Predicting the effects of multiple global change drivers on microbial communities remains challenging

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Abstract

Microbial communities in many ecosystems are facing a broad range of global change drivers, such as nutrient enrichment, chemical pollution, and temperature change. These drivers can cause changes in the abundance of taxa, the composition of communities, and the properties of ecosystems. While the influence of single drivers is already described in numerous studies, the effect and predictability of multiple drivers changing simultaneously is still poorly understood. In this study, we used 240 highly replicable oxic/anoxic aquatic lab microcosms and four drivers (fertilizer, glyphosate, metal pollution, antibiotics) in all possible combinations at three different temperatures (20 °C, 24 °C, and 28 °C) to shed light into consequences of multiple drivers on different levels of organization, ranging from species abundance to community and ecosystem parameters. We found (i) that at all levels of ecological organisation, combinations of drivers can change the biological consequence and direction of effect compared to single drivers (ii), that effects of combinations are further modified by temperature, (iii) that a larger number of drivers occurring simultaneously is often quite closely related to their effect size, and (iv) that there is little evidence that any of these effects are associated with the level of ecological organisation of the state variable. These findings suggest that, at least in this experimental ecosystem approximating a stratified aquatic ecosystem, there may be relatively little scope for predicting the effects of combinations of drivers from the effects of individual drivers, or by accounting for the level of ecological organisation in question, though there may be some scope for prediction based on the number of drivers that are occurring simultaneous. A priority, though also a considerable challenge, is to extend such research to consider continuous variation in the magnitude of multiple drivers acting together.

Introduction

Microbial communities are key components of many ecosystems, and are often exposed to many anthropogenic global change drivers (Christensen et al. 2006; Jackson et al. 2016). Exposure to fertilizer (da Costa et al. 2021) and metal pollution (Xu et al. 2018a), pesticides like glyphosate (Solomon and Thompson 2003; Relyea 2009), antibiotics (Xu et al. 2018b) and temperature increase (Wu et al. 2011) force populations and whole ecosystems to develop in different ways compared to less or unaffected ones (Tylianakis et al. 2008). Studies show that even slight changes in environmental conditions can lead to large taxonomic and functional microbial change, and even to regime shifts, with potential long-term consequences for biogeochemical cycles and ecological function of the affected habitat (Gruber 2011).

While many studies have focused on one global change driver, recent studies (Christensen et al. 2006; Rillig et al. 2019; Suleiman et al. 2021a) indicated that the combined effect of multiple drivers applied simultaneously is often not equal to the sum of the effects of each individual driver. Rather, they demonstrate that interactive effects can occur. These can be synergistic (combined effect greater than the sum of the effect of individual drivers) or antagonistic (combined effect less than the sum of the effect of individual drivers). Furthermore, not just the type of driver, but also the number of factors combined plays a crucial role (Rillig et al. 2019). Nevertheless, the number of studies testing three or more drivers remains very low (Rillig et al. 2019), which highlights the need for further investigations of combined effects. Combining this with the importance of understanding drivers of change in microbial communities is a critical aspect of the emerging research field of climate/global change microbiology (Hutchins et al. 2019).

The limited number of studies with three or more drivers may be in part due to the logistical demands of the required experiments and the complexity of interpreting their

results. For example, to assess all interactions a fully-factorial experimental design is needed which quickly leads to large experiments. Regarding interpretation, it can be difficult to understand the meaning of interactions among more than three drivers, such that even if one would conduct a fully factorial experiment, traditional methods for interpreting interactions (such as interaction plots) may be insufficient. Some studies have instead focused on the effect of variation in the number of drivers, and have applied only a subsample of all possible combinations (e.g. Brennan and Collins 2015; Rillig et al. 2019).

Another gap in understanding is how drivers and combinations of drivers act across levels of ecological organization from individuals to ecosystems (Galic et al. 2018). For example, in a case study of a model of amphipod feeding behavior Galic et al (2018) showed that responses to multiple drivers at the individual level were not consistent with those at higher levels of organization. In their case-study, the nature of this inconsistency would lead to underestimation of effects at population and ecosystem levels if effects at the individual level were assumed to hold across levels of organisation.

These gaps in knowledge are problematic. One reason is that interactions can be source of ecological surprises, i.e., if we assume additivity we can be surprised if there are interactions (Christensen et al 2006). Also, unless there are some generalities about interactions among drivers, we will never be able to predict the effect of a new (previously unstudied) combination of drivers, so interactions will also then be a surprise (Christensen et al 2006). Hence, we and others (e.g., Simmons et al 2021, Rillig et al 2018) are interested in discovering if there are any general patterns that will allow us to predict the effects of combinations of environmental changes. This report is about our search for signals of general patterns in the effects of combinations of environmental drivers. For example, whether the relationship between a biological variable and the number of drivers is dependent on the level

81 of ecological organization of the biological variable and whether the strength and nature of
82 the interaction effects varies with level of ecological organization.

83 Microbial communities in aquatic ecosystems are complex (Chistensen et al. 2006;
84 Davis et al. 2010; Faust and Raes 2012), consisting of feedback among numerous abiotic and
85 biotic interactions (Singh et al. 2009), among and within functional microbial groups (Bush et
86 al. 2017; Richardson et al. 2018). Recent work indicates that these ecosystems are sensitive
87 to environmental change (Christensen et al. 2006; Shade et al. 2011, 2012; Suleiman et al.
88 2021b, a), identifying them as appropriate systems for the study of the influences of global
89 changes.

90 In this work, we applied four different global change drivers (fertilizer, glyphosate,
91 metal pollution, and antibiotics) together with increasing temperatures (20 °C, 24 °C, and 28
92 °C) in all combinations possible, on a recently developed and highly replicable stratified
93 aquatic microbial lab system. Our experiment includes the analysis of several abiotic (oxygen,
94 total nitrogen, total organic carbon, pH) and biotic variables (Shannon index, microbial
95 community composition, genera abundances), since recent studies have shown that various
96 levels of ecosystems can be affected (Weithoff et al. 2000; Shade et al. 2011, 2012; Suleiman
97 et al. 2021b) and in order to examine if effects show predictable variation across levels of
98 organisation. We hypothesize that (i) combinations of drivers will have non-additive effects
99 on system properties, (ii) the non-additive effects will be different depending on the
100 temperature, and (iii) increasing the number of drivers applied will cause a systematic change
101 in system properties.

Material and methods

Experimental ecosystems

Incubation of stratified microbial communities was performed in microcosms consisting of standard glass test tubes (4 mL volume). These test tubes were closed with plastic lids, with a small hole (0.5 mm) that allowed gas exchange between the headspace and the atmosphere. Each microcosm consisted of sediment and water samples taken in May from a small pond in Zurich, Switzerland (47°23'51.2"N 8°32'33.3"E, temperature of 19 °C, pH of 7) at a depth of 25 cm. Sediment was homogenized (30 min mixing) and subsequently supplemented with sterile 0.25 % crystalline cellulose, 0.25 % methyl-cellulose, 0.5 % $CaSO_4$, 0.1% $CaCO_3$, and 0.005 % $NH_4H_2PO_4$. Glass tubes were filled with a 3 mm layer of supplemented sediment and covered with 3.4 mL pond water (with 0.005 % $NH_4H_2PO_4$), resulting in 500 µL headspace. Microecosystems were incubated at room temperature for 2 hours to let sediment settle, before the different treatments were applied. The experiment involved incubation for 23 days at either 20 °C, 24 °C or 28 °C.

Experimental design

The four drivers, namely $NH_4H_2PO_4$-fertilizer (F), Glyphosate (G), metal pollution (M) and antibiotics (A), each had two levels. Furthermore, control microcosms (C) were used without application of any drivers and the experiment was carried out at 20 °C, 24 °C and 28 °C under a dark-light cycle of 8:16 h. The temperature treatment and four driver treatments were applied in a fully factorial design. Five replicates (Burgess et al. 2022) per treatment combination resulted in 240 microcosms, according to the following design:

[“Control","F","G","M","A","FG","FM","FA","GM","GA","MA","FGM","FGA","GMA","FMA", "FGMA"] * 5 replicates * 3 Temperatures = 240 microcosms.

125 For the driver treatments, the following chemical compounds were added to the microcosms
126 two hours after the assembly of the microcosms:

127 - $NH_4H_2PO_4$ in final concentration of 0.1 g/L as fertilizer (F), as used in a previous study
128 (Suleiman et al., 2021)
129 - Glyphosate in final concentration of 0.001 g/L (G) as used in a recent study (da Costa
130 et al. 2021)
131 - Metal pollution: DSMZ Trace element solution SL-10 final concentration 10x higher
132 than recommended
133 (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium320.pdf). The
134 following chemicals with final concentration in the microcosms were 0.015 g/L $FeCl_2$ x
135 4 H_2O,. 0.7 mg/L $ZnCl_2$, 1 mg/L $MnCl_2$ x 4 H_2O, 0.06 mg/L H_3BO_3, 1.9 mg/L $CoCl_2$ x 6
136 H_2O, 0.02 mg/L $CuCl_2$ x 2 H_2O, 0.24 mg/L $NiCl_2$ x 6 H_2O, 0.36 mg/L Na_2MoO_4 x 2 H_2O
137 (M)
138 - Mixture of penicillin and ampicillin (0,05 mg/100 mL each) in final concentration of 25
139 ng/L for antibiotic treatment (A), since comparable maximum amounts were detected
140 in natural aquatic habitats (Xu et al. 2018b)

141 Drivers were applied to the microcosms only on day 0; there were no further press or pulse
142 perturbations. After incubation for 23 days, the whole microcosm was centrifuged for 5 min
143 at 10'000 rpm (sediment and water column together). The pellet was used for DNA extraction
144 the supernatant was used for performing TOC analysis.

145 ## Measurement of abiotic factors

146 Oxygen concentration was measured 1.5 cm below the water/air interphase using an oxygen
147 dipping probe (PreSens, Germany) at the last day of incubation. pH was measured in the

148 supernatant of the centrifuged microcosm. Total nitrogen (TN) and total organic carbon (TOC)
149 were measured in the supernatant using a TOC analyser.

150 ## Microbial community composition

151 DNA was extracted using ZymoBIOMICS DNA Miniprep Kit (ZymoResearch), following the
152 manufacturer's instructions. Full-length 16S rRNA gene amplification was performed using
153 the primer pair 27F forward primer (5'-AGRGTTYGATYMTGGCTCAG-3') and 1592R reverse
154 primer (5'-RGYTACCTTGTTACGACTT-3'), as reported previously (Suleiman et al., 2021).

155 PCR products were visualized on an 1 % (w/v) agarose gel and were pooled for
156 sequencing in equal concentrations. PCR product pools were purified using AMPure®PB beads
157 (PacBio). Sequencing was performed at the Functional Genomic Center Zürich, Switzerland,
158 and performed using SMRT Technology (PacBio) as reported previously (Suleiman et al.
159 2021a) (on three SMRT chips). Sequencing data quality were checked using the PacBio SMRT
160 Link software.

161 Sequencing data were filtered based on primer sequences, length (1300-1600 bp),
162 quality, error rates and chimeras using the R-package *dada2* (Callahan et al. 2016). The final
163 sequence table was aligned using SILVA ribosomal RNA database (Quast et al. 2012), using
164 version 138 (non-redundant dataset 99). A phyloseq object was created using the *phyloseq* R-
165 package (McMurdie and Holmes 2013), consisting of amplicon sequence variant (ASV) table,
166 taxonomy table and sample data. For further analysis, the R-packages *phyloseq* (McMurdie
167 and Holmes 2013) and *vegan* (Oksanen et al. 2019) were used. Overview on number of reads
168 per sample after the dada2 pipeline is shown in supplementary table 1.

169 To compare the effects of the multiple drivers, we analysed various response
170 variables, namely community composition based on non-metric multidimensional scaling

171 NMDS, Shannon index, oxygen, total nitrogen TN, total organic carbon TOC, and abundances
172 of specific genera and functional groups.

173 Microbial community composition was quantified using NMDS based on Bray-Curtis
174 scores with the *metaMDS* function of the vegan R-package (Oksanen et al. 2019), with three
175 dimensions used (k=3, try=100). We chose to use NMDS to better represent the non-
176 linearities that often occur in ecological patterns that lead some other types of ordination
177 (e.g. principal component analysis) to be less desirable. All samples were analysed at once in
178 the NMDS analysis (Stress = 0.11; stress less than 0.2 is generally considered acceptable, e.g.
179 (Dexter et al. 2018). Shannon index was calculated using the *phyloseq* package (McMurdie
180 and Holmes 2013). We included the analysis of the Shannon index since the system is highly
181 dynamic and develops from a small microbe density to a high microbe density. Please note
182 that we can not differentiate between live and dead as well as active and non-active cells.
183 Functional groups were constructed by filtering for cyanobacterial, sulfur-related and typical
184 lake-associated species, which resulted in a reduced dataset consisting of cyanobacterial
185 orders *Cyanobacteriales*, *Synechococcales*, *Leptolyngbyales* and *Limnotrichales*, phototrophic
186 sulfur-bacterial orders of *Chromatiales* and *Chlorobiales*, as well as sulfate-reducing orders of
187 *Desulfobulbales* and *Desulfovibrionales*. Furthermore, we also implemented the order
188 Campylobacteriales, since some members (not all) like *Sulfuricurvum* and *Thiovolum* are also
189 associated with the sulfur cycle. The community of these members were used as a subset for
190 NMDS analysis in this study (stress = 0.10). Detailed scripts and a phyloseq with all sequences
191 are available in the github repository https://github.com/Marcel2907/multiple-stressor and
192 on zenodo (DOI 10.5281/zenodo.5948600).

Data analysis

For every response variable (see above) we carried out two linear models, one to investigate the relation between the response variable and the various drivers and their combinations and one to investigate how the response depended on the number of drivers applied. To ensure that modelling assumptions were met, we carried out the following response variable transformations, which are listed in detail in the deposited R script. For total nitrogen we took the square root, for total carbon we took the squared inverse, for *Tychonema* we used a logit transformation and for *Sulfuricurvum* we took the cubic root. For the variables not listed here no data transformations were necessary. Further, for total nitrogen concentration we excluded 13 data points due to measurement problems (Supplementary Figure 1). For the analyses of the effect of the number of applied drivers, all response variables were further standardized to render them more comparable. Moreover, in the cases of pH and total nitrogen we included the variable fertilizer as a covariate, as it strongly influenced these response variables. In all models we included all possible covariate interactions.

Results

Microbial community and genera abundances

In total, 20’319 unique ASVs were identified after running the dada2 pipeline. After filtering to retain only ASVs with relative abundance of at least 0.1 % in at least one sample, the total number of ASVs decreased to 5’830 taxa. The control microcosms consisted of mainly cyanobacteria (e.g., *Phormidaceae*) and phototrophic sulfur bacteria (specially *Chromatiaceae*) (Fig. 1 a&b, Supplementary Fig. 2). Depending on the driver combination and temperature, the microbial communities and genera abundances showed strong differences compared to the controls. Microcosms with glyphosate and metal pollution, each as a single driver or in combination, were still dominated by members of *Phormidiaceae* and

217 *Chromatiaceae*, but in all remaining treatments the abundance of members of these families
218 was lower (Supplementary Fig. 2).

219 Also on genus level, we observed shifts in microbial composition due to driver
220 combination and temperature (Fig. 1 b, Supplementary Fig. 3). The cyanobacterial genus
221 *Tychonema* was highly abundant in the metal pollution only treatment, and with increasing
222 temperature also in glyphosate+metal pollution treatments (20 % at 28 °C, 7 % at 20 °C).
223 *Chromatium*, in contrast, appeared in several treatments and reached highest relative
224 abundance at 20 °C when confronted with G:M (20 %), but vanished with increasing
225 temperature in all treatments (< 5%). Statistical analysis of the dependence of relative
226 abundance on driver combinations and temperature were performed on genus level for
227 *Tychonema* and *Sulfuricurvum*, as representative species for high-abundance and low-
228 abundance taxa (Fig. 3). Statistical analysis confirmed significant changes of relative species
229 abundance caused by individual drivers, due to combination of drivers, and due to
230 temperature.

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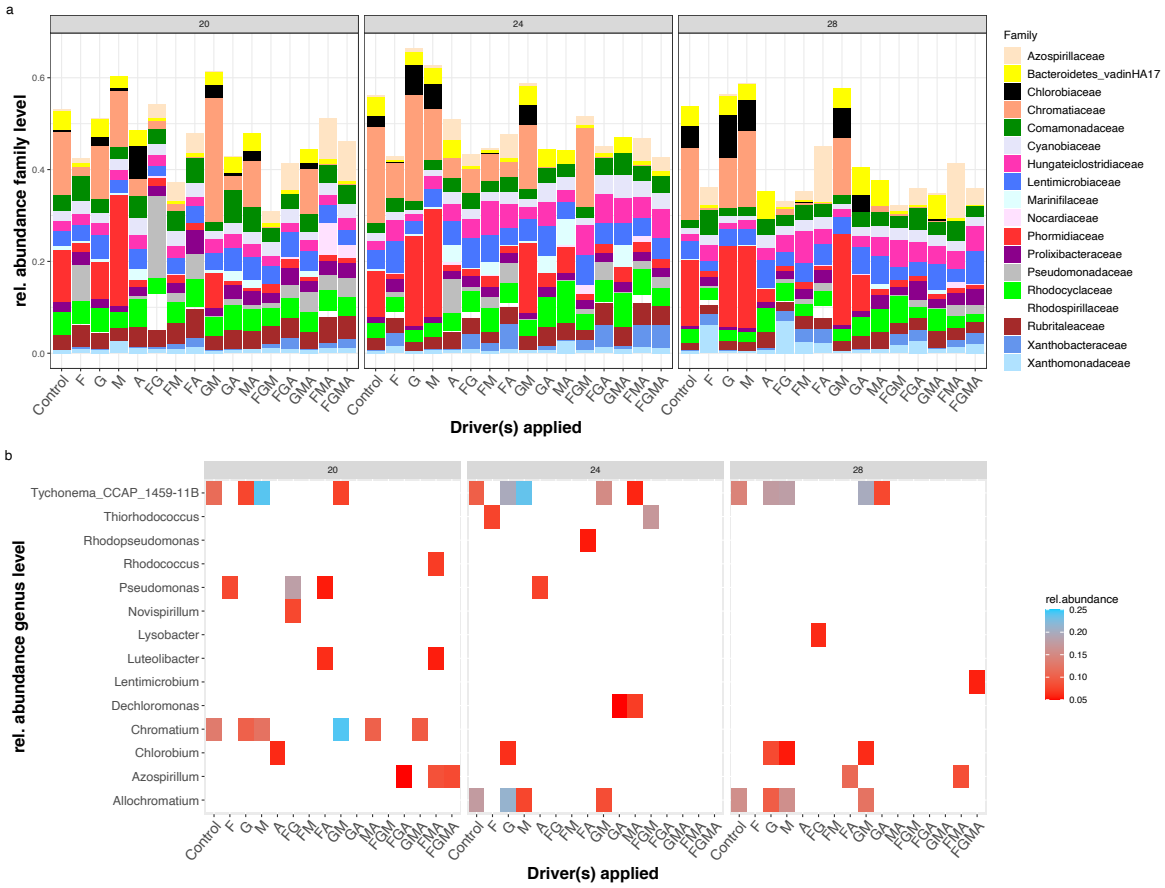

Fig. 1 Effects of drivers and driver combinations on microbial community composition and genera abundance. (a) Microbial community composition (mean per 5 replicates) at family level. Taxa with rel. abundance > 5 % shown. **(b)** Relative abundance of specific genera with rel. abundance > 5 % for each driver combination at each of the three temperatures. White color represents a relative abundance < 5 %. F=Fertilizer, G= Glyphosate, M=Metall pollution, A=Antibiotics.

242 ## Effects of combination and number of drivers on Shannon index

243 Shannon index of the microcosms changed depending on the combination of drivers (Fig.
244 2a+b), resulting in values between 3 and 7, with the majority of samples between 5 and 6,
245 indicating diverse microbial communities within the microcosms. Several significant driver
246 treatments and combinations effects occurred, namely temperature increase (F-statistic =
247 6.8, p-value = 0.001), fertilizer (F-statistic = 16.0, p-value < 0.001), antibiotics (F-statistic =
248 26.3, p-value < 0.001), as well as combination of fertilizer+antibiotics (F-statistic = 17.9, p-
249 value < 0.001) and fertilizer+antibiotics+glyphosate (F-statistic = 4.8, p-value = 0.030) (Fig. 2a).
250 All significant driver combinations had a positive effect on Shannon index, except the
251 temperature increase to 28 °C and the fertilizer+antibiotic combination treatment (Fig. 3, Fig.
252 6). Interestingly, the negative effect of fertilizer+antibiotic was reversed to a positive effect
253 again by adding glyphosate, while the treatment of glyphosate alone had no impact (Fig. 2a,
254 Fig. 3). Number of drivers did not affect Shannon index, while temperature did (Fig. 2b, Fig.
255 4).

256 ## Effects of combination and number of drivers on microbial community structure
257 ## (based on NMDS)

258 Fertilizer addition was a key driver of microbial community composition (quantified via
259 NMDS). (Fig. 2b+c, Supplementary Figure 5). Numerous driver combinations showed
260 significant effects on NMDS score 1 and NMDS score 2 (Fig. 2c for NMDS1, Supplementary Fig.
261 4 for NMDS2, Fig. 3 for both), ranging from single to four-way effects. Different impact
262 directions (positive or negative) can be identified depending on the driver combinations (Fig.
263 2c, Fig. 3). Please note that for clarity we also refer to NMDS scores with “negative” and
264 “positive”, even though the direction is irrelevant for this measure. The three-way interaction
265 of fertilizer+antibiotics+temperature on NMDS1 score is a good example how combination of
266 drivers can change the impact direction (Fig. 5): When no fertilizer was present, the NMDS1

267 score was negative, while in presence of fertilizer, the NMDS1 score was positive. Additionally
268 the combination of antibiotics and temperature changed the magnitude of the NMDS1 score.
269 NMDS1 score was affected by number of drivers (F-statistic = 82.8, p-value < 0.001) (Fig. 2d,
270 Fig. 4), and NMDS2 score was affected by both number of drivers (F-statistic = 12.7, p-value <
271 0.001) and temperature (F-statistic = 27.8, p-value < 0.001, Supplementary Figure 4b, Fig. 4).
272 For both NMDS scores there was no interaction between number of drivers and temperature.
273 Analysis of the community of the subset of cyanobacteria, phototrophic sulfur
274 bacteria and sulfate-reducing bacteria confirmed the trends observed for NMDS of all ASVs
275 (Fig. 2e for NMDS1, Supplementary Fig. 4 for NMDS2, Fig. 3 for both), but showed slight
276 differences on three-factorial and four-factorial driver combinations.

277 ## Effects of combination and number of drivers on abiotic factors

278 Oxygen concentration was highly dependent on the driver combinations used, and various
279 strong effects were identified (Fig. 2g), both positive and negative in direction. All drivers
280 combined had a negative effect on oxygen concentration (F-statistic = 4.3, p-value = 0.040,
281 non-standardised estimate -6.1 %). The number of drivers applied had a strong effect on the
282 oxygen concentration at 24 °C and 28 °C (F-statistic = 18.8, p-value < 0.001), as well as
283 temperature (F-statistic = 17.9, p-value < 0.001), and the interaction between temperature
284 and number of drivers (F-statistic = 3.3, p-value = 0.040, Fig. 2h, Fig. 4).
285 The pH of the microcosms was influenced by numerous treatment combinations, but
286 the strongest influences was identified as fertilizer addition, which decreased the pH from 8.5
287 to 6.5 (F-statistic = 3.2 × 103, p-value < 0.001, Fig. 2i). Driver combinations that included
288 fertilizer addition had lower magnitude though were of the same sign (Fig. 3). Number of
289 drivers had a negative effect on pH (F-statistic = 4.4, p-value = 0.037), with the clearest effect

290 at 20 °C and no fertilizer used, in which case pH decreased the more drivers were applied (Fig.
291 2j, Fig. 4).

292 Effects on total nitrogen were driven by addition of fertilizer (F-statistic = 7.4 × 104, p-
293 value < 0.001), but also numerous other combinations had a significant effect (Fig. 3,
294 Supplementary Fig. 4). Furthermore, number of drivers strongly affected the total nitrogen
295 concentration (F-statistic = 67.8, p-value < 0.001). Total carbon concentration was
296 significantly affected by fertilizer (F-statistic = 31.9, p-value = 0.005), metal pollution (F-
297 statistic = 9.9, p-value = 0.002) and temperature (F-statistic = 23.4, p-value < 0.001), as well
298 as by antibiotics (F-statistic = 9.2, p-value = 0.003) and numerous combinations (Fig. 3,
299 Supplementary Fig. 4). Furthermore, for the concentration of total carbon there was a
300 significant interaction between number of drivers and temperature (F-statistic = 3.5, p-value
301 = 0.032), with TC increasing with number of applied drivers at 28°C (Fig. 4, note that the
302 response variable is the square inverse of total carbon).

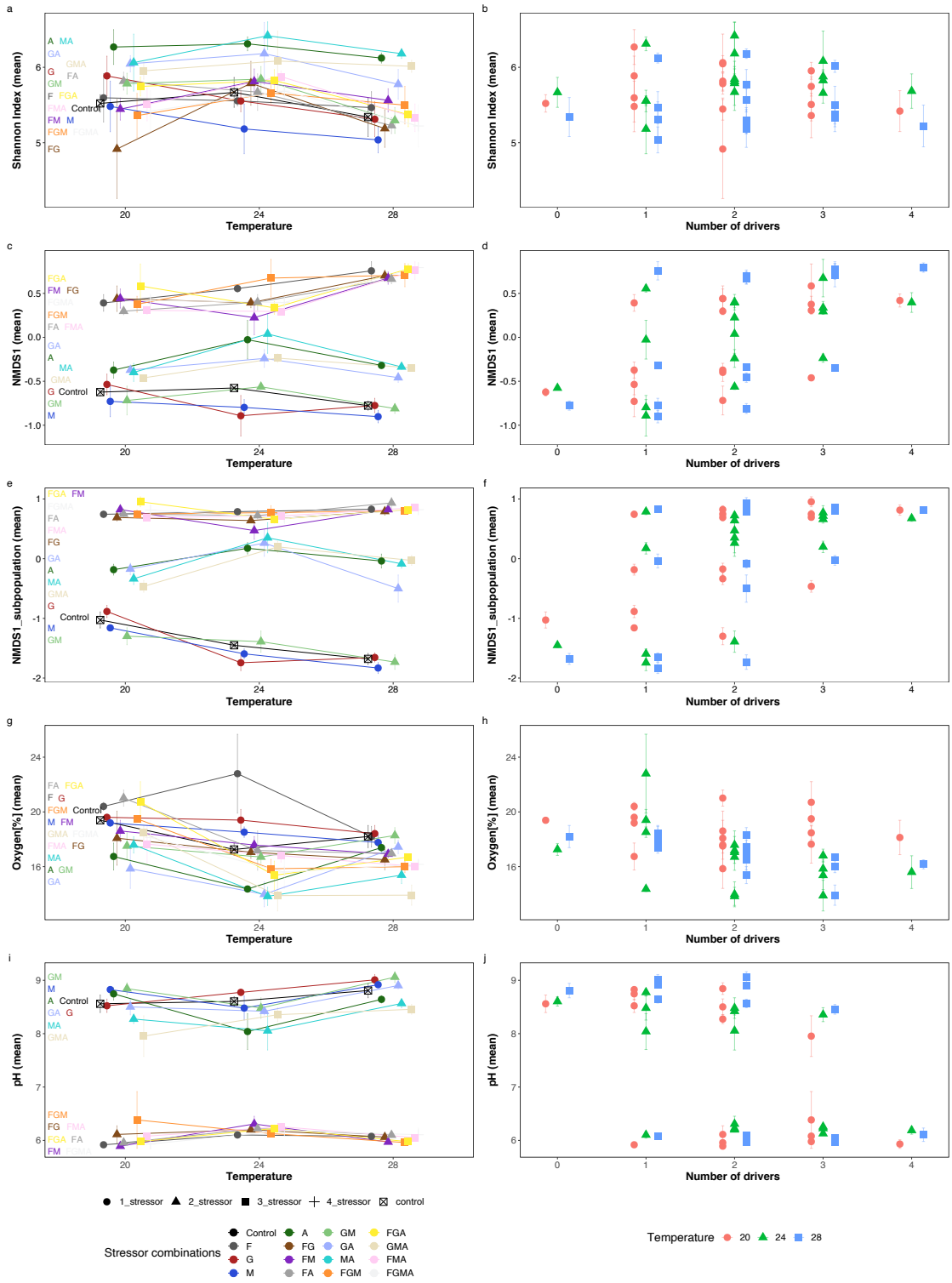

Fig. 2 Effects of driver combination, number of drivers, and temperature on microbial community and ecosystem variables. Each row of the figure contains two graphs, both of which concern the same response variable, e.g., first row (a) and (b) concern Shannon index. The left column of figures shows the effects of temperature and driver combination. The right column shows the effects of temperature and number of drivers. Symbols are the mean of the five replicates, and errors bars show the standard error of the mean.

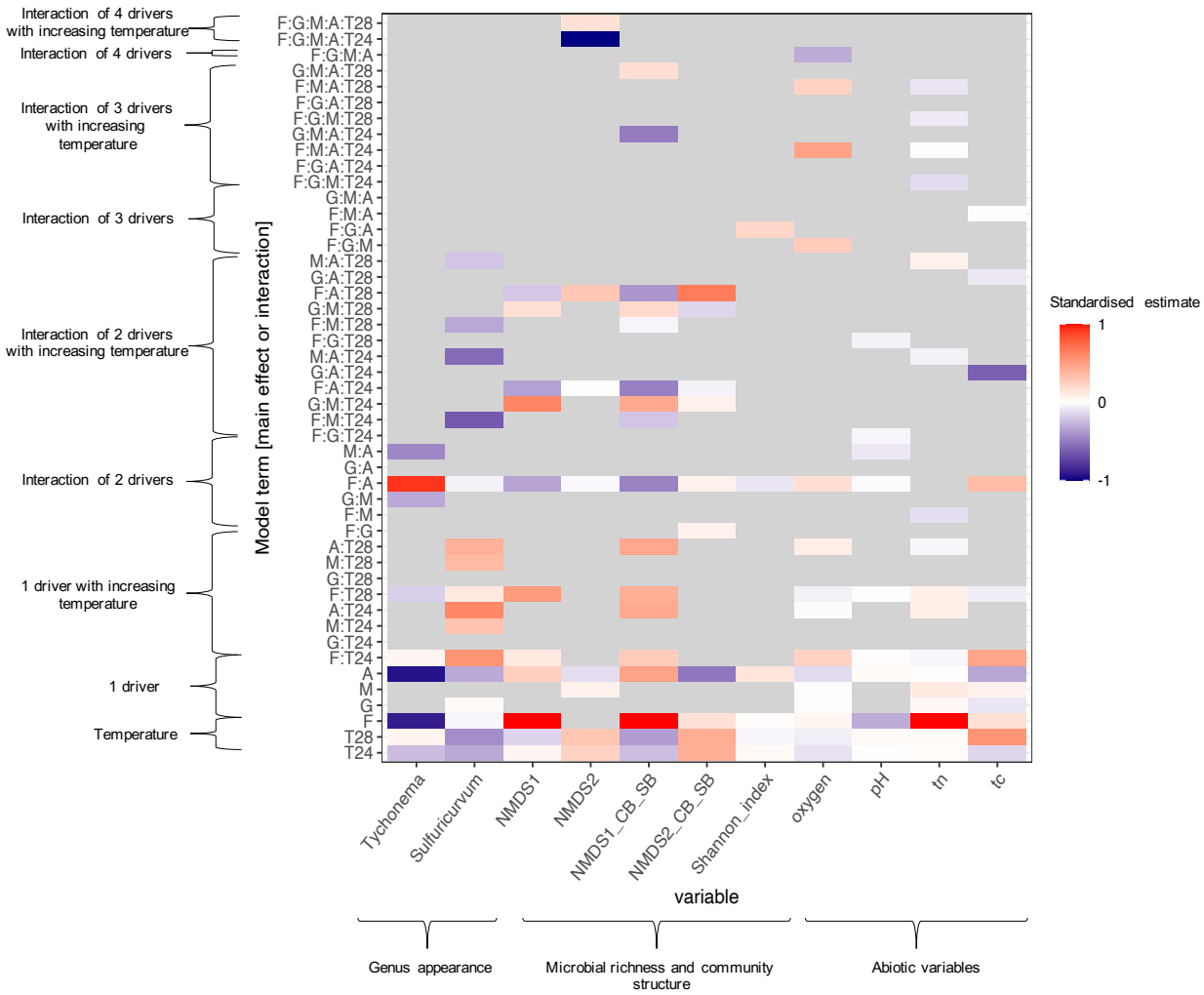

Fig. 3 Summary of statistical analyses of all variables analysed. Eleven variables are shown in the columns of the heat map, (grouped by genus appearance, microbial richness and community structure and abiotic factors). Rows show the estimated coefficients of the single, one-way, two-way, three-way, and four-way interaction terms. Grey cells indicate a response variable and coefficient pairs for which the coefficients were not significantly different from zero (t-test p-value > 0.05), otherwise the diverging colour palette illustrates the direction of the influence by the driver or interaction of drivers (estimates of each variable were standardized by dividing by largest absolute value of the estimates in each variable). See the main text for the applied response variable transformations. tn = total nitrogen, tc= total carbon.

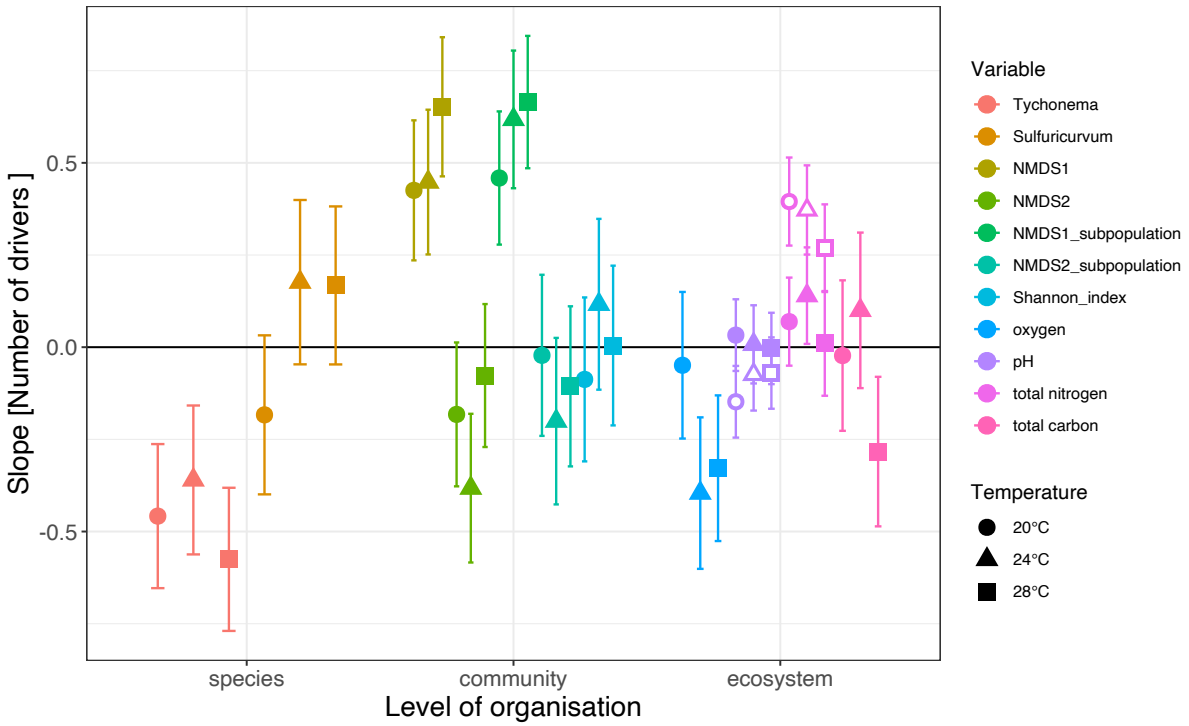

Fig. 4 Effect size of number of drivers for each response variable at each temperature. Means and 95 % confidence intervals are shown. For the variables of pH and total nitrogen, fertilizer was used as a covariate due to its strong influence. Filled total nitrogen and pH labels: fertilizer was used as a driver. Empty total nitrogen and pH labels: fertilizer was excluded as driver. See the main text for the applied response variable transformations.

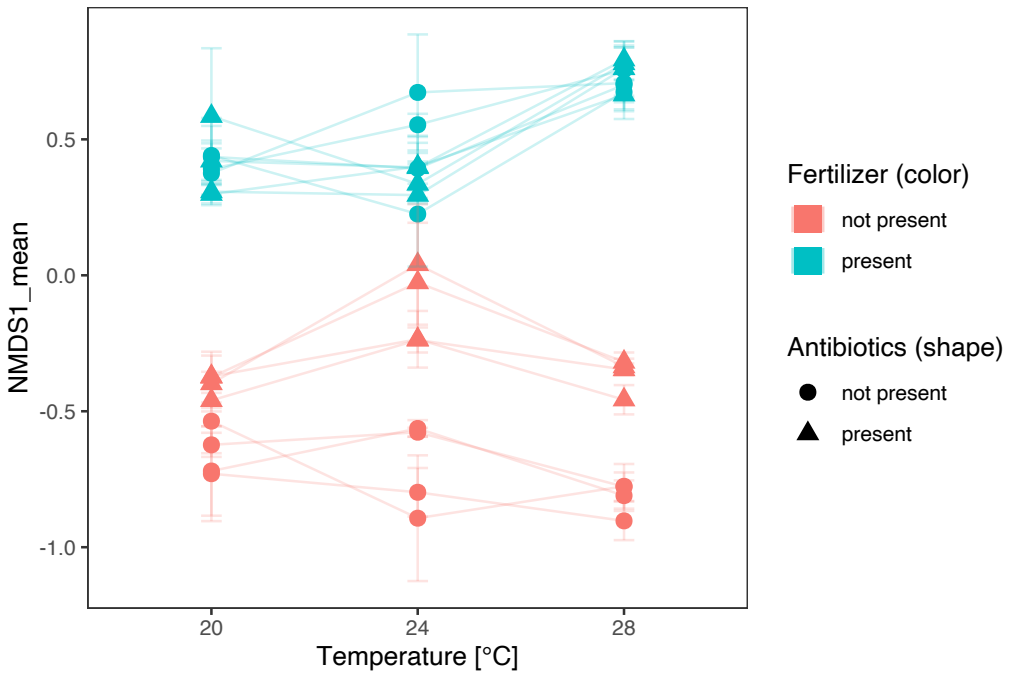

Fig. 5 Visualization of the significant driver interactions of temperature, fertilizer and antibiotics on NMDS1. NMDS1 is affected by the presence of fertilizer, the presence of antibiotics and temperature, but also by the interactions of fertilizer+antibiotics and fertilizer+antibiotics+temperature. Means (n=5) are shown. Error bars represent standard error.

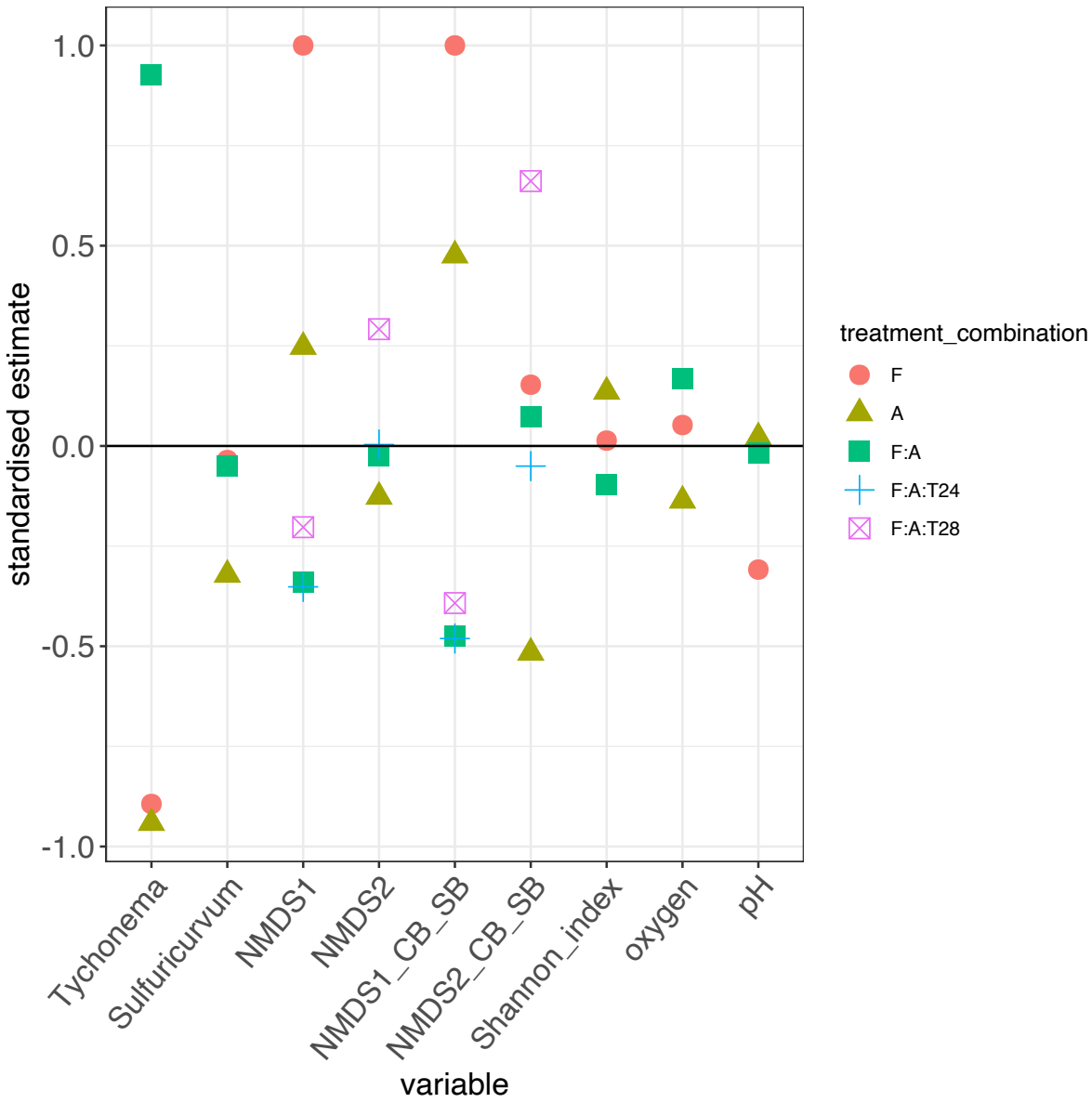

Fig. 6 Effects of fertilizer, antibiotics and their combination at 20, 24, and 28 °C on ASV relative abundance, microbial composition, and ecosystem variables. Only significant treatments / treatment combinations are shown (F-test p value < 0.05). Total nitrogen and total carbon are not listed in this figure because they were not significantly affected by F, A, and F:A.

342 ## Discussion

343 Our study revealed compelling evidence of effects of the combination and the total number
344 of drivers on taxa abundances, community composition, and ecosystem properties, and as
345 such, that multiple drivers acting in combination can have important consequences across
346 levels of ecological organisation (Rillig et al. 2019). Specifically, the addition of a driver, and/or
347 temperature increase, could change the biological consequences of already applied drivers,
348 even when the added driver applied alone had no effect (Fig. 3). We observed three trends
349 here. The addition of a further driver could increase (e.g. F:A:T28), decrease (e.g. F:T28) or
350 reverse the effect of the drivers which were already applied (e.g. F:A on *Tychonema*
351 abundance, Fig. 6). This demonstrates clearly the need of analysing multiple drivers in all
352 combinations. Furthermore, our study also confirmed that the number of drivers occurring
353 together can influence microbial communities and thereby provide some hope that number
354 of drivers can be used as a useful predictor, though individual driver effects can greatly
355 contribute to the effect of number of drivers (Rillig et al. 2019). Furthermore, the effect of the
356 number of drivers can be negative (e.g., *Tychonema* abundance) or positive (e.g. total
357 nitrogen) and of different magnitudes, which highlights again the need of analysing various
358 ecological response variables of species, microbial communities, and the whole ecosystem.
359 While there may be some patterns across levels of organisation, e.g., the largest effects of
360 number of drivers were observed at species and community levels of organisation (greater
361 than 0.5 or less than -0.5 standardised effect size), the absence of strong patterns limits the
362 scope for explanation and prediction of effect of multiple drivers across levels of organisation.

363 It is possible that effects of number of drivers is caused by multiple drivers acting in a
364 similar fashion as a single driver of greater intensity. I.e. two drivers may be considered to
365 typically have a higher effect because the overall intensity is higher. This would imply that

366 drivers combine additively, or at least not completely substitutively. If this is the case, then
367 some of the effects of number of drivers may not be so much number of drivers per se, but
368 rather that total amount of driving being applied. Given that responses can be non-linear (and
369 typically are), we can not exclude that some trends observed may simply be intensity effects.

370 A clear pattern in the results is of less apparent variability at 0 and at 4 drivers applied
371 than with 1, 2, or 3 applied. This mainly because there are fewer combinations possible (1
372 combination possible to be precise: all or none), while at 1 to 3 drivers there are more
373 combination of drivers possible, which leads to more variance. In contrast variance within
374 driver combination is roughly invariant regardless of number of drivers. We are certain if we
375 had a 5th stressor applied, we would see much more variation at 4 stressors applied, again
376 because then we would have (5 choose 4)=5 possible stressor combinations.

377 While fertilizer, metal pollution and antibiotics had detectable effects when applied
378 as single drivers, glyphosate did not. This finding is likely quite specific to the concentration
379 of treatments used and should be regarded with caution. Nevertheless, it is even more
380 interesting that when adding glyphosate to other drivers it did have an effect (see e.g.,
381 Shannon index, Fig. 3). Strong interactions were detected for antibiotics and fertilizer, and
382 interestingly, these drivers revealed in combination different biological consequences
383 compared to applied as a single driver, and temperature could also change the effects of the
384 fertilizer-antibiotic driver duo again (Fig. 6).

385 The effect of temperature is in our view important to note, since it predicts that
386 observed biological consequences caused by global change can change with on-going global
387 warming. An increase of temperature can lead to new interactions among driver
388 combinations (F:A with increasing temperature led again to significant affected variables) or

389 can lead to significant effects that were not observed at 20 °C (G:M, G:M:24 and G:M:28 for
390 NMDS1).

391 Our study also contributes to narrowing the gap of understanding of how drivers and
392 their combination act across various levels of organisation. Our results revealed that
393 individual taxa can be affected differently compared to abiotic factors of the ecosystem or
394 the microbial community composition. Therefore, our study is in line with the findings of Galic
395 et al. (2018) who reported differences of the influence of multiple drivers across levels of
396 organisation. Interestingly, in our study, some treatment combinations, like fertilizer and
397 antibiotics, showed significant influence across most variables and the three levels of
398 organisation (except total nitrogen and total carbon), though even then the sign of the
399 interaction term was sometimes negative and sometimes positive.

400 Effects of drivers, such as those we manipulated, are of course concentration-
401 dependent. In this study, we used only two levels of driver concentration and added the driver
402 in a single pulse treatment. More information about specific concentration thresholds and
403 press disturbances are important to study and may clarify some observed trends, as well as
404 the timing of the driver applied. In addition, responses of functional (metabolic) aspects of
405 the aquatic microbial communities should be investigated in future studies, which could shed
406 light into activated and deactivated pathways and enzymes used in microbial communities to
407 react to changing environmental conditions.

408 One limitation of this study is that the background level of chemical stressor was not
409 analysed prior to stressor addition. This information is important, since prior exposure to
410 pollution would influent the responses to additional exposures. We expect that the small
411 pond which was used to provide the water and sediment sample is low-nutrient loaded, and
412 is due to its location largely free from herbicide, metal-, and antibiotic contamination.

413 Nevertheless, this study showed clearly that combinations of stressors can lead to a new
414 biological consequence, and this finding is very likely not impacted by any pre-exposure of
415 drivers of the small pond.

416 One promising avenue for further research about species responses to multiple
417 environmental drivers is to attempt to relate the patterns of response to features of the
418 species, such as functional traits of the species. A relationship would reveal what traits of the
419 species are determining how they respond to multiple drivers, and then give potential to
420 predict those responses from species traits. This could be done for already present species,
421 and also potentially invasive species. In studies such as ours, this would involve relating
422 response patterns to characteristics of the microbial ASVs, and hence would require mapping
423 of ASVs to trait information, which is not straightforward. Another option would be to use the
424 16S rRNA gene sequences to predict functional characteristics of the ASVs (e.g. (Ling et al.
425 2022).

426 In sum, our study confirmed the need to research the effects of multiple drivers on
427 microbial communities and indicates further that a broad range of levels of organisation
428 (species, community, ecosystem) should be analysed due to unique sets of effect and
429 interactions across these and the different variables within them. Furthermore, our study
430 showed that combination of specific drivers can change the biological consequence and
431 direction compared to single drivers at all levels of organisation and that the effects of driver
432 combinations can be modified by temperature. Overall, the lack of patterns in effect sizes
433 across levels of organisation, and with respect to the number of drivers, represents lack of
434 evidence for these to be useful in the prediction of the effects of combinations of global
435 change drivers on ecological communities.

Finally, it is unclear how transferable our findings are to non-microbes or even to other types of microbial ecosystems. Microbes have some unique characteristics, such as mechanisms by which genetic material can move among individuals (horizontal gene transfer). There may be other important differences, such as strong interspecific interactions, particularly in closed experimental communities. Clearly what is required is a sufficient number of experiments, across a diverse enough collection of ecosystem and organism types, that involve at least four environmental drivers and observation of responses at population, community and ecosystem level, such that a formal meta-analysis is possible. This could be achieved a global network of such multi-stressor factorial experiments studying responses across levels of ecological organization.

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Author contribution

OLP and MS planned the experimental set-up. MS performed all experiments in the lab. MS performed the up- and downstream bioinformatics. MS, OLP and UD performed the data analysis. UD supported the data transformation and statistical models. YC provided technical support and performed the TOC analysis. XZ performed the DNA extractions and provided technical support. OLP, MS and UD drafted the manuscript. All authors confirmed the final version of the manuscript.

Competing interests

The authors declare no competing financial interests.

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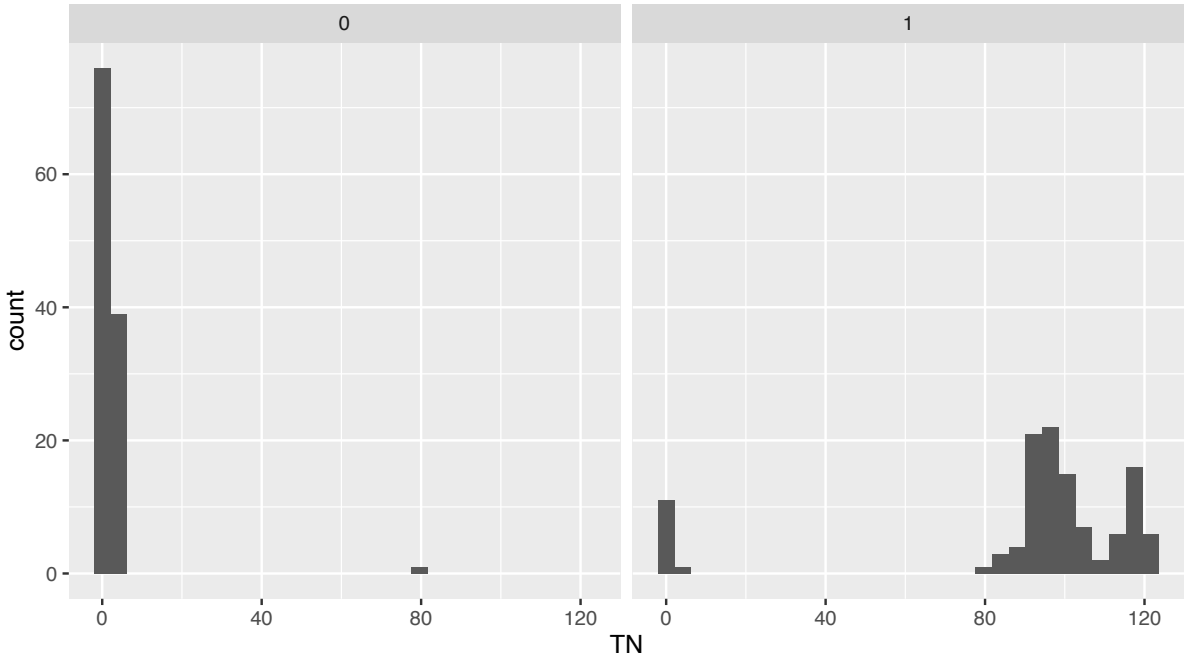
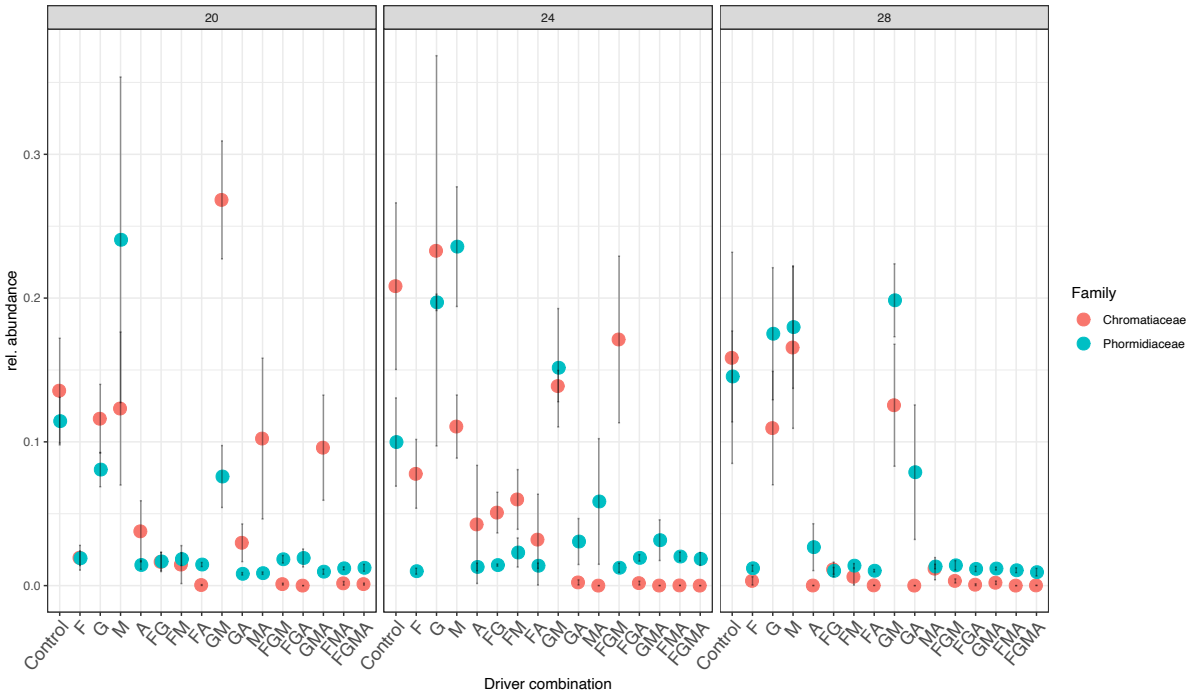
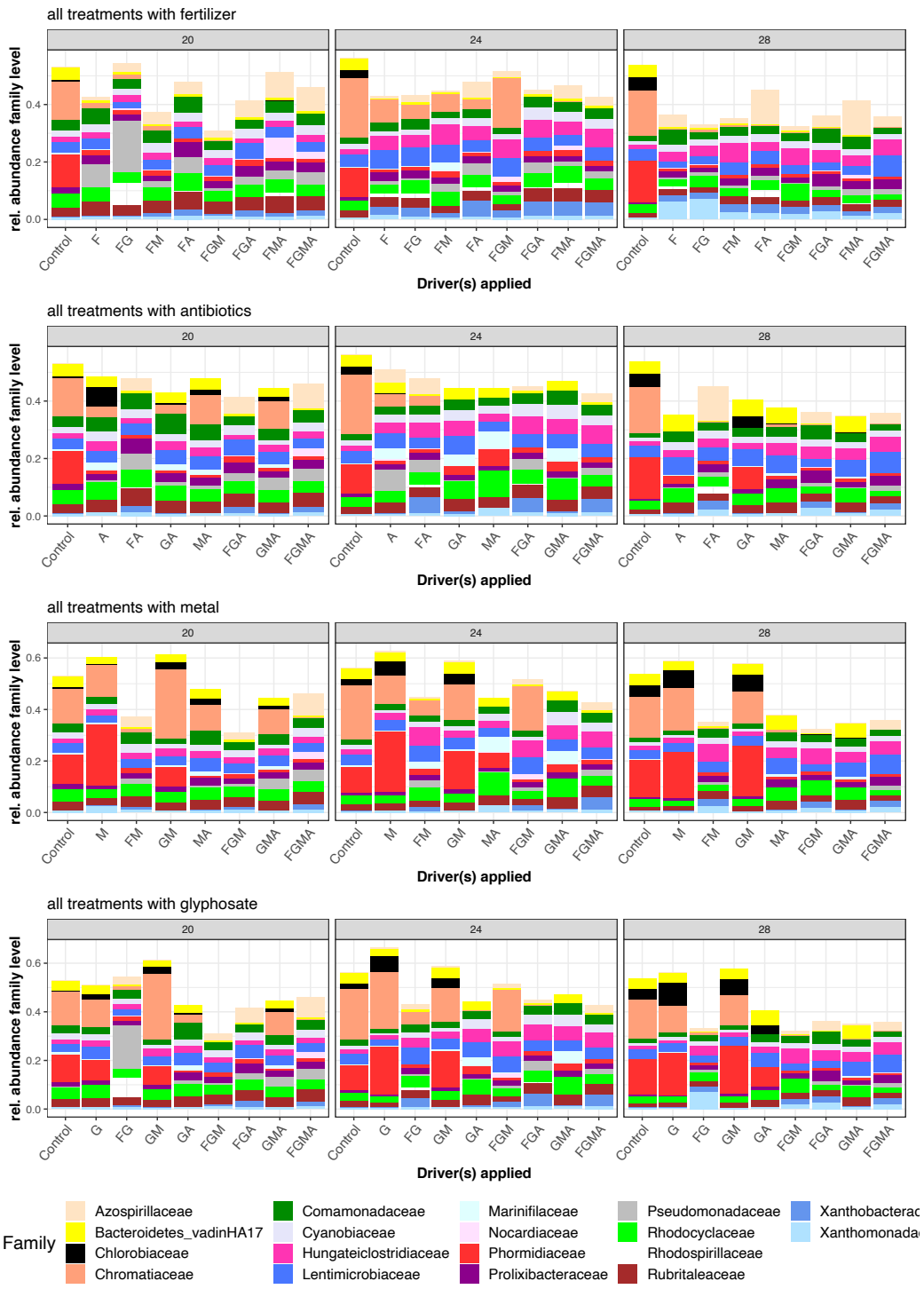
Supplementary Information



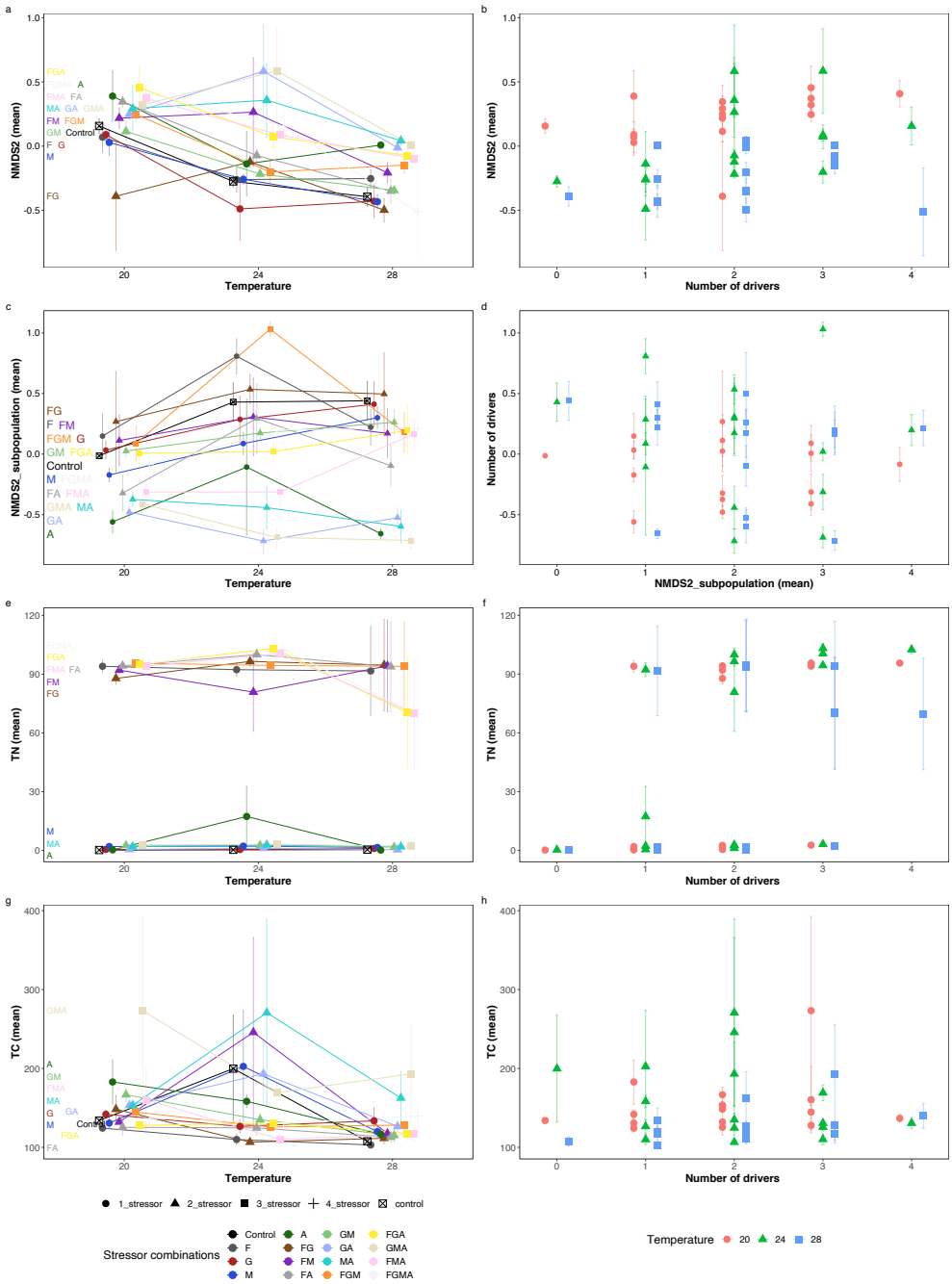
Supplementary Fig. 1 Exclusion of data of the total nitrogen measurement. There was one sample in which high amounts of nitrogen were detected when no fertilizer had been added and 12 samples in which no nitrogen was detected despite the fact that fertilizer had been added to them. Both of these two cases are not possible and were due to a faulty behavior of the measurement apparatus. Based on the measured nitrogen values in the other samples and their distribution we are confident that the measurement problems did not extend to them and there is thus no reason to doubt their validity.



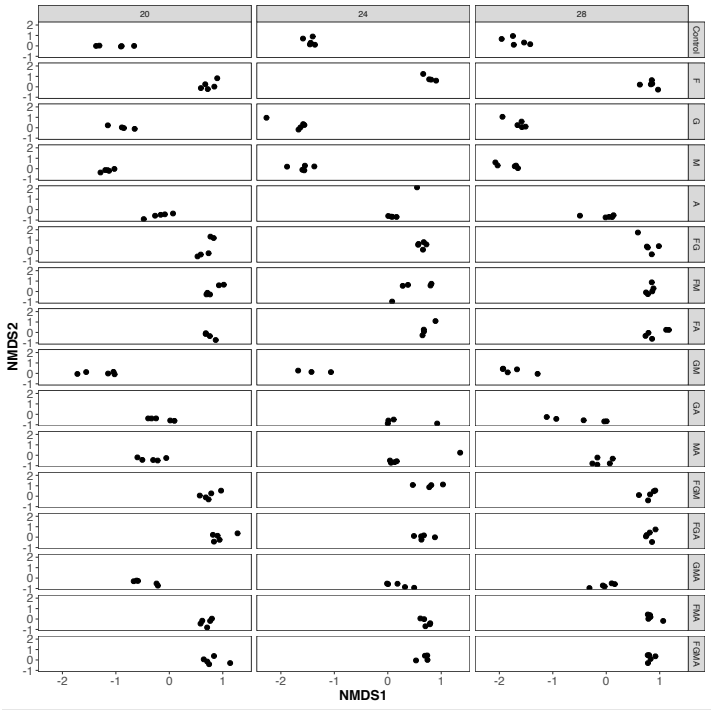
Supplementary Fig. 2 Relative abundance of *Chromatiaceae* and *Phormidiaceae* in the microcosm depending on driver combination and temperature. Mean is shown by the circle, standard errors are shown, n=5)



Supplementary Fig. 3 Effects of drivers and driver combinations on microbial community composition on family level (mean per 5 replicates) per driver category. Taxa with rel. abundance > 5 % shown.



Supplementary Fig. 4 Effects of driver combination and number of drivers on temperature applied for microbial community and ecosystem variables. NMDS2 scores varying with (a) temperature and driver combination and (b) temperature and number of drivers. NMDS2_subpopulation scores varying with (c) temperature and driver combination and (d) temperature and number of drivers. Total nitrogen concentration varying with (e) temperature and driver combination and (f) temperature and number of drivers. Total carbon concentration varying with (g) temperature and driver combination and (h) temperature and number of drivers. Standard errors are shown (n = 5 replicates).



Supplementary Fig. 5 Beta-diversity based on NMDS analysis (Bray-Curtis) in relation to temperature and driver combinations. Stress is 0.16

597 Supplementary Table 1. Number of reads in each replicate.

Combinations	total_reads	Replicate	Temperature
Control	7364	Replicate 1	24
Control	7046	Replicate 1	28
Control	4659	Replicate 1	20
F	9933	Replicate 1	28
F	5912	Replicate 1	20
G	265	Replicate 1	24
G	8662	Replicate 1	28
M	2695	Replicate 1	24
M	8202	Replicate 1	28
M	263	Replicate 1	20
A	4195	Replicate 1	24
A	7550	Replicate 1	28
A	5276	Replicate 1	20
FG	10815	Replicate 1	24
FG	8945	Replicate 1	28
FM	5660	Replicate 1	20
FM	284	Replicate 1	24
FM	8857	Replicate 1	28
FG	4639	Replicate 1	20
FA	4449	Replicate 1	24
FA	8835	Replicate 1	28
FA	3816	Replicate 1	20
GM	7678	Replicate 1	28
GM	6273	Replicate 1	20
GA	7469	Replicate 1	28
GA	6229	Replicate 1	20
MA	10849	Replicate 1	24
MA	11268	Replicate 1	28
MA	18182	Replicate 1	20
FGM	996	Replicate 1	24
FGM	6189	Replicate 1	28
FGM	6489	Replicate 1	20
FGA	2766	Replicate 1	24
FGA	8313	Replicate 1	28
FGA	6763	Replicate 1	20
GMA	9612	Replicate 1	24
GMA	8460	Replicate 1	28
GMA	7501	Replicate 1	20

FMA	11304	Replicate 1	24
FMA	8822	Replicate 1	28
FMA	6340	Replicate 1	20
FGMA	10657	Replicate 1	24
FGMA	14034	Replicate 1	28
FGMA	5819	Replicate 1	20
Control	9426	Replicate 2	24
Control	7438	Replicate 2	28
Control	2301	Replicate 2	20
F	5416	Replicate 2	24
F	9076	Replicate 2	28
F	6040	Replicate 2	20
G	7175	Replicate 2	24
G	8350	Replicate 2	28
G	5573	Replicate 2	20
M	7295	Replicate 2	24
M	6408	Replicate 2	28
M	7580	Replicate 2	20
A	7606	Replicate 2	24
A	7916	Replicate 2	28
A	13579	Replicate 2	20
FG	2733	Replicate 2	24
FG	9212	Replicate 2	28
FG	5454	Replicate 2	20
FM	7872	Replicate 2	24
FM	6398	Replicate 2	28
FM	4005	Replicate 2	20
FA	10016	Replicate 2	24
FA	7423	Replicate 2	28
FA	5710	Replicate 2	20
GM	11100	Replicate 2	24
GM	8294	Replicate 2	28
GM	7579	Replicate 2	20
GA	10206	Replicate 2	24
GA	7078	Replicate 2	28
GA	8674	Replicate 2	20
MA	13163	Replicate 2	24
MA	7927	Replicate 2	28
MA	7229	Replicate 2	20
FGM	10680	Replicate 2	24
FGM	8434	Replicate 2	28
FGM	8146	Replicate 2	20

FGA	12236	Replicate 2	24
FGA	8018	Replicate 2	28
FGA	7086	Replicate 2	20
GMA	10598	Replicate 2	24
GMA	8885	Replicate 2	28
GMA	6503	Replicate 2	20
FMA	5447	Replicate 2	24
FMA	9021	Replicate 2	28
FMA	12484	Replicate 2	20
FGMA	2177	Replicate 2	24
FGMA	7974	Replicate 2	28
FGMA	4190	Replicate 2	20
Control	10159	Replicate 3	24
Control	8105	Replicate 3	28
Control	3981	Replicate 3	20
F	9959	Replicate 3	24
F	7459	Replicate 3	28
F	6840	Replicate 3	20
G	10556	Replicate 3	24
G	8158	Replicate 3	28
G	6546	Replicate 3	20
M	11471	Replicate 3	24
M	12913	Replicate 3	28
M	8389	Replicate 3	20
A	11192	Replicate 3	24
A	10260	Replicate 3	28
A	6407	Replicate 3	20
FG	12182	Replicate 3	24
FG	10109	Replicate 3	28
FG	7141	Replicate 3	20
FM	9788	Replicate 3	24
FM	8356	Replicate 3	28
FM	8224	Replicate 3	20
FA	11486	Replicate 3	24
FA	8163	Replicate 3	28
FA	5961	Replicate 3	20
GM	9096	Replicate 3	24
GM	8953	Replicate 3	28
GM	10665	Replicate 3	20
GA	2887	Replicate 3	24
GA	7543	Replicate 3	28
GA	5011	Replicate 3	20

MA	21214	Replicate 3	24
MA	7612	Replicate 3	28
MA	6836	Replicate 3	20
FGM	10304	Replicate 3	24
FGM	8851	Replicate 3	28
FGM	7718	Replicate 3	20
FGA	11639	Replicate 3	24
FGA	10839	Replicate 3	28
FGA	7296	Replicate 3	20
GMA	14752	Replicate 3	24
GMA	10593	Replicate 3	28
GMA	8784	Replicate 3	20
FMA	12812	Replicate 3	24
FMA	11821	Replicate 3	28
FMA	9476	Replicate 3	20
FGMA	12513	Replicate 3	24
FGMA	12886	Replicate 3	28
FGMA	6091	Replicate 3	20
Control	11749	Replicate 4	24
Control	9920	Replicate 4	28
Control	5989	Replicate 4	20
F	10527	Replicate 4	24
F	9335	Replicate 4	28
F	7371	Replicate 4	20
G	7547	Replicate 4	24
G	8513	Replicate 4	28
G	10282	Replicate 4	20
M	3827	Replicate 4	24
M	6486	Replicate 4	28
M	5361	Replicate 4	20
A	9307	Replicate 4	24
A	8114	Replicate 4	28
A	5193	Replicate 4	20
FG	11085	Replicate 4	24
FG	11630	Replicate 4	28
FG	6776	Replicate 4	20
FM	10109	Replicate 4	24
FM	8542	Replicate 4	28
FM	7170	Replicate 4	20
FA	11529	Replicate 4	24
FA	9826	Replicate 4	28
FA	5165	Replicate 4	20

GM	13030	Replicate 4	24
GM	10250	Replicate 4	28
GM	8414	Replicate 4	20
GA	12392	Replicate 4	24
GA	9148	Replicate 4	28
GA	7635	Replicate 4	20
MA	12252	Replicate 4	24
MA	8859	Replicate 4	28
MA	7882	Replicate 4	20
FGM	11554	Replicate 4	24
FGM	10022	Replicate 4	28
FGM	5482	Replicate 4	20
ori	7942	ori_2	19
FGA	7658	Replicate 4	24
FGA	9903	Replicate 4	28
FGA	4233	Replicate 4	20
GMA	1965	Replicate 4	24
GMA	7948	Replicate 4	28
GMA	4641	Replicate 4	20
FMA	5334	Replicate 4	24
FMA	7455	Replicate 4	28
FMA	3332	Replicate 4	20
FGMA	11071	Replicate 4	24
FGMA	8356	Replicate 4	28
FGMA	5910	Replicate 4	20
Control	13077	Replicate 5	24
Control	7919	Replicate 5	28
Control	6401	Replicate 5	20
F	8301	Replicate 5	24
F	7802	Replicate 5	28
F	5801	Replicate 5	20
G	11899	Replicate 5	24
G	8686	Replicate 5	28
G	2745	Replicate 5	20
M	11401	Replicate 5	24
M	8270	Replicate 5	28
M	2541	Replicate 5	20
A	7766	Replicate 5	24
A	7256	Replicate 5	28
A	497	Replicate 5	20
FG	12676	Replicate 5	24
FG	8439	Replicate 5	28

FG	15008	Replicate 5	20
ori	13295	ori_5	19
ori	3919	ori_3	19
FM	8865	Replicate 5	24
FM	11599	Replicate 5	28
FM	4819	Replicate 5	20
FA	8044	Replicate 5	28
GM	7221	Replicate 5	28
GM	504	Replicate 5	20
GA	229	Replicate 5	24
GA	7704	Replicate 5	28
GA	8461	Replicate 5	20
MA	30	Replicate 5	24
MA	8202	Replicate 5	28
MA	2288	Replicate 5	20
FGM	9054	Replicate 5	28
FGM	3240	Replicate 5	20
FGA	8673	Replicate 5	24
FGA	9333	Replicate 5	28
FGA	392	Replicate 5	20
GMA	488	Replicate 5	24
GMA	8907	Replicate 5	28
GMA	6931	Replicate 5	20
FMA	8137	Replicate 5	24
FMA	9209	Replicate 5	28
FMA	4630	Replicate 5	20
FGMA	8672	Replicate 5	28
FGMA	4883	Replicate 5	20
ori	10488	ori_6	19