Imperial College London

Are regulatory limits on pollutants sufficient to protect from emergent mixture effects?

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science at Imperial College London

Formatted in the journal style of the *Journal of the Total Environment*

Submitted for the MSc in Ecological Applications

Samuel A. Welch

August 2018

1. Declaration

I had a lot of help.

Are regulatory limits on pollutants sufficient to protect from emergent mixture effects?

Samuel A. Welch

Silwood Park, Imperial College London, United Kingdom

Abstract

I did horrible things to bacteria; sometimes several at once. In the end though I think the bacteria may have still come out ahead. Maybe if I pad the abstract out a little more, like so, everything will be much more neatly laid out across the pages.

Keywords: Bacteria, Ecotoxicology, Higher-order effects, Multiple Stressors, Mixture Toxicity

2. Introduction

Any organism today must content with not only a broad range of ecological and biogeographic stressors, but also the presence of more than 100 million known chemicals [1]. Though many of these chemicals have always been naturally present in the world as components of the crust, secreted biocides or waste products, the chemical landscape of ecosystems has never been more complex, with chemical stressors mixing with hard-to-predict effects [2].

Though mixture toxicity has long attracted much academic attention and study [3], criticisms remain of a lack of consensus on experimental design, use of statistics, and even inconsistent interaction definitions [4, 5, 6]. Ecotoxicology still bears the legacy of its largely regulatory past, a preoccupation with declaring any given chemical safe or not, using a limited panel of model species [7]. Equally, existing mixture toxicity regulations in the EU are limited and distinctly human-focused [2], and do not represent the latest in scientific knowledge.

A longstanding criticism of ecotoxicology remains the difficulty of integrating ecotoxicological results into a broader understanding of how ecosystems respond to stress across their component species. [8, 9]. Ecosystems are without exception supported by a broad trophic foundation of microbes that provide vital services as decomposers, chemical engineers, and pathogens [10, 11], but in regulatory and scientific settings a chemical's toxicity to bacteria is typically tested using the luminescent bacteria toxicity assay (LBTA) [7], a 30-minute exposure to *Aliivibrio fischerii*, a bioluminescent marine decomposer. The LBTA is the industry standard due to its simplicity and ease of use, but it suffers from a number of well-documented limitations [12] in addition to the difficult of generalising from one species to an entire ecosystem.

Traditional models of mixture toxicity focus on binary mixtures of stressors. Two chemicals or environmental factors, that known to have a positive or negative effect on some aspect of a species' fitness, are mixed at various concentrations. Their combined effect compared to a null-model prediction of their interaction – often additive, the sum of their parts – in order to determine if in combination they display synergism -– an effect greater than the sum of their parts, antagonism – an effect lesser than the sum of their parts — or simple additivism, when the effect is statistically indistinguishable from the null model.

Though easy to understand and employ, this model is not without its issues. What, for example, is a stressor? The term is often used as a synonym for 'pollutant', 'toxicant', or 'pressure' – a substance or treatment that produces a negative fitness effect in the species of interest [5]. However, not only does

this fail to account for the well-known adage that 'the dose makes the poison'[13] — as true for a rise in temperature or in nutrient availability as it is for toxic pollutants – but also that *the species makes the poison*. Many organisms, particularly bacteria, are are able to tolerate or even thrive on substances that are harmful to other species [14, 15].

Due to the geometrically-increasing complexity of adding additional stressors to interaction experiments, the number of studies that have experimentally examined the interactions of mixtures of three or more chemicals — 'higher order interactions' — has been limited [16]. This has been justified by a long-held assumption that higher-order interactions are negligible compared to lower-order interactions [17], but recent studies examining complex mixtures of antibiotics have shown emergent higher-order effects, three-way interactions not predictable from component two-way effects, meaning that the existing body of binary-interaction based literature may not be scalable over more complex mixtures to understand the effects on ecosystems [18, 19, 20].

Within ecology, research on complex combinations of stressors -– including chemicals — is more common, and suffers from similar uncertainty and confusion in defining synergy and antagonism [21]. Beppler *et al.* also highlight the study of emergent interactions between higher-order mixtures of predators -– Multiple Predator Effects (MPEs) — that has received some attention. One recent review of the literature [22] found overall that species-rich mixtures of predators were more effective at suppressing prey than poorer mixtures, although a considerable number of component studies found no significant relationship.

Parallels can also be drawn with research on inter-species interactions in the vein of food webs, the presence of a species having a positive or negative effect on the fitness of other species in a similar manner to a chemical stressor. Considerable research has explored interaction across food webs and trophic levels, and the cascading shifts in biotic interactions caused by abiotic environmental change [23, 24, 25]. Research has shown that chemical stressors such as pesticides can cause large scale changes over short timescales to ecosystems, mediated through complex webs of interactions [26]. The effect of species interaction on evolutionary responses has also been examined species interaction [27], showing that in bacteria these interactions can over time cause considerable shifts in both communities and the phenotypes and genotypes of component species.

In the Anthropocene, an era where pollution is a greater pressure on ecosystems than ever before, it is vital that we improve our understanding of how a diverse range of wild bacteria respond to different stressors. Greater understanding of these stressors and interactions will enable more targeted and cost-efficient legislation and interventions -– for instance, one stressor has a disproportionate effect on overall response and thus should produce the greatest positive effect when removed.

This study thus aims to address this knowledge gap through the use of automated experimental set-up and observational equipment to examine the effects of complex mixtures of common pollutants on a diverse panel of soil bacteria. It is hypothesised that more complex mixtures of chemicals will produce emergent effects not predictable from their component lower-order interactions, and that single stressor responses and interaction responses will vary across the bacterial panel.

3. Methods

3.1. Isolate Selection

Eight bacterial isolates were selected, sourced principally from the Nash's Field experimental site at Silwood Park, United Kingdom. Since 1991, plots within Nash's Field have been treated three times yearly with metaldehyde at 960 g/ha as part of herbivore exclusion experiments [28] (section B). The bacteria used in this study were isolated and sequenced form plots with different exposure histories (table 1) in 2016 for an earlier study [29].

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

Strain	Species	History	Notes
LUF4_5	*Luteibacter rhizovicinus*	Control	First isolated from the rhizosphere [30].
KUB5_13	*Variovorax paradoxus*	Control	Strains capable of metabolising a wide range of pollutants [31].
NUF1_3	*Variovorax paradoxus*	Metaldehyde	
KUE4_4	*Bacillus simplex*	Control	Strains capable of biosorption of heavy metals and radionuclides [32].
NUE1_1	*Bacillus simplex*	Metaldehyde	
KUE4_10	*Stenotrophomonas acidaminiphila*	Control	First isolated from a waste-acid-treating anaerobic bioreactor [33].
OP50	*Escherichia coli*	Control	Included as a model species.
KUA5 Sample	Soil Community	Control	

Table 1: Bacterial species, strains, and sources.

in a standard R2A broth at room temperature (25.9°C–26.6°C) prior to experiments. Soil communities were frozen in a 60% glycerol solution immediately after extraction and defrosted on an individually for overnight culturing.

3.2. Stressor Selection

Chemical stressors were selected across a diverse range of functional groups (table 2) in order to assemble a panel that had some but not all mechanisms of action in common amongst the stressors, and that have the potential to occur in mixtures in ecosystems.

Nickel ($_{28}$Ni) and Copper ($_{29}$Cu) are heavy metals, common pollutants with a wide range of industrial applications. Copper is an essential respiratory nutrient across all kingdoms of life [34], while nickel is occasionally so in bacteria and fungi [35]. Bacterial resistance to these stressors is thus often nuanced, with a requirement to balance availability in the cell as nutrients with their potential for damage. Copper is a prolific generator of Reactive Oxygen Species (ROS) [36], and damages vital biosynthesis enzymes [37], while nickel is a weak ROS generator that can unbalance iron and zinc homeostasis in the cell [38]. Copper and nickel are resisted through similar pathways [39], including active efflux and membrane modification. Copper is also resisted through chelation and rapid repair of damaged enzymes [37], while nickel can be sequestered inside the cell [40].

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 [41]. Ampicillin, a widespread, broad-spectrum antibiotic from the penicillin family is also widely resisted [42]. 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 [43]. 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 [44]. Research has also found that exposure to ROS from heavy metals, including copper, can co-select for chloramphenicol resistance [45].

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 [46], but has remained the most commonly used herbicide in the US, and acts on plants by disrupting photosynthesis [47], 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 [48]. Atrazine has been shown to be both a food source [49] and ROS stressor [50] to various species of bacteria, but information on metaldehyde's effects on bacteria are limited: one study has examined interactions between bacteria and metaldehyde [51], 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 [52], 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 [52]. 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 [53], although the same study showed *Bacillus* species were capable of growth in highly contaminated soil.

Limited data are available on the prevalence and composition of chemical mixtures in waters in the UK. However, hospital effluent is known to represent an important source of not only anti-bacterial pollution [54] but also heavy metals [55]. Agriculture is also a potential source of complex chemical mixtures, through the simultaneous use of fungicides, herbicides, insecticides, veterinary antibiotics [56], while heavy metals frequently enter the environment through industrial wastewater, mine run-off, and landfill leachate [57]. Where pollution from these sources mix, such as at the interface between different land uses, or in the body of a river that runs through such areas, chemical mixtures are likely to be common.

Stressor	Functional Group	Limit (μg/l)	Form/Product Code	Bacterial mechanisms of action	Bacterial mechanisms of resistance	Sources
Copper	Heavy Metal	2000[t]	$Cu(II)Cl_2$, dihydrate (99%), Alfa Aesar 12458	Essential nutrient, ROS, enzymes	Efflux, chelation, rapid repair, membrane transition	[58, 59, 60, 61]
Nickel	Heavy Metal	20[t]	$Ni(II)Cl_2$, anhydrous (98%), Alfa Aesar B22085	Enzymes, Fe/Zn homeostasis, weak ROS, nutrient	Sequestration, efflux, membrane transition	[58, 62, 40, 35]
Chloramphenicol	Antibacterial	0.05[n]	Powder ⩾98%, Sigma Aldrich C0378	Cell wall synthesis	Membrane transition, mutant ribosomes, anti-AB enzymes	[63, 64, 65, 42]
Ampicillin	Antibacterial	0.12[n]	Ampicillin sodium salt, Sigma Aldrich A9518	Protein synthesis, 50S ribosome subunit	β-lactamase enzymes, efflux	[42, 66]
Atrazine	Pesticide (Herbicide)	0.25[t]	Power, analytical, Sigma Aldrich 45330	ROS, bacterial enzymatic nutrient	Biodegradation	[47, 67, 50]
Metaldehyde	Pesticide (Insecticide)	0.5[t]	Powder, analytical, Sigma Aldrich 63990	Toxicity unknown, possible nutrient	Biodegradation	[68, 69, 51]
Tebuconazole	Antibacterial	1[r]	Ampicillin sodium salt, Sigma Aldrich 32013	Unknown	Biodegradation	[52, 70]
Azoxystrobin	Antibacterial	3[r]	Powder, Analytical, Sigma Aldrich 31697	Unknown	Biodegradation	[71, 72, 73, 53]

Table 2: Summary of stressors including type, target concentration, product information and bacterial interactions.

[t] UK drinking water regulatory limit, Water Framework Directive
[n] No legal limit, typical environmental concentrations used.
[r] Regulatory Acceptable Concentrations, Water Framework Directive

3.3. 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 10 µl of 1-in-100 diluted overnight culture, 80 µl of R2A broth, and 10 µl of stressor stock at either 0.1, 1, 10 or 100 times the target final experimental concentration added to each well. Each well of stressor concentration and isolate was replicated three times. Well OD was read using the cell count protocol below 3.4.

Final experimental concentrations were calculated based on regulatory limits (Limits column, table 2). Calculations are available in Appendix A.

3.4. Metrics of Growth

Microcosm optical density at 590 nm 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 every four hours. At the conclusion of OD readings, wells were checked for contamination by plating the contents of the control plate at 10^{-3} and 10^{-4} dilutions onto a R2A agar plate and culturing at room temperature for 24 hours.

Flow cytometric cell counts, read in an Accuri C6 Flow Cytometer, were plotted against optical density using a linear model in an attempt to transform OD reading into absolute cell counts. However, no significant relationship was found between the two measurements, as at the time of measurement, cell counts were insignificantly high to be detected as changes in optical density (Appendix A).

Optical density was modelled against time in hours using a logistic curve, selected as a well-documented and fitting model [74]. from the package *growthcurver* [75] for the R programming language [76].

$$Nl_t = \frac{K}{1 + (\frac{K-N_0}{N_0})e^{-rt}} \tag{1}$$

Growth data and logistic curve were plotted by well, allowing for visual inspection and the exclusion of models from wells where no growth had occurred as a result of factors other than stressor treatment. A null additive model was assumed for all comparisons, consistent with the use of bacterial growth as a metric of response [5].

A typical bacterial growth curve [77] offers a number of potential parameters for the quantification of bacterial growth, summarised in table 3 below. Of the metrics available, the unitless measure of empirical area under the curve (*auc_e* in *growthcurver)* was selected, as its use did not involve the same loss of exponential phase growth data as in measurement of lag time, carrying capacity, or level of growth at a specific time point. Growth level, which involves the fitting of a Zwietering logistic growth curve to measured growth and the extraction of parameters from the curve, was not considered as a metric due to a lack of awareness during the data analysis phase [78]. Growth score, a refined form of growth level, was rejected for the same reason [79].

3.5. Stressor Exposures

Stressor combinations were formulated by use of a Hamilton MicroLab STARLet, fitted with sterile pipette tips and a laminar flow hood. Seven bacterial isolates and one mixed soil community were exposed to combinations of 8 stressors (n = 255), formulated across twenty four 200 µl flat bottom 96-well plates. Negative controls were added, for a total of 2144 exposures per replicate. 10 µl doses of stressor solution, diluted to regulatory limits (table 2) with R2A broth, were added to the wells, with sufficient R2A broth to bring all well volumes up to 90 µl. Machine-readable worklists were generated from a combination input file for the STARLet using an R script (Appendix A). Experimental for a single replicates typically

Metric	Description	Advantages	Disadvantages	Example/ Citation
Lag time	Time before bacterial growth phase	Provides high level of resolution over initial growth phase	Ignores variation in other phases	[77]
Area under curve (auc)	Area under logistic or empirical growth curve	Accounts for effects at all phases of growth	Loss of growth resolution	[80]
Carrying capacity k	Maximum growth (stationary phase)	Better accounts for variable growth speed between species	Ignores variation in other phases	[81]
Growth_t	Growth as measured at a consistent timepoint	Easy to measure	Considerable loss of resolution	[82]
Growth Level (GL)	Least-squares fitting of Zwietering logistic growth curve model	Effective in differentiating growth speeds	Convoluted, not easily interpretable	[78, 79]
Growth Score (GS)	Simplified and refined interpretation of GL	Easier to interpret, less heavily affected by experiment length	New and untested, complex implementation	[79]

Table 3: Potential metrics of bacterial growth

lasted 20 hours spread across 2 days; plates were stored in sealed containers at 4°C overnight, and from the second run onwards with a dampened paper towel to reduce evaporation. Finally, 10 µl of overnight bacterial culture, diluted to 1 in 1000 in R2A broth, was added to the plates. Plate optical density was read for 48 hours, with one read per hour for replicates 1 and 2 and one read per four hours for replicate 3 and 4, in an attempt to reduce evaporation. In the former case only every fourth read was sampled to produce consistent growth curves.

3.6. Statistical Analysis

All data processing and statistical analysis was conducted in R version 3.5.1 [76], data using the *tidyverse* family of packages [83]. All R code is available in Appendix A, as well as full attribution of all other packages, functions and software used in the production of this report.

In order to provide an initial measure of isolate untreated and single stressor growth rates, optical density for control and single stressor exposures were graphed against time, with a local regression plotted across measured points and error bars added.

The assumption that under an additive null model, fitness would be negatively correlated with mixture complexity was then examined by modelling mean *auc* against the number of stressors applied in a treatment using two linear models with complexity and isolate or species as explanatory variables, which were compared using an analysis of variance (ANOVA).

In order the produce an quantifiable measure of the effects of stressors and stressor mixtures on growth, stressor effect was calculated by subtracting the average growth with stressors from the control growth for a given isolate, producing an additive measure of effect, which was averaged across replicates to produce a measure of mean growth and standard deviation.

$$Effect = Growth_{Control} - Growth_{Treatment} \tag{2}$$

Predicted effect was then calculated using an additive null model, by summing the calculated mean effects of individual stressors on a per-isolate basis and calculating an updated standard deviation from the root of the summed squares of the component standard deviations.

Effect type was classified according to the definitions found in Piggott, Townsend, and Matthaei [5]. If the mean predicted and observed effects were not significantly different (independent two-sample T-test, $p < 0.05$), the interaction was classified as additive, regardless of the absolute distance between the two values. A positive effect on growth larger than the predicted effect was classified as positive synergy (+S), a negative effect larger (in absolute terms) than the predicted effect was negative synergy (-S). An effect between positive synergy and the predicted effect was classified as negative antagonism (-A), while an effect between negative synergy and the predicted effect was positive antagonism (+A) (fig. 1).

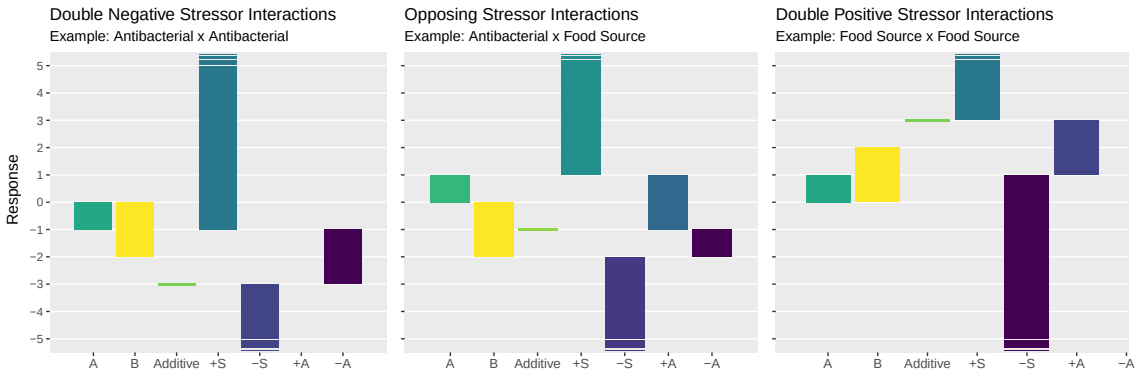

Figure 1: Plots to illustrate the ranges of Piggott, Mattheai & Townsend's reconceptualised interaction definitions [5]. A gradient effect indicates a definition range that continues towards $-\infty$ or $+\infty$, while the range of additivism is technically bounded by statistical significance rather than pure numerical distance.

Eight multiple linear regressions were fitted to the data using a nested set combinations of stressors, from individual stressors to eight-way combinations and all less-complex combinations, to explain variation, and determine statistically what level of interaction was predominant in the observed results. Linear regressions were compared using both a one-way ANOVA to F-test nested models, to apply an F-test to models, and Akaike Information Criteria test (AIC), to compare models with a penalty for number of estimated parameters [84].

A variation of the protocol used in Beppler *et al.* [16] was applied to the data to detect 'emergent interactions', observed effects for an c-complexity stressor mixture that could not be predicted from the combined effects of their component $(c-1)$-complexity mixture observations. Predicted mean, standard deviation and sample size were calculated per isolate as follows:

$$m_c = \sum_{i=1}^{c_n} \left(\frac{m_c - 1}{c_n} \right) \tag{3}$$

$$s_n^2 = \sqrt{\sum_{i=1}^{c_n} \left(\frac{(s_{n-1}^2)^2}{c_n} \right)} \tag{4}$$

$$n_c = \sum_{i=1}^{c_n} \left(\frac{n_c - 1}{c_n} \right) \tag{5}$$

where:

c : complexity of stressor mixture
c_n : number of mixtures of c complexity
m : mean effect of mixture of complexity c
n_c : adjusted sample size of component–predicted effect of mixture of complexity c
s_n^2 : adjusted summed standard deviation of component–predicted mixture of complexity c

4. Results

4.1. Rangefinding

Isolates showed a variable response across the spread of rangefinding concentrations (fig. 2) with KUB5_13, KUE4_4 and the Nash's Field Soil Community generally proving the most able to grow regardless of stressor or concentration. The expected negative correlation between concentration and mean

growth was not apparent. Azoxystrobin–exposed cultures did not appear to grow at any of the concentrations used.

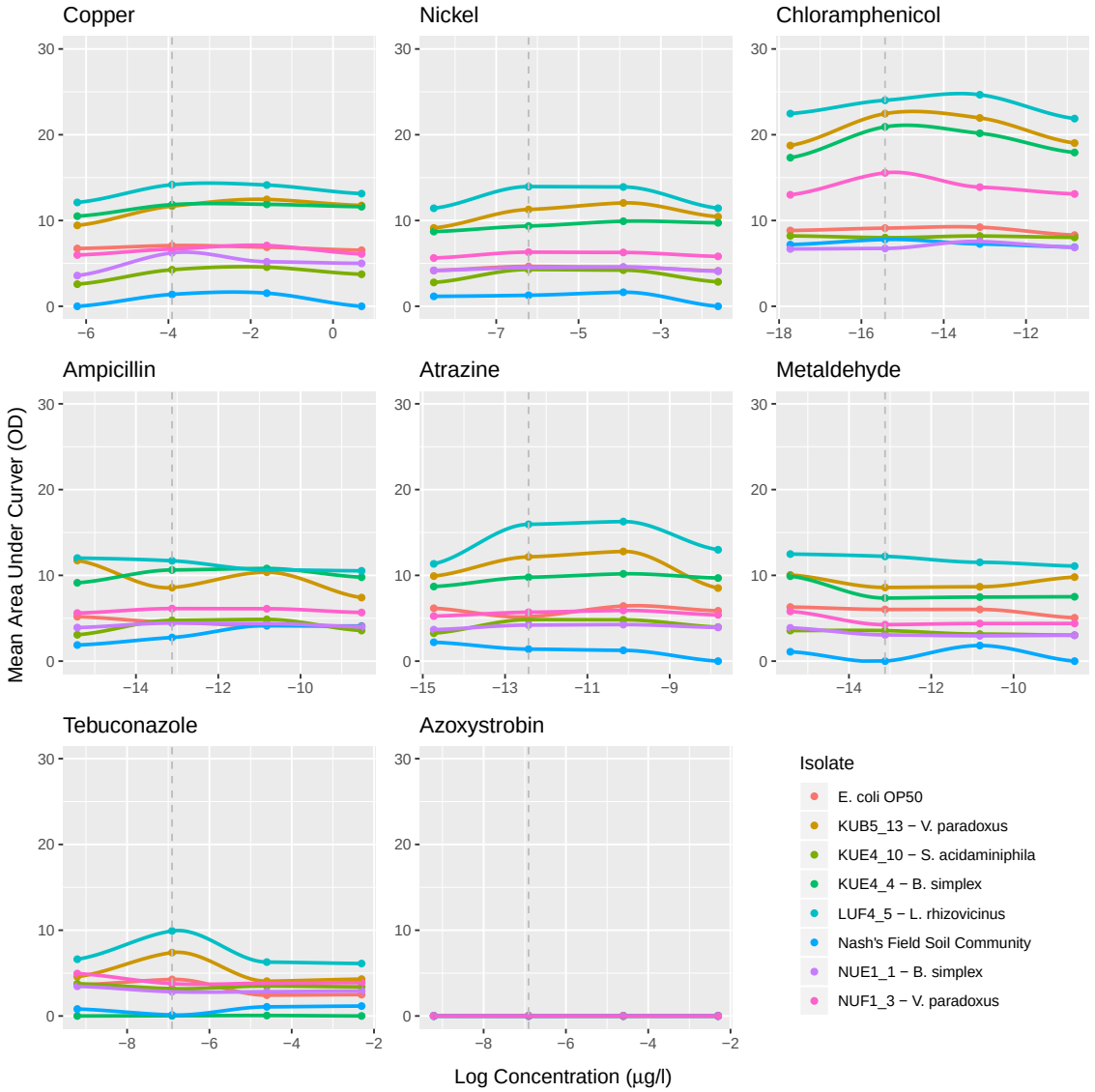

Figure 2: Mean area under growth curve at λ = 590 nm by isolate, across target concentration ÷ 10, target concentration (dotted grey line), target concentration × 10 and target concentration × 100 (see table 2). Local area polynomial regression (LOESS) fitted to data points for illustrative purposes. n = 3 for all combinations of isolate, stressor and concentration.

4.2. Sample Sizes

Due to a number of issues with isolate growth in liquid media and manual pipetting error of bacterial cultures, sample size varies across both treatments and isolates (table 4). These small and variable sample sizes have affected the statistical significance of the data and analysis discussed below.

Isolate	n Mean	n SD
LUF4_5	3.3	1.7
KUB5_13	2.6	1.9
NUF1_3	2.7	1.7
KUE4_4	3.4	1.8
NUE1_1	2.6	1.6
KUE4_10	3.0	1.9
OP50	3.3	2.0
Soil Community	3.0	1.7

Table 4: Sample size mean and standard distribution by isolate.

4.3. Single Stressor Responses

Isolates displayed a variety of responses (fig. 3) to the various stressors, growth in some cases apparently enhanced by the presence of most stressors (NUE1_1, KUE4_10, OP50, KUE4_4, and the soil community) but in some cases diminished (NUF1_3 and LUF4_5) and in one largely unaffected (KUE4_4). A bump in the growth phase generally indicative of diauxic growth was strongly apparent in KUE4_10, and sporadically present in the soil community, KUE4_4, NUF1_3 and NUE1_1.

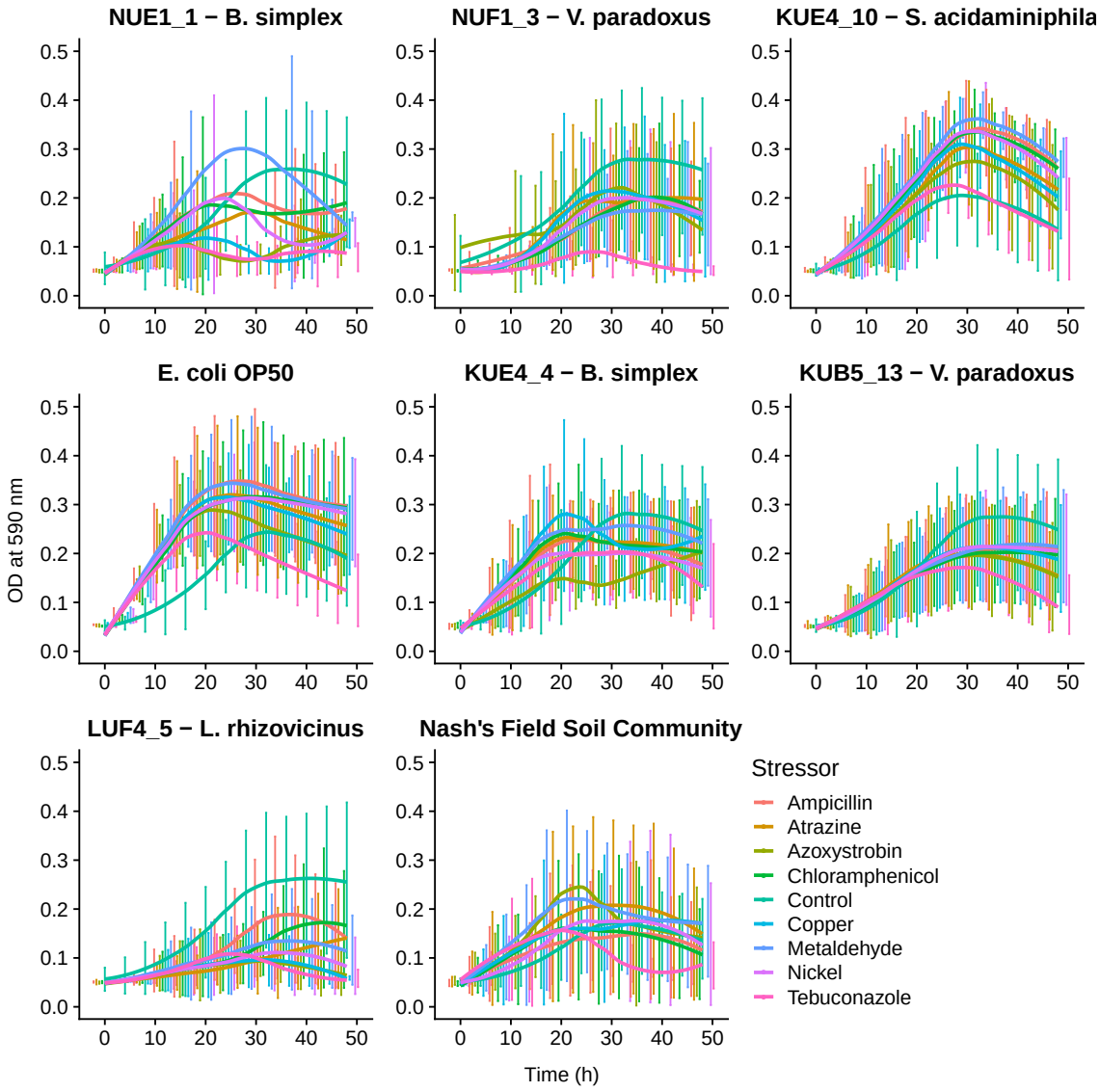

Figure 3: Effects of exposures of eight bacterial isolates to eight individual stressors, as well as a negative control on growth as measured by OD at 590 nm, over 48 hours, with standard deviation. n = 1-4; see table 4

4.4. *Growth by Stressor Complexity*

Modelling mean growth as either isolate or species versus mixture complexity did not reveal a statistically significant relationship between complexity and growth. Species identities *B. simplex*, *E. coli*, *V. paradoxus* and the soil community were good predictors of variation ($p < 0.05$).

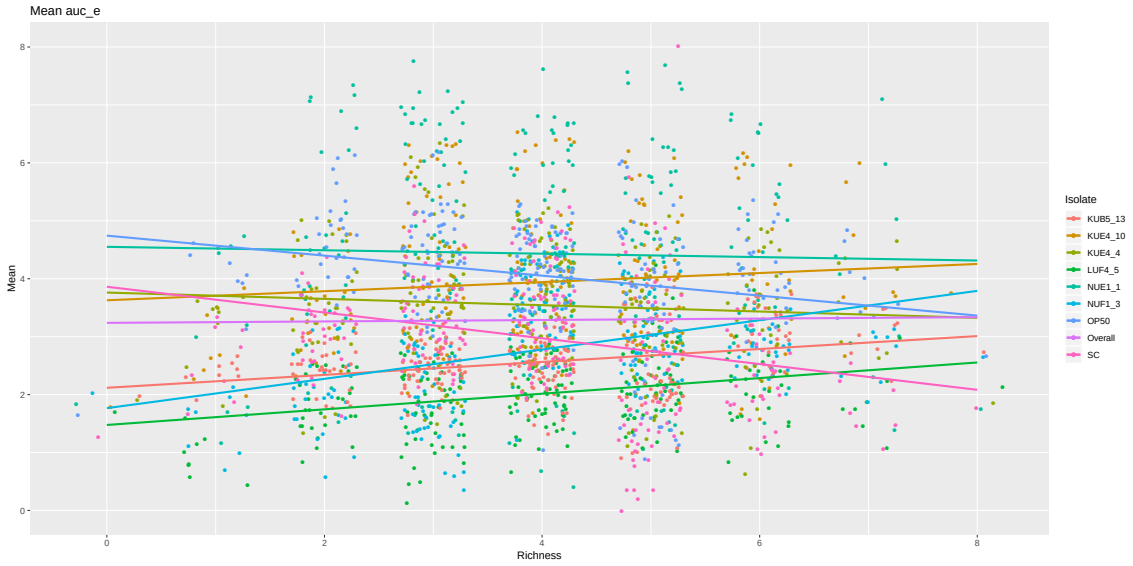

Figure 4: Growth by complexity

4.5. *Predicted Versus Observed Effect*

Patterns of observed versus predicted mean effect varied considerably between the species (fig. 5). KUB5_13, NUF1_3 and LUF4_5's are tight, linear distributions clustered around the 0 marks on both axes, while OP50, KUE4_4 and the soil community's observed effects fell largely below the predicted additive effect, especially at high levels of stressor complexity. KUE4_10 and NUE1_1's patterns are more diffused across the additive line, but display a similar pattern of synergy at lower levels of complexity and antagonism at higher levels. This pattern is repeat across the majority of isolates, although LUF4_5 shows a reversal of this pattern, and NUF1_3 is clustered similarly but with greater x and y overlap between points of different richness.

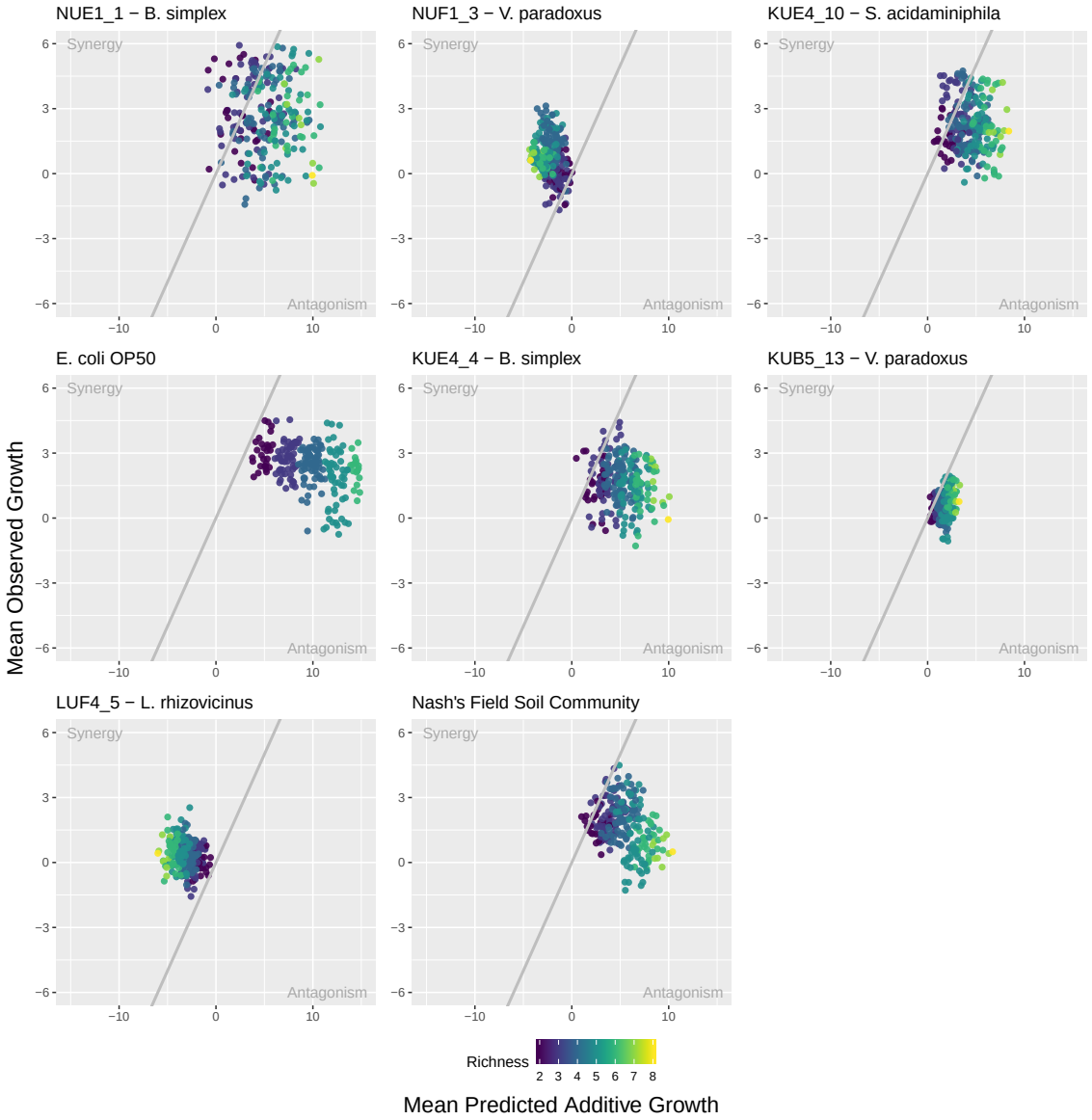

Figure 5: Observed versus predicted effects.

4.6. Comparison of Linear Models of Higher-Order Mixtures

A comparison of linear models of varying complexity (table 5) showed F-values higher than the critical F-statistic (1.08 at p = 0.05) at $p < 0.05$ for the, 2-, 3- and 6-complexity models. Penalised for number of parameters, the 2-complexity model had a significantly lower AIC than other models. We can thus infer that a model of two-way interations and single stressor effects best explains the variation seen in our data.

Complexity	Number of Predictors	Residual Df	Residual Sum of Squares	Df	Sum of Squares	F-value	Pr(>F)	AIC Df	AIC Score
1	8	6154	28546.62	NA	NA	NA	NA	10	26957.56
2	36	6126	27757.15	28	789.47	6.29	0.00	38	26840.72
3	92	6070	27494.60	56	262.55	1.05	0.38	94	26894.15
4	162	6000	26944.72	70	549.87	1.75	0.00	164	26909.64
5	218	5944	26783.04	56	161.69	0.64	0.98	220	26984.55
6	246	5916	26570.95	28	212.09	1.69	0.01	248	26991.55
7	254	5908	26503.86	8	67.09	1.87	0.06	256	26991.97
8	255	5907	26497.05	1	6.82	1.52	0.22	257	26992.38

Table 5: Comparison of the ANOVA and AIC parameters of eight multiple linear models of 1 to 8-way interactions, rounded to 2 decimal places. Calculated critical F-statistic of 1.08 at p = 0.05.

4.7. *Interaction Type Prevalence*

The analysis of interaction type showed a relatively high incidence of T-test errors (133 in 1976; 15%), due to a correspondingly high incidence of observations with a sample size of one and no standard deviation. For most isolates additive effects prevailed (1744 in 1976; 88%), the exception being *E. coli* OP50, where some non-additive effects were seen, especially at a complexity of 4 and above; negative antagonism accounted for slightly under half of the interactions observed (94 in 247; 38%), while negative synergy was observed only at a complexity of 5 and in low quantities (5 in 247; 2%).

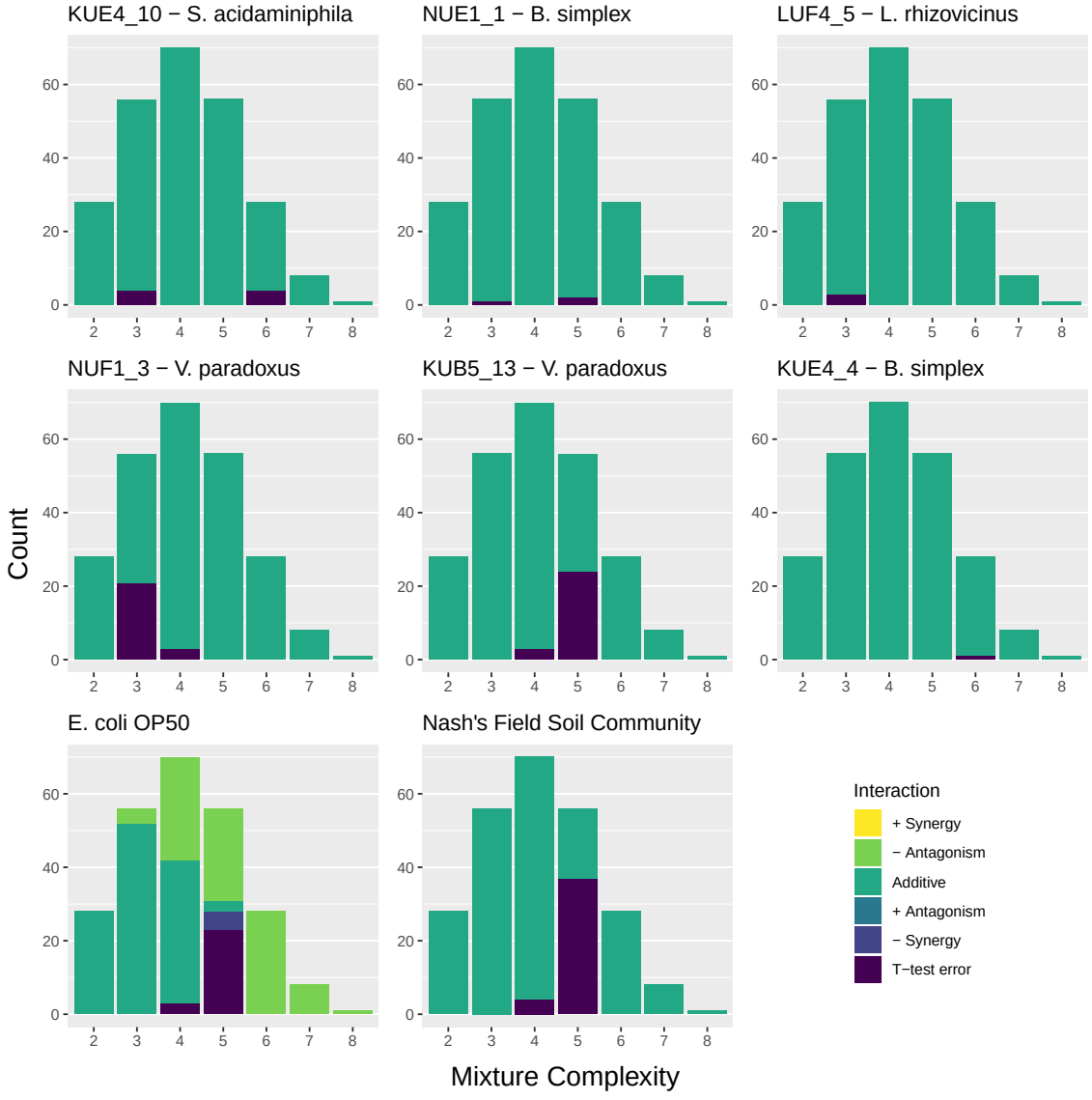

Figure 6: Single stressor additive vs observed interaction

4.8. *Emergent Interaction Type Prevalence*

T-test errors represent a similar proportion of emergent effects observed (161 in 1752, 9%) . Non-emergent effects – "predicted" – dominate (1589 in 1752, 91%). Emergent synergy is non-existent, while two cases of emergent antagonism are present in NUE1_1: a mixture of copper, nickel, metaldehyde and tebuconazole, and a mixture of copper, nickel, chloramphenicol, metaldehyde, tebuconazole, and azoxystrobin.

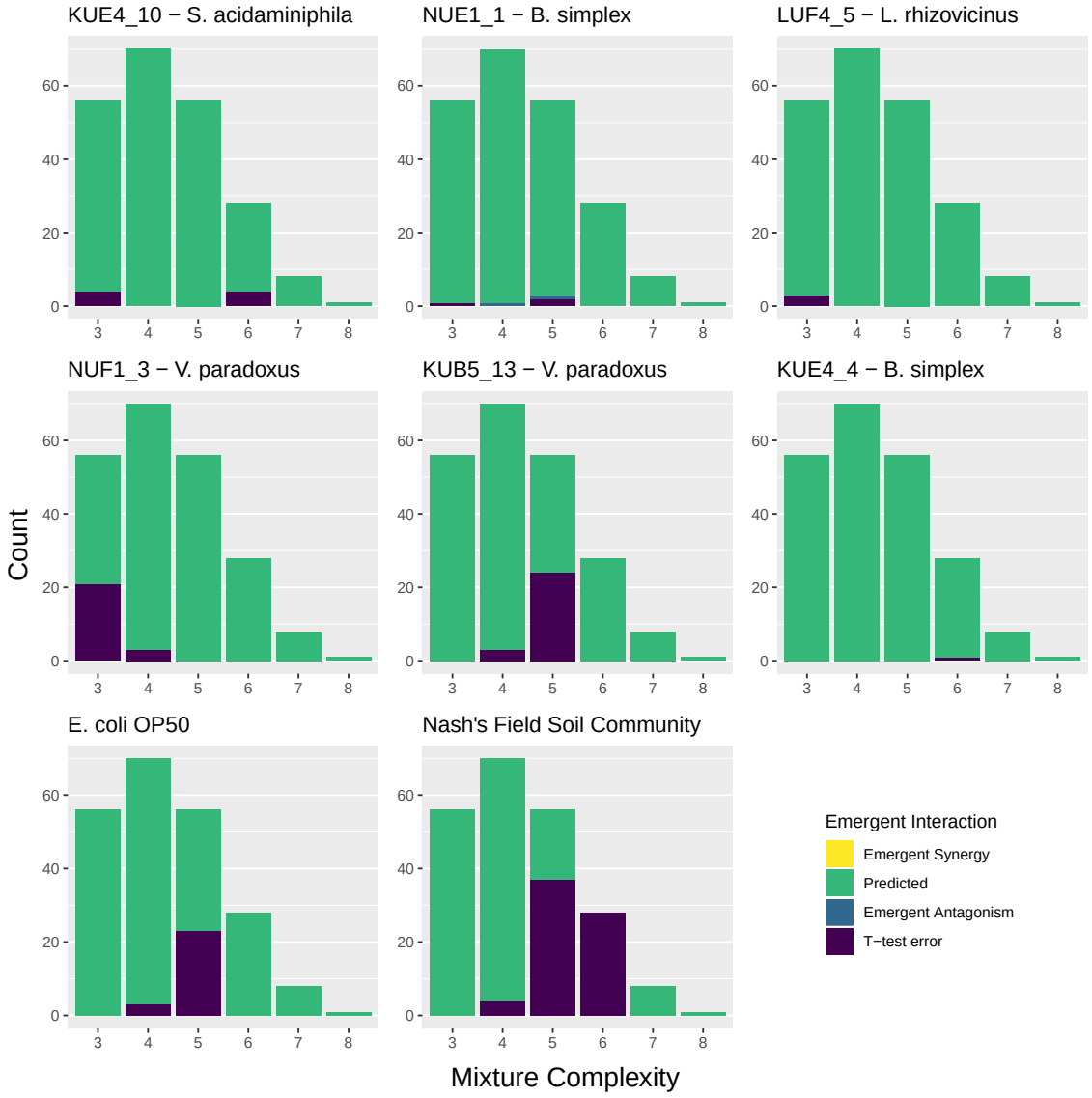

Figure 7: Multiple stressor additive vs observed interaction

5. Discussion

5.1. Novel Findings

Of the two hypotheses laid down in this project's introduction, limited conclusions can be drawn. Figure 2 shows various positive and negative relationships between mixture complexity and growth across the species, although none of these relationships are statistically significant.

Although Figure 4 shows a relatively high incidence of observed effects deviating from the additive prediction, very few of these deviations are significant at $p < 0.05$ (Figure 5), the few cases of synergy and antagonism being limited to *E. coli* OP50, where they were relatively common. A similar lack of emergent interactions (Figure 6) was detected.

However, a degree of interspecies variation at various levels of significance in response and interactive effect was present. The effects of individual stressors on growth varied considerably and frequently reversed between species, and different patterns of clustering between observed and predicted effects were observed in Figure 4. As discussed above, *E. coli* OP50 showed the only significant non-additive interactions detected, although no similar pattern was observed at the emergent level.

5.2. Single Stressor Effects

Although the majority of the stressors used in this study have well-documents impacts on bacteria, a number – including the pesticides atrazine and metaldehyde and the fungicides tebuconazole and azoxystrobin – have little to no literature available on their effects or mechanism of effect on non-target species and indeed may represent a food source of a stressor for different species under different conditions, which appears to have been the case in single stressor exposures.

5.3. Caveats and Limitations

A number of limitations apply to this study if overcome could affect the significance of detected interactions considerably. Due to a combination of mistakes during the lab work phase of the study and the reticence of the strains KUB5_13 and NUF1_3 (*V. paradoxus*) to grow directly in liquid media from cryogenic storage (perhaps unsurprising as the strains in question were sources from soil samples), the sample size in the case of a number of combinations (Table 4) was insufficiently large to detect non-additive effects at all but the highest p-values. Greater replication of a smaller number of isolates would have permitted results to be declared with a greater degree of certainty.

Another issue that may have affected OD readings for strains KUE4_4 and NUE1_1 (*B. simplex*) is the tendency of *Bacillus spp.* to aggregate within the well, creating considerable local variation in optical density across Additionally, due to the long preparation times for exposures, and experimental durations (96 hours from first the addition of media to plates to experiment end), well evaporation posed a non-random but difficult to account for influence on bacterial growth conditions, an additional stressor not part of the experimental design. Evaporation was most evident in peripheral wells (A1-–12, H1-–12, all X1 and X12 wells), and as stressor combination layout on the plates was not randomised between isolates or runs, it is probably that evaporation played a not-insignificant role in the observed effects of various treatments. Were a simpler level of treatment/isolate complexity used in simpler work, it would be possible to mitigate this effect by using only the inner wells of the 96 well plate, and by reduce experimental set-up time.

Lastly, the incorporation of varying concentrations of stressors would have allowed the construction of dose response curves, calculation of inhibitory concentrations (ICs), and correspondingly the modelling of Bliss Independence [3] of stressors, which has been applied with some success to the issue of higher-order and emergent interactions in complex mixtures of antibiotics [16, 17]

5.4. Next Steps

A small but growing body of evidence conducted by researchers at the University of California has suggested that, in lab strains of *E. coli*, the potential for higher-order interactions in complex mixtures of chemicals is far higher than previously assumed [16, 18, 17]. This study has undertaken a similar approach to a more diverse mixture of chemicals with mixed results; however, it is clear that existing attempts to understand the effects of the complex mixtures of stressors that define interactions in real-world ecosystems through simple interactions is insufficient.

With the increasing level of automation available in laboratories, it is crucial that further steps are taken to undertake work that will improve our understanding the role of complex stressor mixtures in contemporary ecosystems and study systems, and to develop effective strategies to protect and restore such effected ecosystems.

6. Conclusions

Recent advances in the understanding of higher-order effects of complex stressor mixtures suggest that effects are not as previous believed negligible. This study found predominantly additive effects at both the interaction and emergent interaction level; with non-additive effects largely limited to negative antagonism for *E. coli* OP50 exposed to more complex mixtures. This suggests that studies that examine only lab species for interactions may not be fully applicable to wild populations, and further study is needed to determine applicability to the diversity of species present in the wild.

7. Acknowledgements

8. References

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Appendix A Data and Code

All data, scripts, and calculations used in this study are available at:

`https://github.com/samawelch/MScProject`

Appendix B Nash's Field Treatments and Isolates

Appendix C Notes on the Operation of Hamilton STARlet