



AFRL-AFOSR-JP-TR-2019-0003

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## The Development of Nitroxide-Containing Anti-Biofilm Agents

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Final Report

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14. ABSTRACT <p>The goal of the project was to develop novel nitroxide-based anti-biofilm agents by incorporating nitroxides into the structures of antibiotics and biocides and which can then be attached to polymer scaffolds using various tethering approaches. The Fluoroquinolone-nitroxide hybrid compounds were prepared and tested for their anti-biofilm properties as well as their use as fluorescent probes. This grant award also had a collaboration with researchers from the Air Force Research Laboratory. The PI has had 2 peer reviewed papers, and 6 conference presentations and 2 manuscripts submitted as a direct result of the grant.</p>						
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## **Final Report for AOARD Grant 16IAO194 (FA2386-16-1-4094)**

### **“The Development of Nitroxide-containing Anti-biofilm Agents”**

**12th December 2018**

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**Period of Performance:** 09/15/2016 – 09/14/2018 (12 month no cost extension granted).

**Abstract:** Bacterial biofilms are a major problem in a number of environmental, industrial and medical applications. They cause significant risks to human health and present an enormous economic burden to society. An emerging strategy to combat biofilm formation and growth is to use small molecules that act through non-microbicidal mechanisms to inhibit and/or disperse biofilms. Nitroxides have shown potential in this regard, demonstrating both biofilm inhibition and dispersal properties. This project aims to develop novel nitroxide-based anti-biofilm agents by incorporating nitroxides into the structures of antibiotics and biocides and which can then be attached to polymer scaffolds using various tethering approaches. Our progress to date has involved the synthesis and characterization of hybrid compounds where nitroxides are linked to an antibiotic (ciprofloxacin), an anti-fungal agent (fluconazole) or a biocide (isothiazolone). The evaluation of the prepared compounds as anti-biofilm agents is on-going.

**Introduction:** The global aim of this project was to develop new anti-biofilm agents which harness the ability of nitroxides to disperse bacterial biofilms. This fundamental work will find application in a number of areas including combating the presence of biofilms in fuel storage tanks. Hydrocarbon utilising micro-organisms (HUM Bugs) degrade fuels, block filters and injectors and corrode fuel storage tanks and pipes. Biocides are currently used to remove bacterial growth present in aircraft fuel systems however this approach is ineffective if the bacteria reside in a biofilm. In this case, tanks must be drained and mechanically cleaned. This process is time consuming, labor intensive and costly. The small molecules developed as part of this research would allow contamination to be controlled before the hydrocarbon utilising bacteria could cause significant problems. This approach should facilitate improved control over biofilm growth and therefore shows promise to have a significant impact in this area of application.

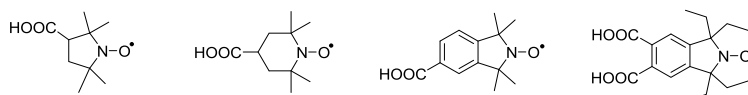
In this work, we initially aimed to gain a deeper understanding of the inhibitory and dispersal activity of small molecule nitroxides in gram positive and gram negative bacterial biofilms. We then planned to explore the synthesis of potential anti-biofilm compounds which combined a nitroxide and an antibiotic or an anti-fungal agent or a biocide into a single molecule. The rationale behind this approach was that the nitroxide would trigger the dispersal of cells from biofilms (which are resistant to antimicrobial action), and the antibiotic/anti-fungal agent/biocide would then kill the dispersed, planktonic bacteria, thus efficiently eradicating the biofilm. Different chemistries to immobilize these compounds to relevant scaffolds would then be explored. Evaluation of both the prepared compounds and surfaces would be undertaken by collaborators at the University of British Columbia (Prof R. E. Hancock) and the Air Force Research Laboratory (Dr Goodson/Ms Bowjanowski).

The five fundamental challenges initially outlined in this work were:

1. Over what range of concentrations do small molecule nitroxides produce bacterial biofilm dispersal? Can small molecule nitroxides induce bacterial growth and biofilm formation and if so, at what concentration does this occur?
2. Do nitroxides have activity in other species of environmental bacterial biofilms (Gram -/+)?
3. Can small molecule nitroxides induce the dispersal of fungal biofilms? If so, over what range of concentrations does this occur and will the use of a nitroxide in combination with an antifungal agent eradicate fungi residing in a biofilm?
4. In order to generate antibiotic-nitroxide conjugates, which position on the antibiotic is best for nitroxide attachment without effecting the activity of the antibiotic? What chemistries can be used to achieve the desired nitroxide linkage at the identified position? How can hybrids bearing several nitroxide units be prepared? Where is the best position to incorporate a surface tether and what chemistries can be used to achieve this?
5. To prepare antifungal-nitroxide conjugates, which position on the antifungal is optimal for nitroxide attachment in order to retain the activity of the antifungal? What chemistries can be used to achieve the desired nitroxide linkage at the identified position? How can hybrids bearing several nitroxide units be prepared? Where is the best position to incorporate a surface tether and what chemistries can be used to achieve this?

**Materials/Methods:** Our approaches used to address each of the fundamental challenges are outlined below:

**Challenge 1:** Dr Wendy Goodson and Ms Caitlin Bowjanowski at the Air Force Research Laboratory to investigate the effect of nitroxide concentration on biofilm dispersal and formation using a selection of small molecule nitroxides in *P.aeruginosa* using a flow chamber biofilm model based on a flow cell system and microscopy.



**Challenge 2:** Collaborators at the Air Force Research Laboratory and Professor Hancock's group at the University of British Columbia to investigate if nitroxides can cause biofilm inhibition and the dispersal of existing biofilms in Gram-negative pathogens (such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*) and Gram-positive (*Staphylococcus aureus* and MRSA, *Listeria monocytogenes*) pathogens in flow cell chambers.

**Challenge 3:** Collaborators at the Air Force Research Laboratory to further investigate the effect of nitroxides in fungal biofilms including *Yarrowia lipolytica*, *Byssoschlamys sp.* [a filamentous fungi] and *Wickerhamomyces sp.* [a yeast]. Additional experiments will be undertaken to determine if nitroxides can be used in combination with an anti-fungal agent to completely eradicate mature fungal biofilms.

**Challenges 4 and 5:** Conjugate molecules composed of nitroxides and antibiotics/anti-fungal agents/biocides will be designed, synthesized and evaluated. Structures containing multiple nitroxide units will also be prepared as our previous studies have indicated that the effective concentrations of

the nitroxide and antimicrobial agent for biofilm eradication are different. Different chemical approaches to attach the most active hybrid compounds to scaffolds will also be explored.

## Results and Discussion:

**Challenge 1:** The AFRL have not provided any data.

**Challenge 2:** The AFRL have not provided any data.

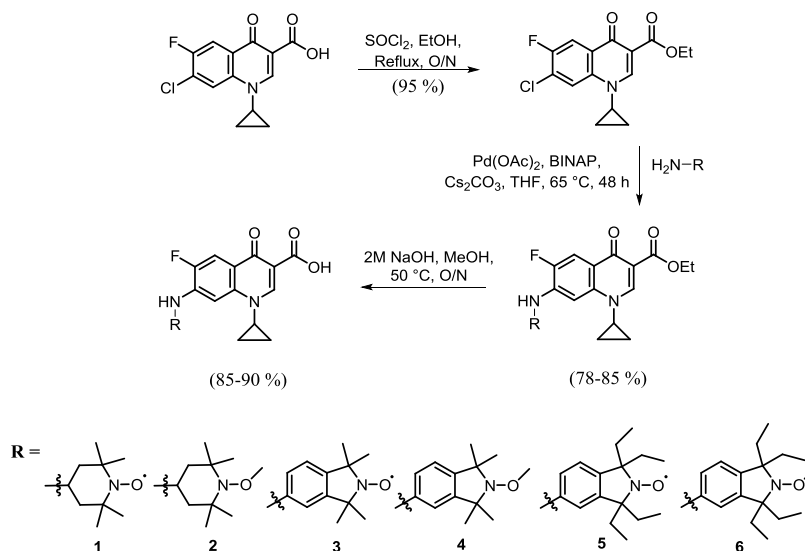
**Challenge 3:** The AFRL have not provided any data.

### Challenge 4:

#### Fluoroquinolone-nitroxide hybrids

We have previously prepared ciprofloxacin-nitroxide hybrids where the two active moieties are linked through an amide or the secondary amine of the piperazine ring (using reductive amination chemistry). Here we proposed to connect the nitroxide to fluoroquinolonic acid (ie the nitroxide ring would replace the piperazine ring of ciprofloxacin). This would generate a potential anti-biofilm agent with profluorescent properties. When a fluorophore is linked to a radical moiety, an intramolecular quenching of fluorescence takes place and the resulting compound exhibits little or no fluorescence. When the radical character of the compound is removed (by radical trapping or change in redox state) the fluorescence of the compound is restored (fluorescence quenching no longer takes place). As the fluoroquinolone core is a known fluorophore and nitroxides are well-documented quenchers of fluorescence, it was envisioned that by linking a nitroxide to the fluoroquinolone core, we could potential develop a dual-acting antibiotic-nitroxide hybrid, which could also act as a probe capable of being tracked, via fluorescence, in biological systems.

Three novel fluoroquinolone-nitroxide hybrids (**1**, **3** and **5**) and their corresponding methoxyamines (**2**, **4**, and **6**) have been prepared in high yield using reductive amination chemistry (Scheme 1). We have evaluated their anti-biofilm properties as well as their use as fluorescent probes.



**Scheme 1:** Synthetic route to fluoroquinolone-nitroxide hybrids

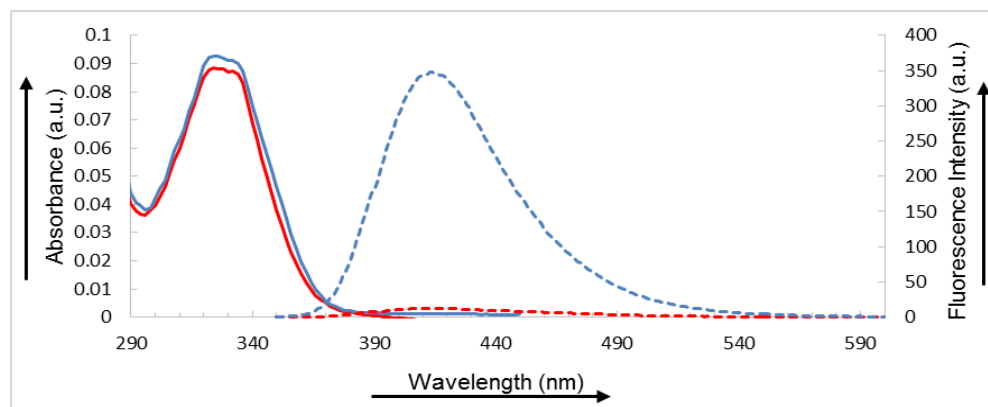
The fluorescence arising from solutions of nitroxide conjugates **1**, **3** and **5** and methoxyamine conjugates **2**, **4** and **6** in chloroform identified a substantial fluorescence suppression (up to 75-fold hybrid **5**) in the presence of the nitroxide moieties (Table 1). In aqueous solution, **1** exhibited a 36-fold fluorescence suppression (Figure 1). Subsequently, nitroxide **1** and methoxyamine **2** were

identified to possess suitable photophysical properties for biological imaging applications.

**Table 1.** Photophysical properties of nitroxides **1**, **3** and **5** and methoxyamine adducts **2**, **4** and **6**.

Compound	$\lambda_{\text{abs}}$ (nm)	$\epsilon$ (M <sup>-1</sup> cm <sup>-1</sup> )	$\lambda_{\text{em}}$ (nm)	Quantum yield $\Phi_F$	$\Phi_F$ ratio NOMe/NO <sup>•</sup>
<b>1</b>	340 <sup>[a]</sup>	10700 <sup>[a,c]</sup>	394 <sup>[a]</sup>	0.0060 <sup>[a]</sup>	27.7 <sup>[a]</sup>
	325 <sup>[b]</sup>	11500 <sup>[b,c]</sup>	413 <sup>[b]</sup>	0.0060 <sup>[b]</sup>	
<b>2</b>	340 <sup>[a]</sup>	9900 <sup>[a,c]</sup>	394 <sup>[a]</sup>	0.1600 <sup>[a]</sup>	36.7 <sup>[b]</sup>
	325 <sup>[b]</sup>	11000 <sup>[b,c]</sup>	413 <sup>[b]</sup>	0.2200 <sup>[b]</sup>	
<b>3</b>	350 <sup>[a]</sup>	16000 <sup>[a,d]</sup>	480 <sup>[a]</sup>	0.0004 <sup>[a]</sup>	67.5 <sup>[a]</sup>
<b>4</b>	350 <sup>[a]</sup>	15000 <sup>[a,d]</sup>	480 <sup>[a]</sup>	0.0270 <sup>[a]</sup>	
<b>5</b>	350 <sup>[a]</sup>	14700 <sup>[a,d]</sup>	515 <sup>[a]</sup>	0.0002 <sup>[a]</sup>	75.0 <sup>[a]</sup>
<b>6</b>	350 <sup>[a]</sup>	11700 <sup>[a,d]</sup>	515 <sup>[a]</sup>	0.0150 <sup>[a]</sup>	

Absorbance and fluorescence spectra recorded in [a] chloroform or [b] water; [c] Measured at 340 nm; [d] Measured at 350 nm. All samples were measured using anthracene as standard (350 nm excitation,  $\Phi_F = 0.27$  in ethanol) with solvent corrections.



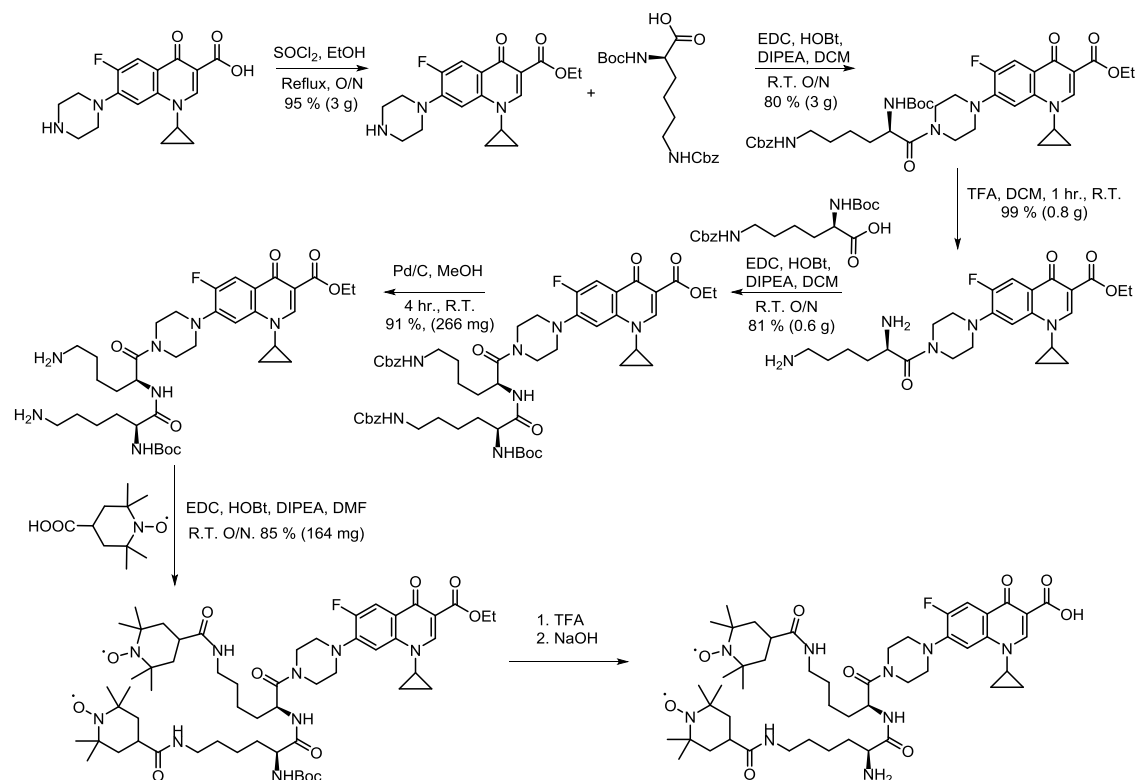
**Figure 1.** Absorption and fluorescence emission spectra of nitroxide **1** (absorbance (—); fluorescence (---)), methoxyamine **2** (absorbance (—); fluorescence (---)). Measured in water, with excitation at 340 nm.

Our biological investigations were initiated with the screening of nitroxides **1**, **3** and **5** and methoxyamines **2**, **4** and **6** in minimum inhibitory concentration (MIC) assays against *S. aureus* ATCC 29213. Ciprofloxacin, a common fluoroquinolone antibiotic with known potency against planktonic *S. aureus* cells, was tested alongside compounds **1-6** to act as a benchmark and confirm the validity of the MIC assays (Table 2). Nitroxide **1** was determined to possess the most potent activity in the assays (MIC  $\leq 0.02$  mM). Interestingly, the methoxyamine derivatives **2**, **4** and **6** exhibited no significant anti-staphylococcal activity even at the highest concentration tested (MIC  $> 1.20$  mM, more than 60-fold less active than the corresponding nitroxides). The next phase of our biological investigation involved efficacy screening of ciprofloxacin, nitroxide **1** and methoxyamine **2** against *S. aureus* biofilms. To achieve this, we utilized the MBEC<sup>TM</sup> device, which allows assays for biofilm susceptibility testing of antibiotics to be conducted. Ciprofloxacin exhibited limited biofilm eradication efficacy, with complete eradication failing to occur at even the highest concentration tested (12.36 mM, MBEC  $> 12.36$  mM). Methoxyamine **2** showed limited to no biofilm-eradication activity (MBEC  $> 1.48$  mM, maximum concentrations tested). Conversely, nitroxide **1** exhibited substantial biofilm-eradication activity (MBEC  $\leq 0.72$  mM) and was at least 17-fold more potent than

ciprofloxacin (device standard). The finding that both methoxyamine **2** and ciprofloxacin were unable to eradicate *S. aureus* biofilms suggests that the addition of a nitroxide moiety to the fluoroquinolone considerably improved the biofilm-eradication activity of the antibiotic. We are currently investigating the use fluorescence imaging to visualise nitroxide **1** in *S. aureus* biofilms in order to gain a deeper understanding of its mode of action.

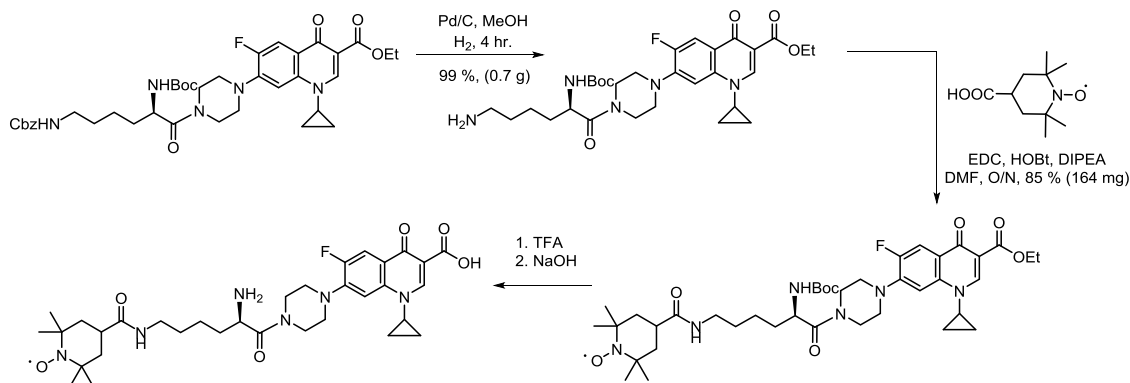
Compound	MIC <sup>[a]</sup> (mM)	MBEC <sup>[b]</sup> (mM)
<b>1</b>	≤ 0.02	≤ 0.72
<b>2</b>	> 1.200 <sup>[c]</sup>	> 1.48 <sup>[c]</sup>
<b>3</b>	≤ 0.170	-
<b>4</b>	> 1.200 <sup>[c]</sup>	-
<b>5</b>	≤ 0.020	-
<b>6</b>	> 1.200 <sup>[c]</sup>	-
<b>ciprofloxacin</b>	≤ 0.0015 <sup>[d]</sup>	> 12.36 <sup>[c]</sup>

### Ciprofloxacin conjugates bearing multiple nitroxide units



### Ciprofloxacin-nitroxide hybrids with potentially improved potency

We envisioned that the lysine-based linker could also be used to explore the effect of positioning the nitroxide moiety in the ciprofloxacin-nitroxide hybrids further away from the antibiotic whilst still providing a free amine moiety. We have synthesized a novel ciprofloxacin-nitroxide hybrid (Scheme 3) and its corresponding methoxyamine derivative and will shortly evaluate their biological activity.

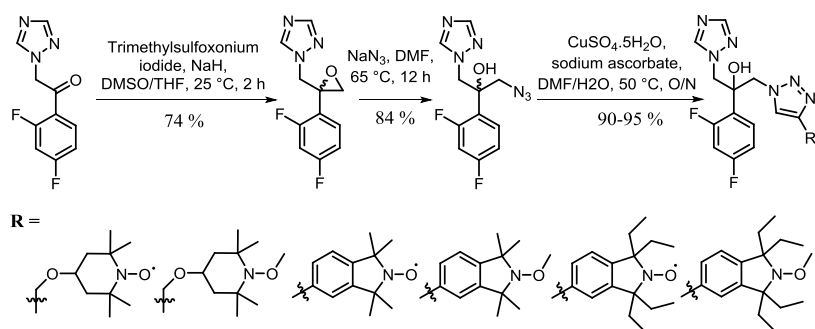


**Scheme 3:** Synthetic route to ciprofloxacin-nitroxide hybrids linked via a lysine unit.

### Challenge 5:

#### Fluconazole-nitroxide hybrids

To prepare anti-fungal based nitroxide conjugates, the azole based anti-fungal agent, fluconazole, was selected as the compound of choice as it represents a well-known broad spectrum anti-fungal agent, which has demonstrated biological activity against most yeasts and filamentous fungi. Fluconazole, much like other azole based anti-fungal agents, exerts its activity through inhibition of cytochrome P450 via the azole rings present within the molecule. Thus, functionalization of the fluconazole core is generally achieved via a triazole linker. Accordingly, we chose to incorporate three different nitroxides into the fluconazole core using a triazole linker. The corresponding methoxyamine derivatives were also synthesized in high yield (Scheme 4).



**Scheme 4:** Synthesis of fluconazole-nitroxide hybrids.

#### Isothiazolone-nitroxide hybrids

Isothiazolones are biocides with documented activities against both bacteria such as *Escherichia coli* and *Staphylococcus aureus*, and fungi such as *Aspergillus niger*. The activity of these heterocyclic compounds is believed to arise from their ability to diffuse through either bacteria or fungal cell wall membranes and react with important sulfur containing proteins ultimately causing the cell's function to be impaired. Two novel isothiazolone-nitroxides and their methoxyamine analogues were prepared via the cyclisation of the corresponding sulfoxide to the desired unsubstituted isothiazolone hybrids.



Whilst additional derivatives of both the fluconazole and isothiazolone conjugates are planned, we will first await preliminary biological results to ensure that the prepared hybrids have the desired activity. In the absence of ARFL data, we have built another collaboration with A/Prof Makrina Totsika and her group at QUT, where we are beginning to investigate the antifungal effects of the fluconazole- and isothiazolone-nitroxide hybrids.

**List of Publications and Significant Collaborations that resulted from your AOARD supported project:** In standard format showing authors, title, journal, issue, pages, and date, for each category list the following:

**a) papers published in peer-reviewed journals,**

1) Verderosa, A. D.; de la Fuente-Núñez, C.; Mansour, S. C.; Cao, J.; Lu, T. K.; Hancock, R. E. W.; **Fairfull-Smith, K. E.**, “Ciprofloxacin-Nitroxide Hybrids with Potential for Biofilm Control”, *European Journal of Medicinal Chemistry*, **2017**, 138, 590-601.

2) Boase, N., Torres, M. D. T., Fletcher, N., de la Fuente-Núñez, C., **Fairfull-Smith, K. E.**, “Polynitroxide Copolymers to Reduce Biofouling on Surfaces”, *Polymer Chemistry*, **2018**, 9, 5308-5318.

**b) Papers published in non-peer-reviewed journals or in conference proceedings,**  
N/A

**c) Conference presentations,**

1) Kathryn E. Fairfull-Smith, invited oral presentation, “Nitroxide-containing Agents for Biofilm Remediation”, 8<sup>th</sup> Pacific Symposium on Radical Chemistry, Brisbane, July 2017.

2) Kathryn E. Fairfull-Smith, oral presentation, “Nitroxide-containing Agents for Biofilm Remediation”, Royal Australian Chemical Institute Centenary Chemistry Congress, Melbourne, Victoria, Australia, July 2017.

3) Kathryn E. Fairfull-Smith, invited oral presentation, "Nitroxide-containing Scaffolds with Potential for Biofilm Control", World Polymer Congress – MACRO18, Cairns, Australia, July 2018.

4) Anthony Verderosa\*, Kathryn E. Fairfull-Smith, Makrina Totsika, oral presentation, “Nitroxides: a promising therapeutic strategy for the treatment of *Staphylococcus aureus* biofilm-related infections”, IHBI Inspires Annual Conference, QUT, August 2018.

5) Kathryn E. Fairfull-Smith, oral presentation, “Controlling Bacterial Biofilms with Nitroxides”, Royal Australian Chemical Institute Medicinal Chemistry and Chemical Biology Conference, Brisbane, November 2018.

6) Kathryn E. Fairfull-Smith, plenary lecture, “Controlling Bacterial Biofilms with Nitroxides”, Queensland Annual Chemistry Symposium, Brisbane, November 2018.

**d) Manuscripts submitted but not yet published**

1) Verderosa, A. D., Dhouib, R., Totsika, M., **Fairfull-Smith, K. E.**, “Multifunctional Fluoroquinolone-Nitroxides: ‘Switch On’ Fluorescent Antibiotics”, *Eur. J. Org. Chem.*, **2018**, to be submitted.

2) Verderosa, A. D., Dhouib, R., **Fairfull-Smith, K. E.**, Totsika, M., “Nitroxides Enhance the Efficacy of the Antibiotic Ciprofloxacin Against *Staphylococcus aureus* Biofilms”, *Antimicrobial Agents and Chemotherapy*, **2018**, to be submitted.

**e) Provide a list any interactions with industry or with Air Force Research Laboratory scientists or significant collaborations that resulted from this work.**

We are building a collaboration with Dr Caitlin Bowjanowski and Wendy Goodson (AFRL). PI Fairfull-Smith visited the AFRL (Dayton, Ohio) in February 2016 on a Windows on Science grant.

Please note: The current assessment of small molecules and functionalized surfaces provided to AFRL for anti-fungal evaluation in 2016 has stalled as AFRL scientist Dr Caitlin Bowjanowski had been devoting her time to completing her PhD and she is now on maternity leave. In order to start to evaluate the antimicrobial and anti-biofilm activities of the newly synthesized molecules, we have built another collaboration with A/Prof Makrina Totsika and her group at QUT.