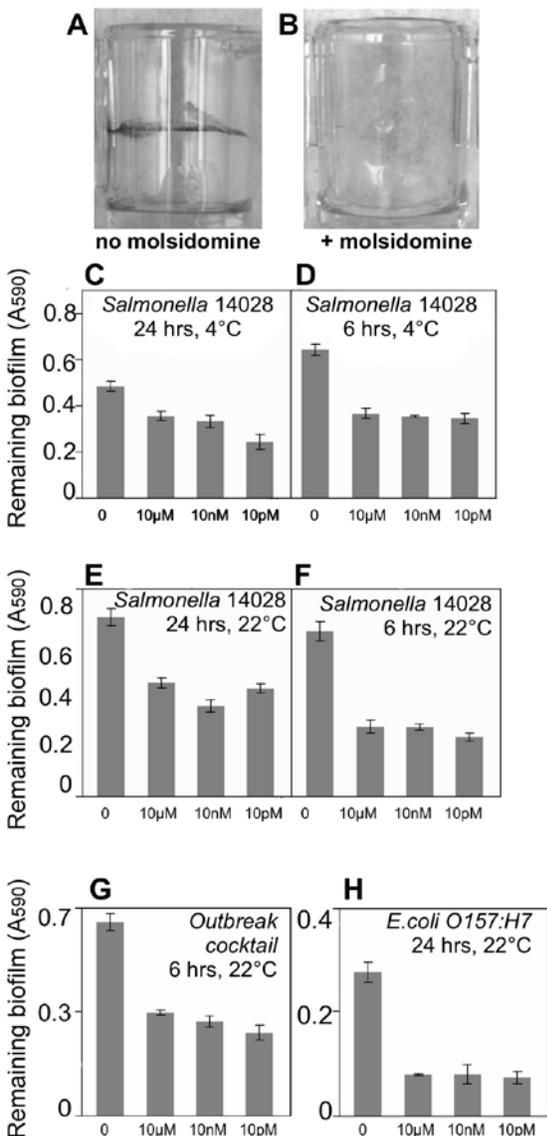


Figure 1. Biofilm dispersal by molsidomine.

Exposure to molsidomine doses ranging from 10µM to 10pM induced biofilm dispersal as measured by staining of the attached cells with crystal violet. “0” indicates control, an existing biofilm not treated with molsidomine. **A.** Biofilm formed by *S. enterica* sv. Typhimurium 14028 in wells of a microtiter plate before treatment with molsidomine. Attached cells are visualized with crystal violet staining. **B.** Residual biofilm after treatment with 10 nM of molsidomine. **C→H.** Induction of biofilm dispersal by molsidomine. Biofilms were pre-formed on polystyrene. Contact time and temperature at which biofilms were exposed to the chemical are listed above each panel. Concentrations of molsidomine are indicated on the x-axis. Error bars are standard errors.

Biofilm dispersal by diethylamine NONOate diethylammonium

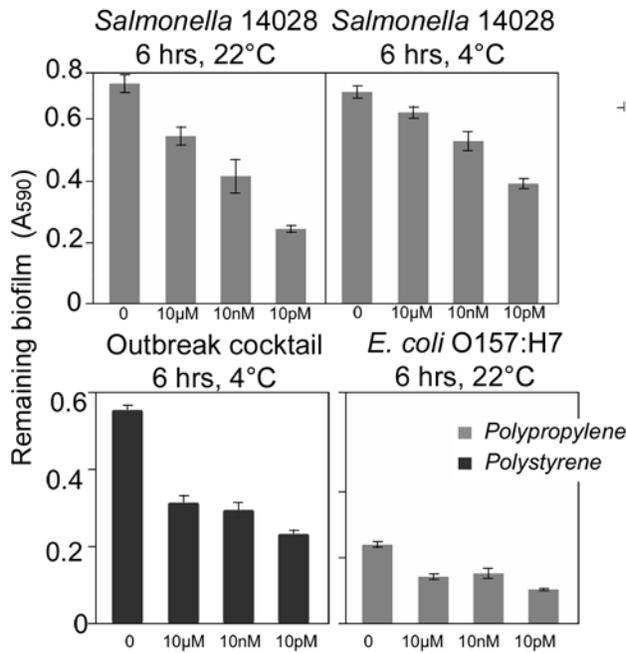


Figure 2. The effect of diethylamine NONOate diethylammonium on biofilms. Remaining biofilms were measured by crystal violet staining, which was solubilized in diluted acetic acid and the absorbance at 590 nm was measured. Concentrations of the nitric oxide donor are on the x-axis. Contact time and temperature at which pre-formed biofilms were treated with diethylamine NONOate diethylammonium are indicated above each panel.

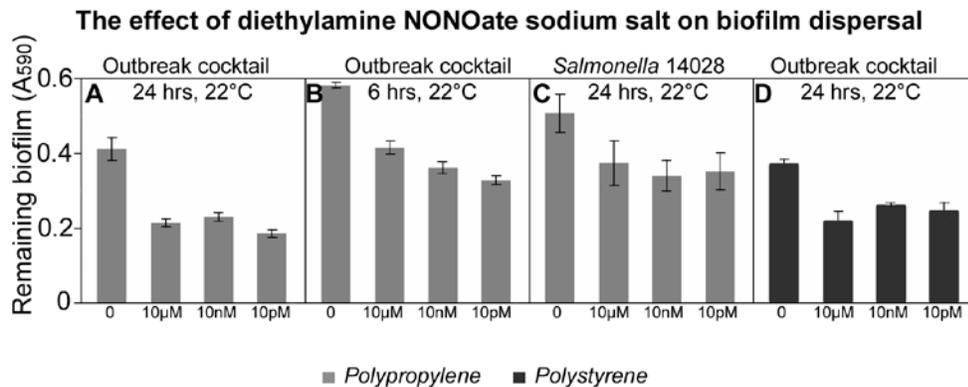


Figure 3. Dispersal of Salmonella biofilms by diethylamine NONOate sodium salt. Biofilms were formed overnight by either *S. enterica* sv.

Typhimurium ATCC14028 or a cocktail of six *Salmonella enterica* strains (sv. Braenderup 04E01347, Braenderup 04E01556, Braenderup 04E00783, sv. Montevideo LJH519, sv. Javiana ATCC BAA-1593 and sv. Newport C6.3) linked to produce-associated outbreaks of gastroenteritis. Biofilms were pre-formed on either polystyrene (black bars) or polypropylene (grey bars) prior to the treatment with diethylamine NONOate sodium salt. Concentrations of the nitric oxide donor are on the x-axis. Contact time and temperature at which the treatment

took place are indicated above each panel. Residual biofilms were quantified by staining with crystal violet. Error bars are standard errors.

Dispersal of biofilms by MAHMA NONOate

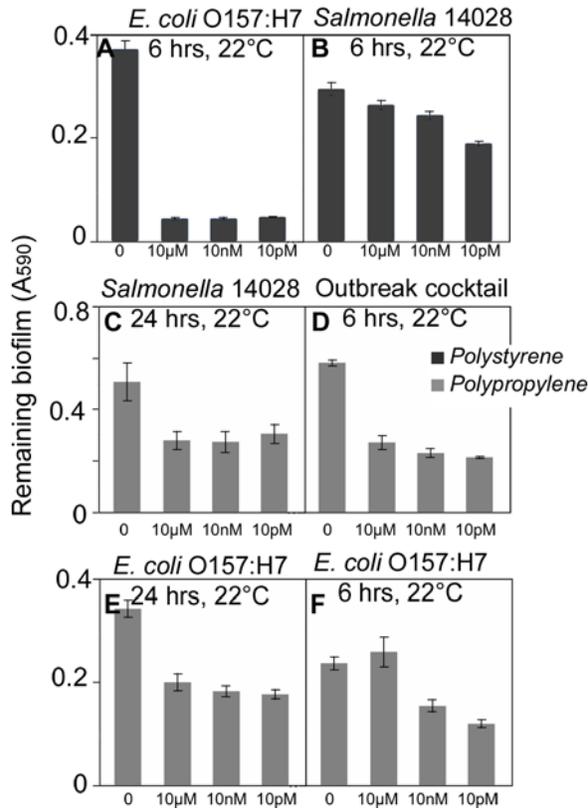


Figure 4. Biofilm dispersal by MAHMA NONOate. Biofilms were formed by *E. coli* O157:H7, *Salmonella* sv.

Typhimurium 14028 and a cocktail of the six *Salmonella* strains linked to human produce-related gastroenteritis outbreaks on polystyrene (black bars) or polypropylene (grey bars) prior to the treatment with MAHMA NONOate.

Contact times and temperatures at which biofilms were exposed to the nitric oxide donor are listed above each panel. Concentrations of the nitric oxide donor are on the x-axis.

Effect of the microbiocide SaniDate 12.0 and selected nitric oxide donors on biofilm dispersal

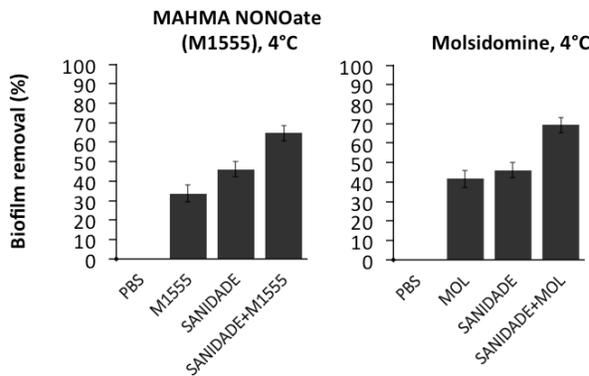


Figure 5. Additive effect of the microbiocide SaniDate 12.0 and nanomolar concentrations of Molsidomine and MAHMA NONOate. Bars represent the standard errors. Different letters represent significant different means ($p=0.05$).

The effect of the association of cellulose nanocrystals with selected nitric oxide donors on 24-hour old biofilms

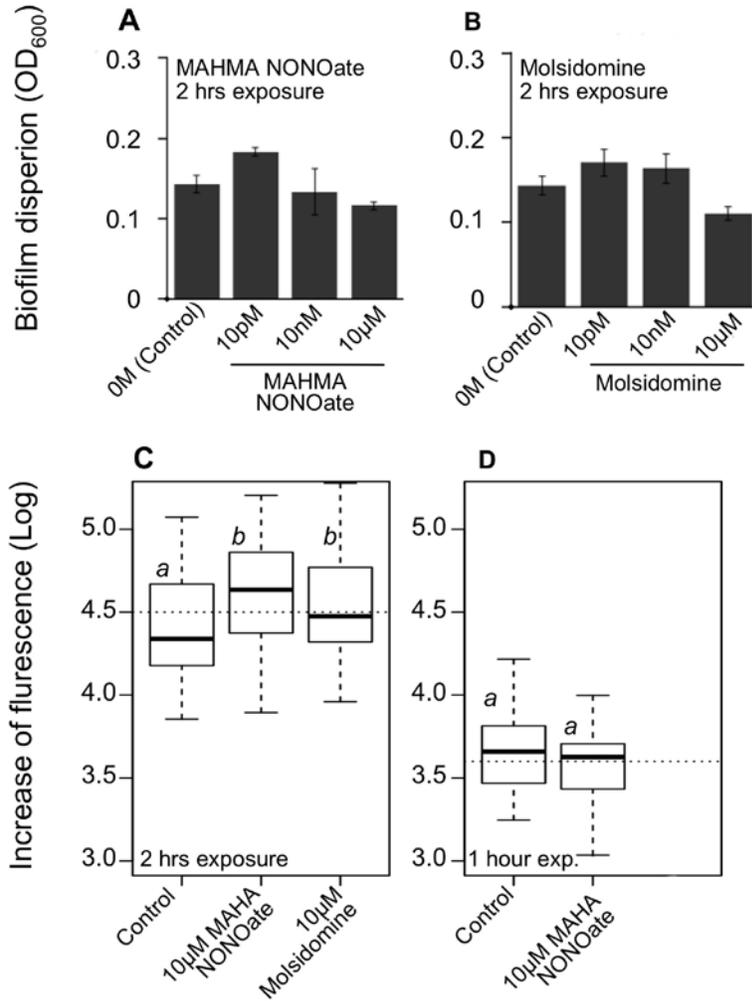


Figure 6. 24-hour old *Salmonella* biofilm dispersal by MAHMA NONOate dissolved in BPS and CNC.

Biofilms were preformed by *S. enterica* Thyphimurium 14028 for 24 hours on polystyrene prior to the treatment with MAHMA NONOate and molsidomine. Contact time at which biofilms were exposed to the nitric oxide donor is listed on each panel. Panels A and B, show the decreasing of biofilm formation measured by using the technique of crystal violet staining. Panels C and D, a fluorescent *Salmonella* 14028 pGFP-ON biofilm was treated with MAHMA NONOate and molsidomine dissolved in CNC. Increase in fluorescence of the planktonic cells was measured at 485nm/535nm. The box-plots encompass the lower and upper quartiles, thick lines within the box are the median values, and the whiskers indicate the degree of dispersion of the data. Dotted

line represents the grand mean. Letters above each box represent the Tukey means separation: different letters are significant different means.

The effect of the association of cellulose nanocrystals with selected nitric oxide donors on 1-week old biofilms

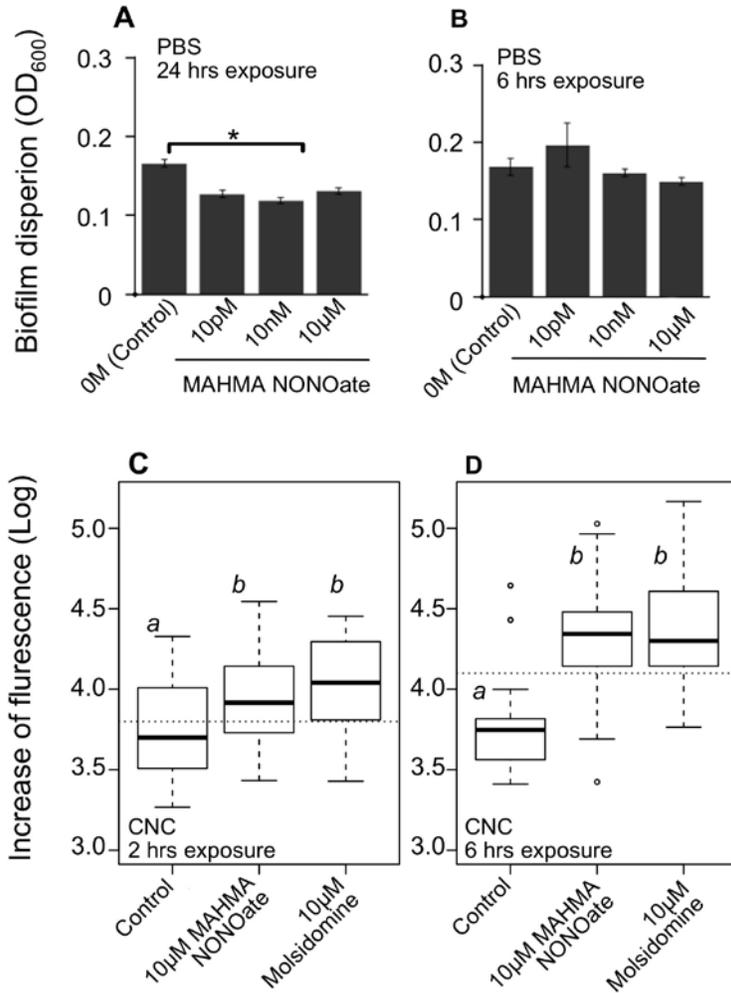


Figure 7. One-week old *Salmonella* biofilm dispersal by MAHMA NONOate dissolved in BPS and CNC.

Biofilms were preformed by *S. enterica* Thyphimurium 14028 for 1-week on polypropylene prior to the treatment with MAHMA NONOate and molsidomine. Contact time at which biofilms were exposed to the nitric oxide donor is listed on each panel. Panels A and B, decreasing of biofilm formation measured by using the technique of crystal violet staining. Panels C and D, a fluorescent *Salmonella* 14028 pGFP-ON biofilm was treated with MAHMA NONOate or molsidomine dissolved in CNC. Increase in fluorescence of the planktonic cells was measured at 485nm/535nm. The box-plots encompass the lower and upper

quartiles, thick lines within the box are the median values, and the whiskers indicate the degree of dispersion of the data. Outliers are shown as dots. Dotted line represents the grand mean. Letters above each box represent the Tukey means separation: different letters are significant different means.

Nitric oxide diffusion in cellulose nanocrystals enriched with MAHMA NONOate

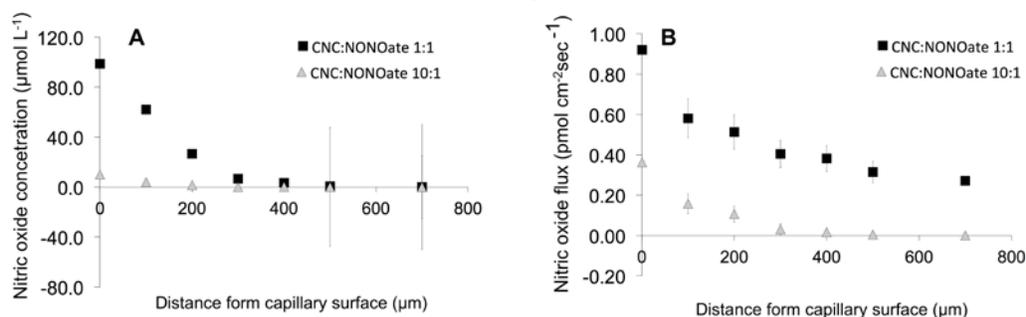


Figure 8. Nitric oxide diffusion in CNC. MAHMA NONOate was mixed with cellulose nanocrystals (whisker type) and a hydrogel was formed with a final concentration of 1mM. A microcapillary was tapered with a tip diameter of ca. 10µm using a glass puller. Consequently the hydrogel-NONOate was injected into the microcapillary and the microcapillary was placed into a phosphate buffer solution (PBS). A nitric oxide microsensor was immediately used to measure release of NO (both concentration and flux). Panel A) Nitric oxide concentrations from the capillary surface to the electrode. Panel B) Variation in nitric oxide flux. 10:1 represent a 10 times dilution of the MAHMA NONOate dissolved in CNC. Error bars represent the standard error of three replicas.

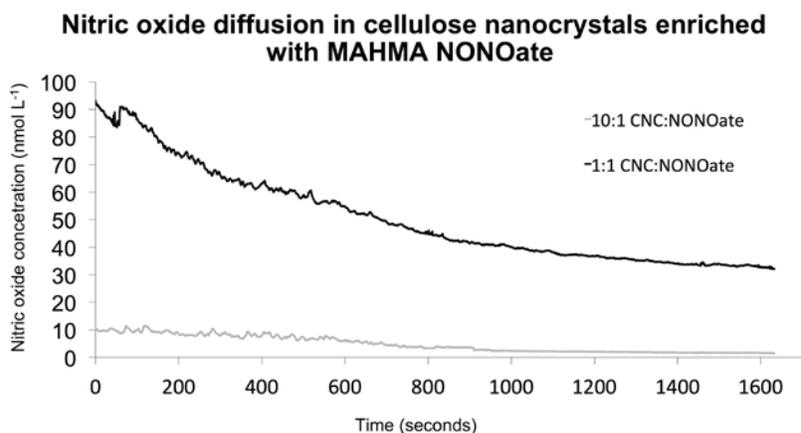


Figure 9. Nitric oxide release profiles of hydrogel-NONOate. Time 0 represents the introduction of the electrode in the CNC:NONOate. 10:1 represent a 10 times dilution of the MAHMA NONOate dissolved in CNC.

Salmonella recA-hydN region is involved in nitric oxide signalling

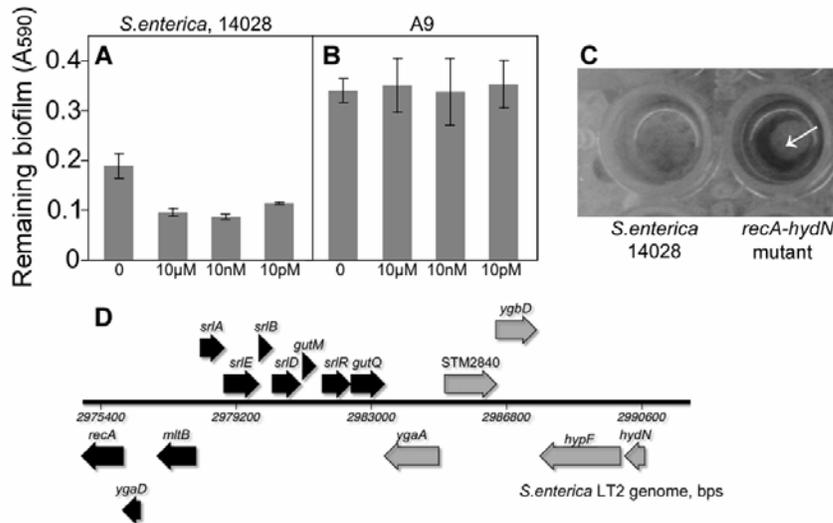


Figure 10. Involvement of the *Salmonella recA-hydN* genomic region in the nitric oxide-mediated signaling. *Salmonella* sv. Typhimurium A9 (mutant lacking a 15kB fragment spanning *recA-hydN* genes) formed more abundant biofilms, which were not responsive to treatment with nitric oxide donor molsidomine.

Detachment of the wild type biofilms in response to molsidomine (**A**). Lack of response to molsidomine by the A9 mutant (**B**). Appearance of the biofilms formed by the A9 mutant and the wild type (**C**). Genomic context of the A9 deletion (**D**). Thick arrows are annotated ORF. Genes with putative functions in nitric oxide signaling are shaded grey.

Suggestions to CPS (optional)