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MICROBIOLOGY, PARASITOLOGY & VIROLOGY | RESEARCH ARTICLE

Low-concentration diffusible molecules affect the formation of biofilms by mixed marine communities

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Abstract: Biofilm formation is a major concern in any venture in the marine environment and often precedes the establishment of fouling by macro-organisms. In this study, the effects of three known cell-to-cell signalling molecules, nitric oxide (NO), *cis*-2-decenoic acid (CDA) and patulin, on the formation of marine biofilms were investigated. Each of the molecules has been shown to affect biofilms and this is the first study to investigate their effect on mixed communities of marine biofilm-forming micro-organisms. Studies of the biomass of those biofilms grown in the presence of the molecules showed that all three reduced biofilm formation by marine communities, with both NO and patulin reducing biofilm formation by more than 90% at the highest concentrations studied. However, colony counts revealed that the effect of patulin is likely due to toxicity. Analyses of the biofilm communities were also carried out using DGGE to determine whether there was any variation in the effects of each molecule on different bacterial taxa. No effect was seen on the communities and the biofilms appeared to form according to a neutral community model. Further investigations are recommended to study biofilms at a functional level.

Subjects: Biofilms; Marine Biology; Microbiology

Keywords: marine; biofilm; nitric oxide; *cis*-2-decenoic acid; patulin; DGGE; non-toxic

ABOUT THE AUTHORS

David Ian Walker's research interests include microbial ecology, biofouling and biosensor technologies. The research in this paper formed part of Walker's PhD thesis which examined the potential of non-toxic antifouling technologies for *in situ* marine sensors.

This work was undertaken under the supervision of Keevil who is the director of the Environmental Healthcare Unit. His research group investigates the detection, survival, physiology and eradication of micro-organisms in environmental, freshwater, seawater, food and clinical samples, including polymicrobial biofilms. Advanced light microscopy techniques have been developed for these studies, which have also aided work into the antimicrobial mechanisms of copper alloy surfaces and with prion diseases, with a particular focus on hospital decontamination procedures of surgical instruments and endoscopes.

PUBLIC INTEREST STATEMENT

The accumulation of bacterial communities or biofilms on man-made structures in the marine environment is of great concern, and can affect the functioning of scientific equipment, increase the speed of degradation of structures and increase fuel consumption by ships. In this research, three chemicals previously shown to be effective at reducing biofilm accumulation in very low concentrations were investigated for the first time using mixed species marine biofilms. Using chemicals that are effective at low concentrations, there is a reduced environmental and economic impact in reducing the accumulation of biofilms.

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1. Introduction

The formation of biofilms in the marine environment is an ubiquitous process which can have significant impacts on the functioning of marine equipment. Biofilms alone can increase the frictional resistance of a ship's hull through the water by up to 18% (Haslbeck & Bohlander, 1992), leading to significant increases in fuel consumption. Marrs, Head, Cowling, Hodgkiess, and Davenport (1999) showed that marine biofilms absorbed up to 65% of light shone through them and so can seriously reduce the effectiveness of optical marine sensors. It is clear that marine biofilms are a serious concern to any human venture in the marine environment, and as such, their formation and its remediation are of great interest.

Biofilms are complex structures with highly regulated biological processes. Many of the processes involved in the life cycle of a biofilm utilise chemical signalling to allow synchronisation between cells and so increase the efficiency of the biofilm for gathering nutrients and avoiding predation or attack from harmful agents.

Over the last 15 years, many studies attempting to understand chemical signalling in biofilms have been published (Nobile & Mitchell, 2005; Sudarsan et al., 2008; Teasdale, Liu, Wallace, Akhlaghi, & Rowley, 2009; Vejborg & Klemm, 2008; Xavier & Bassler, 2005) and as a result, many biofilm signalling molecules are now known. Unlike antibiotics and other forms of bactericides, many of these chemicals modify the formation of biofilms by altering physiological processes by, in essence, tricking micro-organisms into detecting certain environmental conditions that do not in fact exist. This means that micro-organisms may form less biofilm or disperse from biofilms prematurely. Due to the low concentration at which these molecules have an effect, they will be referred to as low-concentration diffusible molecules (LCDM) herein.

One such LCDM is nitric oxide, the addition of which has been shown to trigger dispersal of both single species and mixed species biofilms at sub-toxic concentrations. This dispersal response has been shown to be linked to the cyclic-di-GMP intracellular signalling system and alters the expression of several genes involved in the life cycle of biofilms (Barraud et al., 2006; Barraud, Storey, et al., 2009).

Another LCDM that has been found to induce dispersal of biofilms is *cis*-2-decenoic acid (CDA) (Davies & Marques, 2009). CDA is a short-chain, mono-unsaturated fatty acid produced by *P. aeruginosa* which induces dispersal of several single species biofilms. It has also been shown to reduce the formation of *P. aeruginosa* biofilms.

Patulin, a mycotoxin produced by fungi of the genera *Penicillium* and *Aspergillus* (Bergel et al., 1943), has been shown to disrupt quorum sensing in both the medically important bacterial species *P. aeruginosa* (Rasmussen et al., 2005) and the marine species *Halomonas pacifica* (Liaqat, Bachmann, & Edyvean, 2014). Liaqat, Bachmann, Nasim Sabri, Edyvean, and Biggs (2008), Liaqat, Bachmann, Sabri, and Edyvean (2010), Liaqat et al. (2014) studied the effect of patulin on *Klebsiella* sp., *B. subtilis*, *Bacilus cereus, P. aeruginosa* and the marine species *H. pacifica* and *Marinobacter hydrocarbonoclasticus* biofilms. It was found that patulin increased the formation of *B. cereus* and *P. aeuruginosa* biofilms at low concentrations but only effected *B. subtilis* biofilm formation when used in combination with EDTA. Patulin was shown to have no effect on *M. hydrocarbonoclasticus*, but increased the formation of biofilms by *H. pacifica* in concentrations up to 10 µM and inhibited biofilm formation over 25 µM.

In natural ecosystems, biofilms usually do not exist as single species structures, but instead contain several different species. Some LCDMs such as nitric oxide and CDA have been shown to affect the formation and dispersal of mixed species biofilms; however, no investigations have been carried out to investigate the effects of these molecules at a community level. Given that such molecules affect different species to different extents (Barraud, Storey, et al., 2009; Davies & Marques, 2009; Liaqat et al., 2014), it may be hypothesised that different LCDMs will result in a shift in the species present in biofilm communities.

In this study, three LCDMs, nitric oxide, CDA and patulin, were investigated for their effect on the formation and community structure of biofilms by mixed marine communities grown from natural microbial assemblages taken from the sea. The effects on biofilm formation were measured using optical density. Additionally, their effects on biofilm communities were investigated using denaturing gradient gel electrophoresis (DGGE). In a literature search, no studies were found where any of the three LCDMs were tested on mixed marine communities.

2. Materials and methods

The effects on biofilm formation of NO, CDA and patulin were investigated in a titreplate microcosm using a standard inoculum and filter-sterilised seawater as the growth medium. All chemicals were supplied by Sigma-Aldrich (UK) unless otherwise stated. These experiments were independently repeated on three occasions.

2.1. Preparation of a marine inoculum

Forty litres of seawater were collected from Southampton Water, Southampton, UK (50°53′28.36″N, 1°23′37.56″W, WGS 84) and filtered through 100-μm pore-size sterile filter membranes (Millipore, USA) to remove large particulate matter. Micro-organisms were collected on a sterile 0.2-μm pore-size, 120-mm diameter sterile filter membrane (Millipore, USA) and resuspended in some of the sterile sea water (SSW). This suspension was centrifuged for 30 min at 4800 RCF; the supernatant was removed and centrifuged a second time to increase recovery. The pellets were combined and resuspended in filter-sterilised seawater before sterile glycerol was added to a final concentration of 15% (w/v) in a total volume of 40 ml. This mixture was separated into 300-μl aliquots in sterile cryotubes and stored at −80°C until needed.

2.2. Preparation of LCDM solutions

The effect of each LCDM was tested over five different concentrations. Patulin and CDA (Advanced Synthesis Technologies, USA) were tested between 5 nM and 50 μM, and the NO-donor sodium nitroprusside (SNP) was tested between 500 nM and 5 mM. Stock solutions of 100–0.01 mM CDA and patulin were created in neat ethanol or ethyl acetate, respectively, and stored at −20°C until needed. CDA stocks were stored under argon. One micro litre of stock CDA or patulin solution was added to 2 ml of SSW to create working solutions. To control for the effects of the carrying solvents, ethanol and ethyl acetate controls were also created using 1 μl of pure solvent in 2 ml of SSW. SNP solution was created in SSW daily as needed by serially diluting 50 mM SNP (in SSW) with SSW to create the five final concentrations of SNP.

2.3. Growth of biofilms

Marine inoculum was diluted 100 times in each of the LCDM solutions described above and 180 μl was added to the wells of a 96-well titreplate (Greiner Bio-one, UK), giving five repeats for each treatment and control. The plate was incubated at room temperature for 4 h under static conditions and in the dark to allow the attachment of cells. SSW with no inoculum was used as a negative control.

After attachment, the wells were emptied and rinsed carefully with SSW before new LCDM solutions were added. The plate was then left in the same conditions as for attachment for 5 days, with solutions being changed every 24 h. After the 5-day growth period, the solutions were removed and the wells were rinsed with SSW before being fixed with 4% (w/v) paraformaldehyde and further rinsed with 0.2-μm filter-sterilised deionised water.

2.4. Measurement of biomass by optical density

To determine the relative amount of biomass in each well, a crystal violet assay adapted from Stepanović, Vuković, Dakić, Savić, and Švabić-Vlahović (2000) was used. To each well, 180 μl of crystal violet (2%) was added and left for 10 min. The crystal violet was then removed and the plate was rinsed under slowly running water to remove excess crystal violet. The plate was left to air dry before 180 μl of 33% (v/v) acetic acid in water was added to each well. This was left for 30 min on an orbital

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shaker to allow the bound crystal violet to be fully eluted into the acetic acid and reading the optical density at 540 nm. Data were standardised between experiments against the negative controls. Two-way ANOVA tests showed no significant differences between experiments for any of the treatments and subsequent one-way ANOVA and Tukey HSD tests were performed using the combined results of all three experiments.

2.5. Toxicity of LCDMs

To rule out the possibility of any reduction in biofilm formation being due to toxicity, colony counts of inocula treated with the LCDMs at concentrations, shown to significantly reduce biofilm formation, were carried out. Samples of marine inoculum were diluted 1000 × and treated with a final concentration of 500-μM SNP, 50-μM CDA or 5-μM patulin in SSW or 100% SSW as control. These were incubated for 24 h and then diluted 100×, 1000 × and 10000 × in SSW before 100 μl of each was spread on to 90-mm Zobell's 2216 marine agar plates (Zobell, 1941) and incubated for 3 days. SSW was used as a negative control. Differences in CFU counts from the control were tested using the Student's *t*-test for each treatment.

2.6. Community analysis

2.6.1. Sample collection and DNA extraction

Biofilms were grown in the presence of SNP (500 μM), CDA (50 μM) and patulin (5 μM) as above, but with three repeats for each treatment. After 5 days growth, the wells were rinsed with SSW. DNA was extracted and purified using a modified protocol for the Qiagen DNeasy blood and tissue kit in which approximately 0.1 g of sterile 100-μm diameter glass beads and 180 μl of enzymatic lysis buffer were added to the wells and shaken for 15 s. The plates were then left at 37°C for 15 min. The shaking and heating processes were repeated thrice, with an additional shaking step after the final heating. The solution was pipetted into clean microfuge tubes and any remaining biofilm was removed from the wells using sterile cotton swabs. The swab tips were placed into the microfuge tubes and further lysis and DNA purification continued following the standard procedure for the Gram-positive protocol as per the manufacturer's instructions.

2.6.2. PCR

Universal bacterial PCR primers U968f-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and U1401R (5′-CGG TGT GTA CAA GAC CC-3′) were used to amplify partial 16S rDNA sequences from the V6 region (Nubel et al., 1996; Piquet, Bolhuis, Meredith, & Buma, 2011). The first 40 bases of the primer U968f-GC were a GC clamp to stop complete denaturation of dsDNA strands during denaturing gradient gel electrophoresis (DGGE). PCR was run for each sample using the following conditions. Each reaction contained Promega PCR master mix (1x), 3 pmol of each primer, 10-μl DNA template and water in a total volume of 25 μl. The PCR was run in a MJ Research Tetrad 2 thermal cycler with a denaturation at 94°C for 5 min followed by 10 touchdown cycles, reducing the annealing temperature by 0.5°C per cycle from 60 to 55°C for 1 min and an extension at 72°C for 2 min. This was followed by a further 20 cycles with an annealing temperature of 55°C. The amplification was finished with a final extension step of 72°C for 30 min to reduce artifactual double bands in DGGE gels (Janse, Bok, & Zwart, 2004). PCR products were visualised on agarose gel stained with ethidium bromide and DNA concentrations were calculated using the ImageJ software.

2.6.3. DGGE and sequencing

DGGE was performed using the BioRad Dcode system. Three hundred nanograms of each PCR product were run through a 6% polyacrylamide gel with a denaturant gradient from 46 to 60% (where 100% denaturant is 40% formamide (v/v) and 42% urea (w/v)) for 18 h at 60°C at 100 V. Three lanes in each gel contained a standard created from bands extracted from previous DGGE to allow comparisons between gels.

The gels were stained in a 0.5 mg ml^{-1} ethidium bromide/TAE solution for 15 min and then destained with fresh TAE buffer for a further 15 min. The stained gels were imaged on a Syngene G:Box UV transilluminator. Dominant bands were extracted, reamplified and run through a second DGGE gel to confirm their position and that they were single bands. Products with multiple bands were re-extracted, amplified and run through DGGE again until a single band was obtained. Single-band PCR products were purified using the Qiagen QIAquick PCR purification kit before being sequenced by Source Bioscience (Oxford). Those bands that gave poor sequence data were cloned using the Qiagen PCR cloning kit and resequenced to obtain good-quality sequence data. These sequence data have been submitted to the EMBL database under accession No. HE818046 to HE818071.

2.6.4. DGGE data analysis

DNA sequences were aligned using the NAST program on the GreenGenes database website (DeSantis, Hugenholtz, Keller, et al., 2006; DeSantis, Hugenholtz, Larsen, et al., 2006) and classified using the Classify program.

The banding patterns of the gels were compared using the Phoretix 1D software to obtain a matrix of bands present in each sample and their relative intensities. These data were further analysed using DGGEstat software (van Hannen, 2000) to create a qualitative Dice similarity matrix. The similarity data were visualised with a multidimensional scaling (MDS) plot using the ALSCAL program within the SPSS 19 software. Species richnesses were calculated for each sample, and one-way ANOVA was used to compare the species richnesses between treatments.

3. Results

3.1. Biofilms

Biofilms were grown from a complex marine bacterial inoculum in the presence of LCDMs to determine their effect on biofilm formation. Each of the three LCDMs tested were used at five concentrations to determine the effective dosage. The biomass of biofilms was measured semi-quantitatively by the optical density of redissolved crystal violet which had been bound to the biofilms. Alterations in biomass were measured against biofilms grown in untreated sterile seawater as control. As CDA and patulin were initially dissolved in ethanol and ethyl acetate, respectively, before being added to aqueous solution, the effects of these carrying solvents were measured by growing biofilms in the presence of these solvents without any LCDM. No effect of either carrying solvent was seen on the biomass of biofilms ($p = 0.609$).

The SNP nitric oxide donor molecule significantly altered biofilm formation ($p < 0.001$, Figure 1). At 50 μM, SNP significantly ($p < 0.001$) reduced biofilm formation by 33% compared with the control and was the lowest concentration at which SNP had a significant effect. SNP significantly reduced biofilm formation at all concentrations above 50 μM and at 5,000 μM SNP, the highest concentration tested for this LCDM, the formation of biofilm was significantly ($p < 0.001$) reduced by 90% compared to the control.

CDA also significantly altered the amount of biofilm formed ($p < 0.001$, Figure 1). At 0.005 μM, the lowest concentration of CDA tested, there was a significant ($p < 0.001$) reduction in biofilm formation by approximately 27% compared with the control. Above this concentration, biofilm formation increased and this increase became significant at 0.5 μM ($p = 0.031$) with 15% greater biomass than the control. Above this concentration, biomass was reduced and a significant ($p = 0.022$) reduction of 18% relative to the control was seen at 50 μM CDA.

At concentrations below 0.5 μM, patulin had no significant effect on the formation of biofilms. At concentrations of 0.5 μM and above, patulin significantly reduced biofilm formation ($p < 0.001$). At 50 μM, the highest patulin concentration tested, biofilm formation was reduced by approximately 95%.

Figure 1. Optical density (absorbance) at 540 nm for crystal violet bound to biofilms and eluted into acetic acid indicates the relative biomass of mixed species biofilms grown in the presence of five concentrations of three LCDM. Those concentrations at which ANOVA test showed biofilm formation to significantly differ from the control are marked with one ($p < 0.05$) or two ($p < 0.001$) stars. Error bars are standard deviation from the mean of 15 repeats.
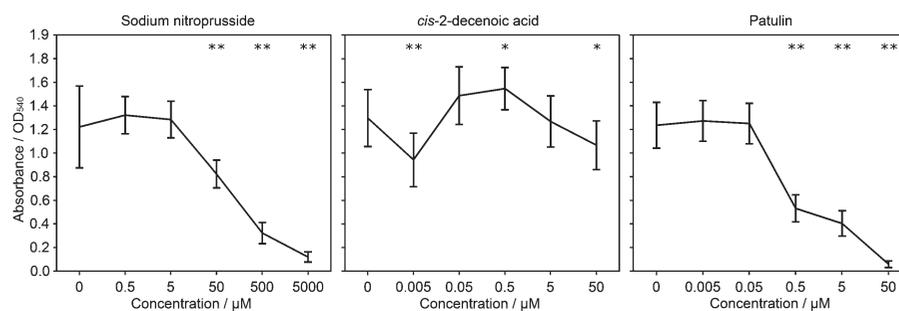
Figure 2. Colony forming unit (CFU) counts for marine bacteria treated with one of the three LCDMs, SNP (500 µM), CDA (50 µM) or patulin (5 µM) or sterile seawater (control). Those molecules that had a significant ($p < 0.001$) antimicrobial or toxic effect relative to the control are marked with two stars.
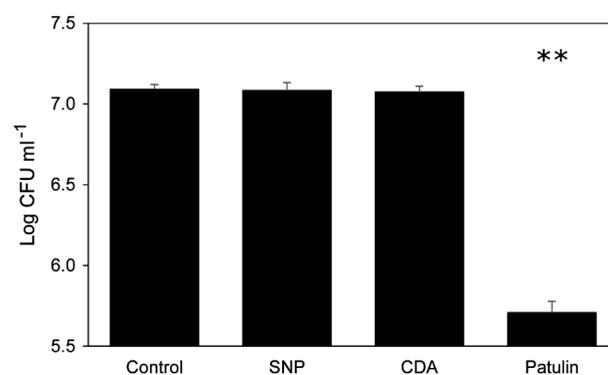
To determine whether the effects of LCDMs were due to antimicrobial activity, samples of the bacterial inoculum were treated with each of the three LCDMs or sterile seawater as control, and grown on marine agar. No differences in CFU counts (Figure 2) were observed between the control, SNP and CDA. However, treatment with 5-µM patulin reduced colony formation from approximately 1.2×10^7 CFU ml^{-1} to an average of approximately 5.2×10^5 CFU ml^{-1}.

3.2. Community analysis

To determine whether LCDMs altered the community structure of mixed species marine biofilms, the 16S rRNA profiles of biofilms grown in the presence of LCDMs were compared using DGGE and sequencing. A representative DGGE gel is shown in Figure 3.

A total of 28 unique DGGE bands were found in analyses of samples treated with different LCDMs. An average of 6 bands was found in each sample, but this number ranged from a minimum of 1 to a maximum of 12 bands. However, comparisons of the species richnesses of the marine biofilm communities by ANOVA tests revealed no significant differences ($p = 0.055$) between treatments.

There were no bands that occurred in all of the samples and three bands were unique to a single sample. One band was unique to the patulin and two bands were unique to CDA. The band unique to patulin was band 17 and was identified as belonging to the γ-proteobacteria genus *Marinobacter* or the β-proteobacteria genus *Delftia*. Of the two bands unique to CDA, only one was successfully sequenced and identified as containing both γ-proteobacteria *Pseudoalteromonas* species and β-proteobacteria *Delftia* species.

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Figure 3. A representative DGGE gel image run with bacterial 16S rRNA PCR amplicons from biofilms grown in the presence of three LCDMs or a sterile seawater control. Numbers on the gel represent banding positions that were extracted and sequenced for phylogenetic analyses (Table 1). Bands marked "U" were not successfully sequenced but were included for Dice similarity analyses (Figure 4). The flanking and middle lanes were a standard sample constructed from several samples and used for comparison of banding positions across and between gels.
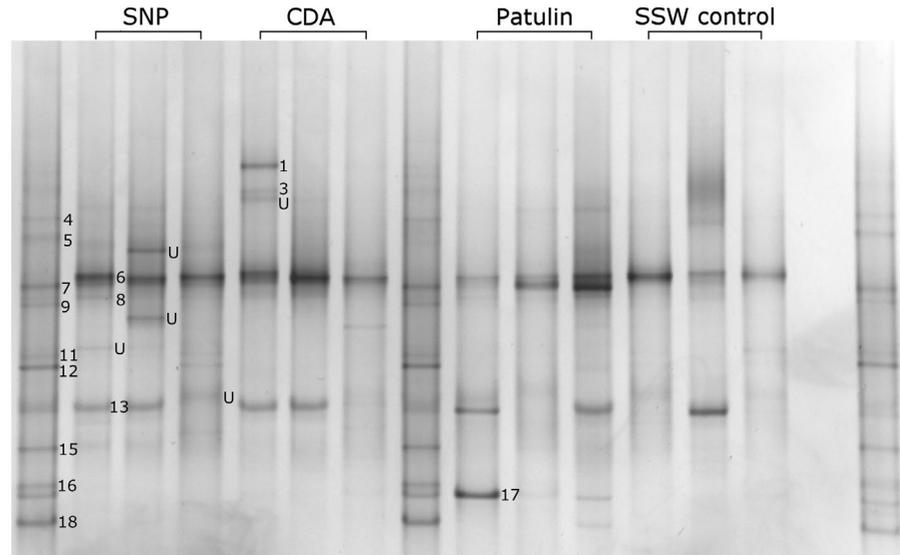

MDS plots of Dice similarity data (Figure 4) showed that the DGGE profile patterns did not cluster based on similar treatments as would have been expected. This was even true for the control profiles, which did not cluster any more with each other than with the profiles of those communities treated with LCDMs.

Sequences of bands extracted from DGGE gels (Figure 3) were compared with the GreenGenes database. Phylogenetic analyses of extracted DGGE bands indicated that 19 of the 26 identified biofilm-forming organisms in this system were in the class γ-proteobacteria (Table 1). Other organisms included members of the α- and β-proteobacteria, and only one organism, *Luteolibacter*, was identified that did not belong to the proteobacteria phylum. Bands 02, 07, 11, 16, 17 and 18 (Figure 3) were represented by more than one sequence type and showed that the same banding position could represent more than one species. Bands 02, 16 and 17 contained species from different classes (β- and γ-proteobacteria), while band 07 contained different genera from the same class, γ-proteobacteria.

Comparisons of the community structures between treatments at the taxonomic level of class showed that the majority of the community was made up of γ-proteobacteria (Figure 5). Notably, however, samples treated with SNP did not contain β-proteobacteria or Verrucomicrobiae.

Figure 4. MDS plot of Dice similarities of species presence/absence data from 16S rRNA DGGE analyses of biofilms grown in the presence of three LCDMs or a sterile seawater control. Molecules with which the biofilms were grown are indicated by different marker shapes and indicated in the legend. Axes scales are arbitrary distance units calculated by the ALSCAL program within the SPSS 19 software.
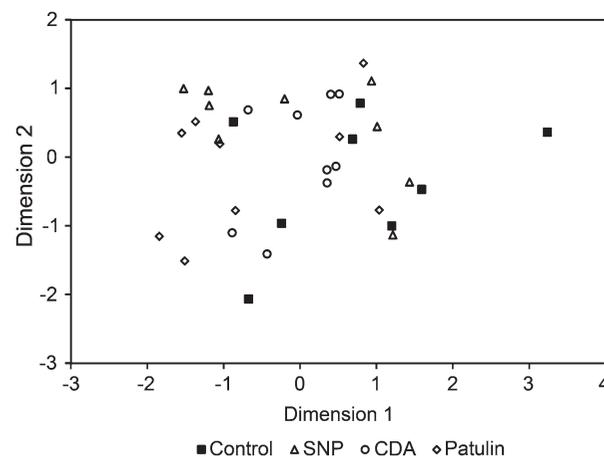
Table 1. DGGE bands were sequenced and compared to the GreenGenes database to identify the taxonomy of the organisms present in the system			
Band	**Identification**	**Taxonomic level of identification**	**Class**
05	Rhodobacteraceae	Family	α-proteobacteria
06	*Loktanella*	Genus	α-proteobacteria
13	*Hyphomicrobium*	Genus	α-proteobacteria
02b	Alcaligenaceae	Family	β-proteobacteria
16b	*Delftia*	Genus	β-proteobacteria
17b	*Delftia*	Genus	β-proteobacteria
01	*Colwellia*	Genus	γ-proteobacteria
02a	*Pseudoalteromonas issachenkonii*	Species	γ-proteobacteria
03	Altermonadaceae	Family	γ-proteobacteria
04	*Pseudoalteromonas*	Genus	γ-proteobacteria
07a	*Glaciecola*	Genus	γ-proteobacteria
07b	*Pseudoalteromonas issachenkonii*	Species	γ-proteobacteria
07c	*Marinobacterium*	Genus	γ-proteobacteria
07d	*Marinomonas mediterranea*	Species	γ-proteobacteria
08	Oceanospirillaceae	Family	γ-proteobacteria
09	*Colwellia*	Genus	γ-proteobacteria
10	*Pseudomonas pachastrellae*	Species	γ-proteobacteria
11a	Thiotrichacaea	Family	γ-proteobacteria
11b	Sinobaceraceae	Family	γ-proteobacteria
12	*Pseudoalteromonas*	Genus	γ-proteobacteria
14	*Simidua*	Genus	γ-proteobacteria
16a	*Pseudoaltermonas*	Genus	γ-proteobacteria
17a	*Marinobacter*	Genus	γ-proteobacteria
18a	*Marinobacter*	Genus	γ-proteobacteria
18b	*Marinobacter*	Genus	γ-proteobacteria
15	*Luteolibacter*	Genus	Verrucomicrobiae

Notes: Four organisms were identified to species level and most organisms were identified to the level of genus. Some banding positions were represented by more than one sequence (indicated by letters), and revealed that the same banding position could represent more than one species and in some cases those different species were from different classes.

Figure 5. Proportions of 16S rRNA DGGE bands from biofilms grown in the presence of three LCDMs or a sterile seawater control that were identified as belonging to the bacterial class Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria or Verrucomicrobiae. Class identifications were obtained by comparing DNA sequences of extracted DGGE bands to the GreenGenes database.
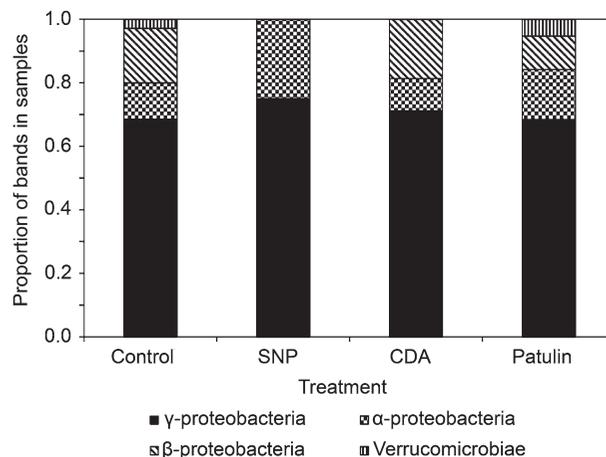
4. Discussion

4.1. Biomass

Previous studies have shown that nitric oxide is involved in the regulation of monoclonal biofilm formation for marine species (Arora & Boon, 2012; Henares, Xu, & Boon, 2013; Liu et al., 2012). This study has shown that the NO-donor SNP reduced the growth of biofilms when it was present from the beginning of biofilm development. It is interesting to note that the SNP concentrations that caused a reduction in biofilm formation in this study were much greater than those concentrations found to be effective in previous studies (Barraud, 2007; Barraud et al., 2006; Barraud, Storey, et al., 2009). While Barraud and colleagues reported dispersal events occurring at SNP concentrations of 500 nM, no significant decrease in biofilm formation was seen in this study until an SNP concentration of 50 μM was used. The reason for this increase in the amount of SNP needed for an effect on biofilms is not clear, but may be due to the difference in the nature of biofilm formation as studied here, and biofilm dispersal as studied by Barraud and colleagues. Preliminary studies by R. Howlin (personal communication) suggest that in order for SNP-derived nitric oxide to trigger dispersal of *P. aeruginosa* biofilms, the biofilm must first be well established. If this is also true of marine biofilms, then we would not expect nitric oxide to trigger dispersal in this system where it was present from the beginning of the biofilm growth. However, the lack of toxicity shows that there could be another physiological effect causing the reduction in biofilm formation. Studies by Thompson, Taylor, Brownlee, Callow, and Callow (2008), Thompson, Callow, and Callow (2010) showed that elevated concentrations of nitric oxide can reduce the adhesion of diatoms and zoospores to surfaces.

Another possible explanation for the difference in concentration at which SNP is effective between previous studies and the present study is the difference in media in which SNP was diluted: seawater in this study and M9 medium in Barraud's studies. The products of SNP decay can differ depending on the chemical conditions of an SNP solution (Smith & Dasgupta, 2002). For example, at higher pH, the level of nitroxyl anion (NO^-) tends to be higher relative to nitric oxide radical ($NO\cdot$). The measured pH of the seawater medium in this study was 8.2, which could have resulted in a reduction in $NO\cdot$ production and therefore a reduction in the effect of SNP. The use of alternative nitric oxide donors may therefore result in a greater reduction in biofilm formation in a marine environment.

The effect of CDA on the formation of biofilms, while statistically significant, was much lower than that of SNP and patulin. The reduction in biofilm formation appeared to begin at the lowest concentration of 0.005 μM. Davies and Marques (2009) showed that CDA was effective at inducing dispersal of many single species biofilms as well as an undefined mixed culture of airborne bacteria. The study by Davies and Marques did not show any data relating to the effects of biofilm formation across different concentrations of CDA, but dispersal events were shown to occur in a range of 0.001 μM–10 mM. As with this study, Davies and Marques' investigations showed an effect of CDA at the lowest concentration used, and so it is possible that it is effective at lower, unstudied concentrations.

In addition to a significant decrease in biofilm growth, CDA was also found to significantly increase biofilm growth at 0.5 μM. While this may indicate that CDA has two, concentration-dependent modes of action on biofilm growth, the lower level of significance found at 0.5 μM indicates that the increase in biofilm formation may not occur reliably and so may not represent a real effect in such a variable system as a marine biofilm.

The reduction in biofilm formation caused by patulin at concentrations from 0.5 μM was somewhat surprising because previous studies have shown that it increases the formation of biofilms at this concentration range (Liaqat et al., 2008, 2010, 2014). However, Liaqat et al. (2014) also showed a drop in biofilm formation by the marine bacterium *H. pacifica* at 25 μM, a similar concentration range to the maximum effect seen in this study at 50 μM. In addition, the range of concentrations at which patulin had an effect on the formation of biofilms was similar to those concentrations that have previously been shown to have an inhibitory effect on bacterial growth (13–650 μM) (Kavanagh, 1947; Klemmer, Riker, & Allen, 1955; Raistrick, 1943). It is therefore probable that patulin had an

antibacterial effect on the marine bacteria, reducing their ability to grow, as opposed to a cell signalling effect. This is supported by colony counting tests which showed that the inhibitory effect of patulin also occurred in the planktonic phase.

4.2. Biofilm communities

Previous studies of marine biofilms have shown that it is the Gamma and Alphaproteobacteria that tend to predominate. However, a study by Lee, Nam, Kim, Lee, and Lee (2008) showed that the while the Gammaproteobacteria are most prominent after 3 h of exposure of an acrylic surface, they become less prominent as the biofilm becomes established. After 6 h of exposure, Alphaproteobacteria begin to dominate the biofilm. The importance of both Alpha and Gammaproteobacteria is not supported in this study, with Gammaproteobacteria being greatly dominant over Alphaproteobacteria which were present at similar frequencies to Betaproteobacteria. Betaproteobacteria are also commonly found in marine biofilms (Edwards et al., 2010; Jones, Cottrell, Kirchman, & Dexter, 2007; Lee et al., 2008), but occur at much lower levels than Alpha and Gammproteobacteria. In this study, all three DGGE bands that were identified as belonging to Betaproteobacteria occurred at the same position as other bands which were identified as Gammaproteobacteria.

The MDS plots of DGGE banding similarity showed no clustering among treatments. This indicates that the LCDM molecules did not have an effect on the species present in biofilm communities. This is an unexpected result, as biomass analyses revealed differences in biofilm formation due to treatment with LCDMs. This suggests that the LCDMs are effective at reducing biofilm formation, irrespective of the species present. The lack of effect on the species within biofilms treated with different LCDMs was further supported by the richness data, which showed that none of the treatments significantly altered the richness of species in biofilms. Again, this was unexpected, as it might be expected that the LCDMs would have a greater effect on some species than on others, resulting in an increase in the dominance of some species over others.

While little community effect was seen using DGGE analysis, the true nature of the biofilm ecosystem may not be at the community level. In traditional models of the formation of biofilm communities, members of certain taxonomic groups will colonise a surface in a particular order to fulfil a specific niche (Hansen, Rainey, Haagensen, & Molin, 2007; Hassell, Comins, & May, 1994). The niches will vary depending on the physical and chemical environment, thereby modifying the structure of the biofilm community. In neutral community models (NCM), however, the membership of a community is determined largely by chance (Bell, 2000; Hubbell, 2001). Niches are colonised randomly by whichever species are present that can occupy that niche. In any given system, there may be a large number of different species that are capable of exploiting the same resources, and this group of species is known as a guild (Root, 1967). Members of guilds may not be taxonomically related, which means that in two communities grown under the exact same conditions, the species composition may vary considerably due to chance, while the community function may remain similar. This concept was demonstrated in biofilm communities by Burke, Steinberg, Rusch, Kjelleberg, and Thomas (2011) with bacterial species associated with the green macroalga *Ulva australis*. The assemblage of bacterial communities on *U. australis* was observed to vary significantly even under similar conditions. However, profiling of the functional systems of the bacterial biofilms showed a large degree of similarity.

Application of NCM to this study explains why there were no distinct biofilm communities based on 16S rDNA profiling, even when the effects of the physiological treatments were so great. It is possible that had functional genes been profiled, there would have been a much greater degree of similarity among treatments.

For this reason, future investigations of biofilms at a functional level may serve to increase the understanding of biofilm biology more effectively than community profiling. With the increasing availability of next-generation sequencing and protocols such as RNA-seq (Siezen, Wilson, & Todt, 2010; van Vliet, 2010; Wang, Gerstein, & Snyder, 2009), elucidating the functionality of groups of

micro-organisms within complex communities is a now realistic undertaking that can be carried out by many reasonably equipped laboratories. Using methods to investigate community function along with traditional 16S community analysis will provide invaluable information about the way in which mixed species biofilms function.

It is clear that much work is still needed to understand the processes involved in marine biofilm formation, the interactions between organisms in mixed communities and the effect that LCDM molecules such as NO and CDA have. However, this study has shown that NO in particular can be used to indiscriminately reduce biofilm formation by mixed communities at the physiological level instead of relying on toxicity.

Competing interests
The authors declare no competing interest.

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