# Bacterial Isolates

8 sets of bacterial isolates (Table A) were selected, sourced principally from the Nash’s Field experimental site at Silwood Park. Plots N and P (see Appendix X) of Nash’s Field have since 1991 been treated thrice yearly with metaldehyde at 960 g.ha-1 as part of herbivore exclusion experiments (Allan and Crawley, 2011), while bacteria were isolated and sequenced in 2016 for an earlier study (Mombrikotb, 2016). Isolates were stored on CryoBeads in a standard Brucella Broth with Glycerol solution (Hardy Diagnostics, CB12) at -80°C until needed for experiments, when they were cultured overnight on a rotary shaker in a standard R2A broth at room temperature (25.9 – 26.6 °C over a 48h period) prior to experiments. Soil communities were frozen in a 60% glycerol solution immediately after extraction and defrosted on an individual basis for overnight culturing.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Strain | Species | Species Notes | Source | Exposure History |
| SBM\_R2A\_LUF4\_5 | *Luteibacter rhizovicinus* | First isolated from rhizosphere | Nash’s Field | Control |
| R2A\_KUB5\_13 | *Variovorax paradoxus* | Strains capable of metabolising a wide | Nash’s Field | Control |
| R2A\_NUF1\_3 | *Variovorax paradoxus* | range of pollutants. | Nash’s Field | Metaldehyde |
| R2A\_KUE4\_4 | *Bacillus simplex* | Strains capable of biosorption of heavy metals and radionuclides | Nash’s Field | Control |
| R2A\_NUE1\_1 | *Bacillus simplex* |  | Nash’s Field | Metaldehyde |
| R2A\_KUE4\_10 | *Stenotrophomonas acidaminiphila* | First isolated from a waste acid-treating anaerobic bioreactor. | Nash’s Field | Control |
| *E. coli* OP50 | *Escherichia coli* | Included as an example of a well-studied lab model species | *C. elegans* feeder | Control |
| Nash’s  Field Community | Multiple | ??? | Nash’s Field | Control |

*Table A: Bacterial species, strains, and sources.*

# Stressor Selection

Stressors were selected across a diverse range of functional groups (see Table 1) in order to assemble a panel that had some but not all mechanisms of action in common amongst the stressors.

Nickel (28Ni) and Copper (29Cu) are heavy metals, common pollutants with a wide range of industrial applications. Copper is an essential respiratory nutrient across all kingdoms of life (Babcock and Wikström, 1992), while nickel is occasionally so in bacteria and fungi (Zamble, 2015). Bacterial resistance to these stressors is thus often nuanced, with a requirement to balance availably in the cell as nutrients with their potential for damage. Copper is a prolific generator of Reactive Oxygen Species (ROS) (Bal and Kasprzak, 2002), and damages vital biosynthesis enzymes (Macomber and Imlay, 2009), while nickel is a weak ROS generator that can unbalance iron and zinc homeostasis in the cell (Samland and Sprenger, 2006). Copper and nickel are resisted through similar pathways (Mykytczuk *et al.*, 2011), including active efflux and membrane modification. Copper is also resisted through chelation and rapid repair of damaged enzymes (Macomber and Imlay, 2009), while nickel can be sequestered inside the cell (Nishimura, Igarashi and Kakinuma, 1998).

Chloramphenicol and ampicillin are broad spectrum antibacterial agents, used in decreasing amounts in healthcare applications due to their severe side effects and growing resistance, but nevertheless well-studied environmental pollutants. First isolated from *Streptomyces venezuelae*, a soil-dwelling bacterium, chloramphenicol is a broad-spectrum antibiotic to which resistance in the wild and areas under antibiotic pollution is particularly common (Allen *et al.*, 2010). Ampicillin, a widespread, broad-spectrum antibiotic from the penicillin family is also widely resisted (Ruiz *et al.*, 1999). Chloramphenicol acts bacteriostatically by inhibiting protein synthesis in the 50S ribosomal subunit, while ampicillin inhibits cell wall synthesis. Drug interaction studies have shown that chloramphenicol is negatively antagonistic towards ampicillin due to their competing modes of action (van Bambeke *et al.*, 2017). It has been suggested that many of the genes that provide resistance to common antibiotics including chloramphenicol also provide tolerance to environmental stress in non-pathogenic species (Groh *et al.*, 2007). Research has also found that exposure to ROS from heavy metals, including copper, can co-select for chloramphenicol resistance (Harrison *et al.*, 2009).

Metaldehyde and atrazine are two distinct chemical pesticides; the first a molluscicide, the second a herbicide. Atrazine, a triazine pesticide has been banned in the EU since 2004 (EU, 2004), but has remained the most commonly used herbicide in the US, and acts on plants by disrupting photosynthesis (Shimabukuro and Swanson, 1969), while metaldehyde is rapidly converted within the body of molluscs to aldehyde, which damages mucus producing cells, causing excessive mucus production, dehydration, and eventual death (Triebskorn, Christensen and Heim, 1998). Atrazine has been shown to be both a food source (Wackett et al., 2002) and ROS stressor (Zhang et al., 2012) to various species of bacteria, but information on metaldehyde’s effects on bacteria are limited: one study has examined interactions between bacteria and metaldehyde (Thomas et al., 2017), showing only that *Variovorax* and *Aceinetobacter* strains can be isolated from metaldehyde-treated soil and can degrade the molluscicide.

Tebuconazole is a triazole fungicide that acts against a broad range of pathogens by inhibiting fungi-specific membrane synthesis pathways. Tebuconazole is known to be toxic to a range of non-target species (Sehnem *et al.*, 2010), but information on effects on bacteria is limited to the knowledge that some bacteria (not including any of the species used in this study) are capable of degrading tebuconazole (Sehnem *et al.*, 2010). Azoxystrobin is a systemic fungicide in heavy use due to its broad-spectrum inhibition of respiration across major groups of fungal pathogens. Azoxystrobin has been shown to inhibit bacterial growth in mixed fungal-bacteria communities (Baćmaga, Kucharski and Wyszkowska, 2015), although the same study showed *Bacillus* species were capable of growth in highly contaminated soil.

# Rangefinding and Concentration Calculations

Initial dose-response exposures were conducted at concentrations above and below regulatory limits to obtain an overview of individual stressors’ effects on isolates. Eight 96-well microtiter plates were prepared, with each well being aliquoted with 10 μl of 1-in-100 diluted overnight culture, 80 μl of R2A broth, and 10 μl of stressor stock at either 10-1, 1, 101 or 102 times the target final experimental concentration. Each well of stressor concentration and isolate was replicated three times. Well OD was read using the cell count protocol below (3). Data is available in Appendix X.

Final experimental concentrations were calculated based on well volumes and regulatory limits. Calculations are available in Appendix X.

# Cell Counts

Microcosm optical density was used as a metric of cell count over time. Immediately after isolates were exposed to stressors, plates were placed in an automatically-fed BioTek Synergy 2 microplate reader for 48 hours, agitating the wells for 5 seconds then reading absorbance at 590 nm every hour. Optical density readings were converted into cell counts through the use of calibration curves generated by prior analysis performed on a BD Accuri C6 flow cytometer.

# Stressor Exposures

Stressor combinations were formulated by use of a Hamilton MicroLab STARLet, fitted with sterile conductive pipette tips. 255 combinations of 8 stressors across 8 bacterial isolates were formulated across 24 2uL 96-well plates, with additional controls for a total of 2144 exposures per replicate. 10 μL doses of stressor solutions at environmentally relevant concentrations (Table 1) were added to the wells, in addition to 10 μL of overnight bacterial culture diluted to 1 in 1000, and sufficient R2A broth to bring all well volumes up to 100 μL. Machine-readable worklists (Appendix Y) were generated from a combination input file for the STARLet using an R script (Appendix Z).

# Statistical Analysis

I processed and manipulated raw data using the *tidyverse* family of packages (Wickham, 2017). Optical density at 509 nm was models against time in hours using a logistic curve from the package *growthcurver* (Sprouffske and Wagner, 2016). I plotted growth data and logistic curve by well in order to visually inspect goodness of fit and exclude inappropriate data. A null additive model was assumed for all comparisons, consistent with the use of bacterial growth as a metric of response (Piggott, Townsend and Matthaei, 2015).

All R code is available in Appendix R.

## Stressor Complexity versus Growth Parameters

I plotted fastest growth rate, carrying capacity (highest growth rate), empirical area under the raw growth curve and area under the logistic growth against stressor richness. None of the models fit the data well yet so I didn’t bother checking goodness of fit/statistical power.

## Single Stressor Growth Curves by Isolate

I plotted growth curves for single stressors by isolates. The graphs aren’t very good, and this probably doesn’t need its own section.

## Binary Stressor Interactions by Isolate

Interaction between binary combination of stressors was calculated by comparing individual and binary stressors growth rates to unexposed. I may end up removing this.

## Prevalence of Effect Type

This is going to be the graph Tom recommend I make.

## Emergent Interactions in Higher-Order Stressors

Beppler *et al.* (2016) is very interesting, but a lot to take in. I will be adapting their methods to my higher order stressors as I go.

I will also be ANOVA-ing models of 1-8 way interactions between stressors to determine the most important order of interactions.

## Other Statistical Treatments

Who knows?