GROUNDWATER FLOODING: ECOSYSTEM STRUCTURE FOLLOWING AN EXTREME RECHARGE EVENT

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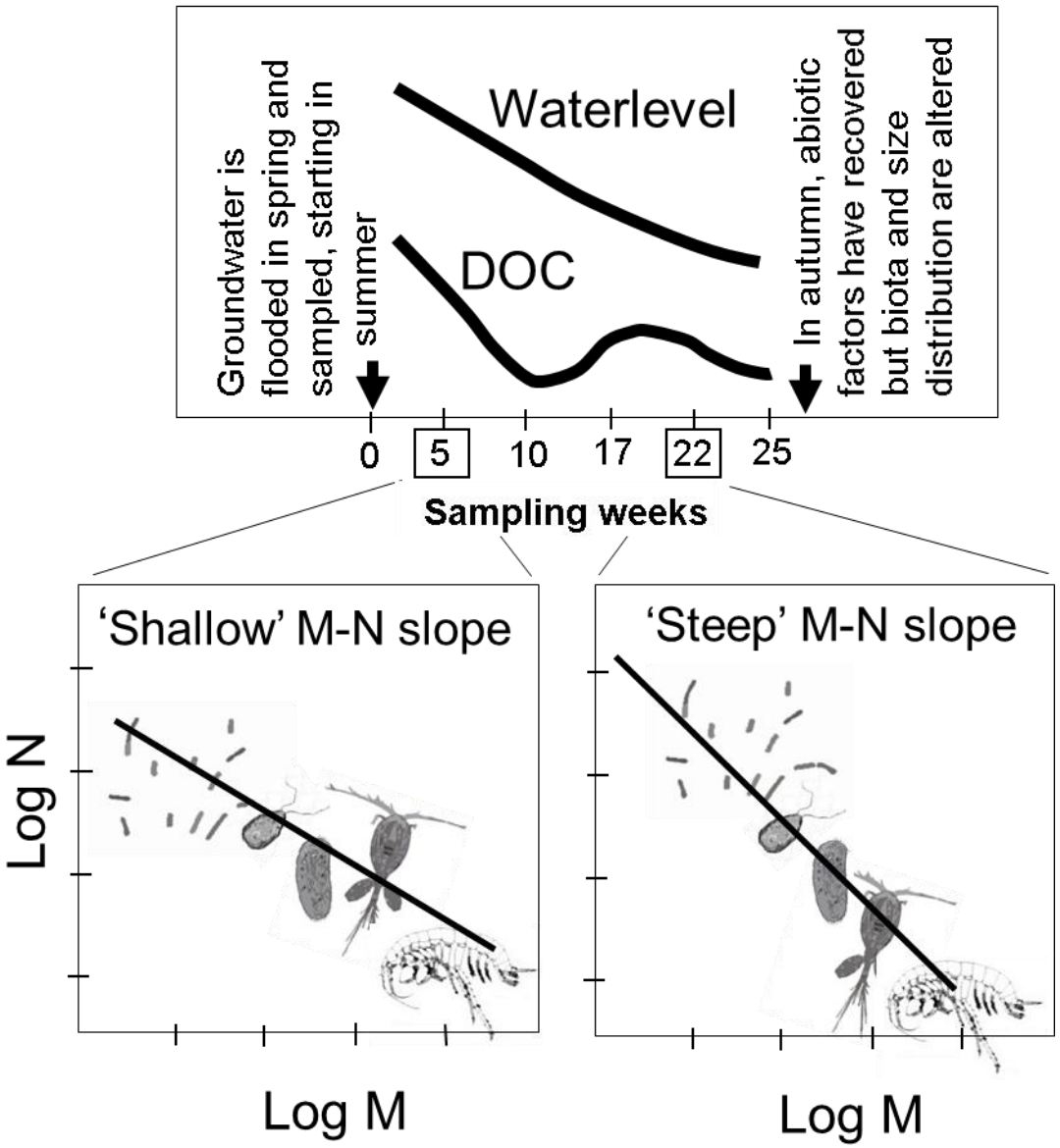
Conceived and designed the study: AR, JR, SK. Performed the study: KF, AR, JR, SK, CC, PR. Analysed the data: DP, JR, SK. R-codes and Figures: DP. Wrote the paper: JR, KF, DP, SK, CC, AR.


Groundwater is flooded in spring and sampled, starting in summer
Waterlevel
DOC
In autumn, abiotic factors have recovered but biota and size distribution are altered
0
5
10
17
22
25
Sampling weeks
'Shallow' M-N slope
'Steep' M-N slope
Log N
Log M
Log M

HIGHLIGHTS

- **Groundwater that had been flooded after an extreme rainfall event was sampled**
- **The flooding had resulted in high DOC levels that were tracked by bacteria**
- **Overall, small organisms increased in abundance while larger ones did not**
- **This altered the size distribution of the community towards steeper M-N slopes**
- **The deeper aquifer was less affected by the flooding**

ABSTRACT

1) Aquifers are recharged by surface water percolating through soil and rock and by connections with surface streams and rivers. Extreme rainfall can cause extensive flooding of surface waters and, eventually, of groundwaters. However, how the resultant changes in nutrients impact groundwater organisms and the structure of groundwater food webs is largely unknown.

2) We monitored abiotic (nutrients, temperature and more) and biotic (all organismal groups except viruses) conditions in eight groundwater boreholes in two locations in a chalk aquifer over the course of 25 weeks (ten sampling occasions), following an extreme rainfall- and groundwater-flooding event in the UK.

3) We show that groundwater flooding can cause substantial nutrient fertilisation of aquifers – nutrient concentrations (especially dissolved organic carbon) in the groundwater were highest when we started the sampling campaign, directly following the flood event, and then decreased over time while groundwater levels also declined back to their baseline.

4) Bacteria in the open water (i.e. bacteria not associated with sediment) became more abundant as the water table and DOC concentrations decreased. Importantly their functional richness tracked the DOC patterns, illustrating that bacteria were responsible for respiring DOC. Microbial metabolic activity and bacterial respiration, measured using smart tracers, supported this finding; DOC and microbial respiration showed a positive correlation.

5) The other biota (protists, micro- and macro-metazoans) showed different abundance patterns over time, but importantly, the entire sediment community, ranging from bacteria to macrofaunal species, showed a strong community size structure (mean size spectra slope: -1.12). Size spectra changed gradually through time towards steeper slopes, except in the very deep aquifer.

6) Our approach allowed us to demonstrate that groundwater communities track extreme changes in their usually stable environment, highlighting that they potentially buffer environmental change, although we still do not know what the limits of this ‘service’ might be.

Keywords: DOC, protozoan, bacteria, recharge, stygobite, metabolism

1. Introduction

Aquifers hold >97% of our world's unfrozen fresh water and more than two billion people world-wide rely on groundwater for their daily supply of drinking water (see review on groundwater services by Griebler and Avramov, 2014). Aquifers are constantly recharged with water from the land's surface, as groundwater ecosystems are tightly linked with surface waters via a hydrological continuum with complex recharge–discharge processes (Boano et al., 2014; Boulton et al., 1998; Boulton and Hancock, 2006; Brunke and Gonser, 1997; Krause et al., 2017, 2011). However, extreme rainfall can result in the flooding of inland waters and these events are expected to become increasingly more frequent in the northern hemisphere (Taylor et al., 2012), driven by the increase of greenhouse gas concentrations in the atmosphere (Min et al., 2011). Apart from the devastating impact on the economy and human livelihoods (Munro et al., 2017), flooding has the potential to detrimentally impact on freshwater ecosystems, both, above- and below-ground (Taylor et al., 2012).

Surface water flooding can induce increases in groundwater levels with variable time lags. As water percolates from the surface, groundwater levels rise and fill sediment pore-spaces and fractures (groundwater flooding). During such extreme recharge events, infiltrating rainfall can potentially transport pollutants and nutrients, including carbon, from the surface to the aquifer (Taylor et al., 2013; Van Halem et al., 2009), affecting the delicate groundwater ecosystem and water quality. Background groundwater quality in general depends on the underlying geology of the aquifer (e.g. Weitowitz et al., 2017), however, to truly understand groundwater quality, we must recognize that groundwater is an ecosystem: in addition to the chemical and physical environment, it comprises a wide range of organisms which have the ability to mediate biogeochemical processes and to immobilise or transform pollutants and nutrients (Griebler and Lueders, 2009).

Groundwater in Europe contains organisms that span > 9 orders of magnitude in terms of size, from microbes such as bacteria, through microscopically small fauna (e.g. protists, rotifers and copepods) to macrofaunal crustaceans (e.g. *Niphargus* and *Proasellus* species) but we still know very little about groundwater biota and their interactions (e.g. within the food web). There is extreme endemicity in this ecosystem (Gibert et al., 2009; Griebler and Lueders, 2009; Marmonier et al., 1993); most groundwater organisms are unique to this habitat (the macroscopic species are called 'stygobites') and are adapted to the low energy levels and lack of light that prevail. In mainland Britain they include the oldest inhabitants by millions of years (McInerney et al., 2014), forming distinctive truncated food webs that cannot be found in surface waters (Gibert and Deharveng, 2002).

Aquatic communities in surface waters, and especially invertebrate communities, are often described and compared to assess ecosystem health, using a range of approaches such as score systems with indicator species (e.g. Armitage et al., 1983), estimations of energy flow (e.g. Reiss and Schmid-Araya, 2010) or comparisons of community structure (e.g. Petchey & Belgrano 2010). For instance, stream invertebrate communities are strongly size-structured: small organisms are very abundant while larger ones are rare (Schmid, 2000) and the decrease in abundance from small to large organisms reflects the energy flow within the community. The size-structure of natural communities can be quantified by constructing size spectra (White et al., 2007), the frequency distribution of individual sizes within a community, which is depicted by plotting the total number of individuals occurring within 'body size bins' (White et al., 2007). Typically, this relationship is negative and linear on logarithmic axes and is quantified by the slope (Yvon-Durocher et al., 2011a). Since size spectra slopes provide integrated measures of trophic structure (Yvon-Durocher et al., 2011a), they can be used to gauge ecosystem-level responses to environmental stressors (Petchey and Belgrano, 2010) such as pH (Layer et al., 2011; Mulder and Elser, 2009) and

warming in fresh waters (Dossena et al., 2012; O'Gorman et al., 2012). Whether such an approach can be applied to groundwater ecosystems is unknown given that, to our knowledge, no groundwater study has estimated the body size and abundance of all groundwater groups and we do not know how these communities respond to stressors such as flooding.

We monitored abiotic and biotic conditions in a chalk aquifer following an extreme rainfall and river flooding event in the UK, by monitoring eight groundwater boreholes in two locations (Berkshire and Dorset) over the course of 25 weeks. The flooding had resulted in the elevation of water level in all boreholes by at least 10 m, and in two boreholes water levels had reached the land surface (Fig. 1). This extreme event permitted a uniquely important case study to track a pulse of energy through groundwater.

We characterized changes in the groundwater environment after the flooding by analysing its physical (groundwater levels, temperature), biogeochemical (nutrients such as carbon) and biotic (microbial biomass, metabolic activity, functional- and species richness) conditions. We then investigated the size structure of groundwaters by constructing size spectra (these included bacteria, protists and metazoans) and assessed temporal variability in the scaling of these relationships.

Our main objective was to measure the impact of this extreme flooding across the groundwater assemblage, from the energy supplied by nutrients (especially dissolved organic carbon) to prokaryotes through to macrofauna; and to track the recovery of the system after this exceptional groundwater recharge period through space and time.

We hypothesized that nutrients would have been washed into the groundwater and that this pulse would be tracked by the organisms in terms of abundance and (metabolic) activity. We expected that the response of the community would be reflected in changes of the size distribution (e.g. because small organisms such as bacteria respond more rapidly to

environmental change compared to larger ones such as macrofauna). Lastly we anticipated the deeper aquifer (>100 m deep) to be less affected by groundwater flooding, in terms of changes within the chemistry and biology, because both nutrient- and water load would have been attenuated within the underground layers above the deep groundwater.

2. Methods

2.1. The sites

We sampled the chalk aquifer in two locations (in west Berkshire and east Dorset) in southern England from May to October 2014 (10 sampling occasions over 25 weeks, see Fig. 1, Appendix A, Tables A.1 and A.2) following a major storm period with subsequent groundwater flooding (Fig. 1). In each location, we selected 4 boreholes of similar topographic location (interfluves). In Berkshire we sampled: Bottom Barn (BB), Briff Lane (BL), Calversley Farm (CF) and Greendown Farm (GF); and in Dorset we sampled: Marley Bottom (MB; on 7 out of the 10 sampling occasions), Milborne (M), Newfield (NF) and Winterborne (WB). Boreholes enable access to the aquifer and, while faunal abundances are usually higher in boreholes than in fractures in the aquifers, the same species are found, and it is also possible to track abundance trends in the aquifer via sampling boreholes (often dubbed 'windows to the aquifer'; Hahn and Matzke, 2005; Sorensen et al., 2013).

The water table in the boreholes was still unusually high when we started our sampling regime - from 2 to 7 m above baseline levels (Fig. 1, Table A.1). The first sampling was on 06/05/14 ('week 0'), followed by another 9 sampling occasions. Counting from week 0, we sampled in weeks 3, 5, 7, 10, 13, 16, 19, 22, and week 25 (see Table A.2 for dates). We used short sampling intervals to capture the expected rapid responses of the community to gradual flooding cessation. We also obtained historical data on waterlevels in all boreholes from the Environment Agency, which we combined with our measurements (Fig. 1).

Two sub-habitats were sampled in the boreholes: the open water (for abiotic parameters, bacteria and metabolic activity) and the sediment (biota). Further, sterile cotton strips were exposed in the boreholes for two weeks at a time to estimate fungal densities (90 cotton strips were evaluated but we did not detect any fungal biomass, see methods in

Appendix A). Across boreholes, the sampling resulted in 77 samples for open water and sediment bacteria, 69 sediment samples for sediment protists and 77 samples for meiofauna and macrofauna (Table A.1).

2.2. Open water sampling: chemistry and bacteria

On each sampling occasion we measured the depth of the groundwater table, dissolved oxygen (see Table A.3), pH and conductivity *in situ* with a YSI sonde. Borehole temperatures were monitored with automatically logging thermistors; temperature was stable in all boreholes and there was little inter-borehole variation (10.31 ±0.011°C over 7 months). On every sampling occasion, groundwater was collected with a bailer from each borehole and analysed for dissolved organic carbon (DOC; 77 samples, see Table A.2) and eight other nutrients (see Appendix A) by catalyst aided combustion on a Shimadzu TOC analyser.

We further took samples of bacteria in the open water column of the borehole (270 mL of water sampled with a bailer from the top of the water column). Bacterial abundances were expressed as individuals per L of open water. To estimate the abundance of bacteria, we took a 1ml sub-sample of the borehole sample that had been brought back from the field. For open water bacteria, we subsampled the 270 ml open water bailer sample and for sediment bacteria, we sub-sampled the sediment bailer sample (sediment suspended in 270 ml filtered borehole water). The 1 ml sub-samples were frozen and roughly half of that sample (0.495 ml) was subsequently analysed by flow cytometry (Gasol and Del Giorgio, 2000) using an Accuri C6 Flow Cytometer (BD Biosciences). A threshold of 8000 on the forward scatter (FSC-H), 2000 on the side scatter (SSC-H) and slow Fluidics setting was used. Each sample was measured for 1 min. To stain the DNA of living cells, 200 µL of PicoGreen dye solution (Quant–iT™ PicoGreen™ dsDNA Assay Kit, Sigma–Aldrich) was added to 1 mL filtered water and incubated at 4°C for 15 min. Sediment particles were identified through the FL1-H

channel (with values < log 3.1). The list of individual events returned by the flow cytometer was extracted using the R packages flowCore and flowViz (Ellis et al., 2009; Sarkar et al., 2008).

We used Biolog EcoPlates to assess the functional capacity of the open water microbial communities (e.g. Baho et al., 2012; Christian and Lind, 2006; Korbel et al., 2013; Stefanowicz, 2006), for each borehole and sampling occasion. Water samples were filtered through a 40 μm sterile sieve and 100 μL was pipetted into each of the 96 wells on a Biolog Eco-Plate: each EcoPlate contains 31 carbon substrates plus a no-substrate control in triplicate as well as a redox dye that turns purple if it is reduced when a given carbon source is metabolized (e.g. Stefanowicz, 2006). Plates were incubated at 10 °C for 7 days in the dark, after which time colour change was quantified by measuring optical density at 595 nm using a bench-top microplate photometer (Multiskan® EX, Thermo Scientic). We estimated the functional richness of bacteria communities as the % of substrates used (100 * number of positive substrates / 31). We scored a carbon source as positive when two out of three wells reached a predetermined optical density (after Roger et al., 2016), which we set at 0.1 after subtraction of the mean blank from all wells.

2.3. Open water: microbial metabolic activity

On the second sampling occasion only (28/4/14), we took samples to characterise the metabolic activity in the boreholes (except for MB) so that we could relate these measurements to DOC levels at that time. Microbial metabolic activity was measured by applying the “smart tracer” Resazuring/Resorufin (Raz/Rru) system (see Appendix A) that was first used in ecohydrological applications by Haggerty et al. (2009).

Samples were cooled and transferred to the laboratory without filtering to preserve the microbial community. The sample (250 ml) was incubated in duplicate in 1000 ml

microcosms with a concentration of 100 µg/l Raz for 24 h. Two microcosms were prepared with only deionised water to act as control treatments. A detailed overview of these methods is available in Appendix A.

2.4. Sediment: sampling of biota, abundance and community composition

We sampled the borehole sediment with a bailer (one 270 ml sample; to sample bacteria and protists in the sediment) and a net (microscopic and macroscopic metazoans; net diameter was 21, 11 or 4 cm depending on the borehole). The bottom bailer, used for bacteria and protists (Table A.2), sampled sediment with an area of 0.11 dm^2 (bailer radius was 0.19 dm) and the net samples emptied the entire area of the borehole bottom (this area varied depending on the borehole from ~0.2 dm^2 to ~7.3 dm^2; see Table A.1). With the net, we removed the sediment in the borehole (3 net hauls that were pooled in the field). All sediment samples were initially stored in 270 ml filtered borehole water for the transport back to the laboratory. This way, all subsampling in the laboratory followed the same method.

Abundances of all sediment biota were expressed as individuals per dm^2. The number of bacteria was estimated using flow cytometry as described above. Protists were measured, counted and identified alive in 1 ml (subsampled from 270 ml), using a Fuchs-Rosenthal counting chamber under a light microscope using 100 times and 400 times magnification (after Reiss and Schmid-Araya, 2010). Sediment samples for hard bodied meiofauna and macrofauna were preserved in formaldehyde for identification, measurement of body size and enumeration. For meiofauna and macrofauna, we did not sub-sample but counted individuals in the entire sample. For protists and metazoans, we assigned individuals to a taxonomic group (species in most cases) and measured the size (length and width) of individuals. This also gave a record of abundances in the (sub-) samples (Table 1). For all groups, we calculated biomass by multiplying body mass by abundance.

2.5. Sediment community size structure

The measurements of individual sizes from bacteria to meiofauna and the estimates of abundances allowed us to construct community size spectra. Bacteria cell size was estimated using calibration beads to convert forward scatter to average diameter of bacterial cells (see Fig. A.1; after Schaum et al., 2017). Cell carbon content (C) was estimated from bacterial cell volume, assuming 0.10 pgC /μm^3 (Norland et al., 1987) - this gives a very similar result to a different method used by Fuhrman and Azam (1980).

Body length and width (for protist and meiofauna individuals) was converted to biovolume assuming a spherical shape. Individual dry mass (mg) was estimated by assuming a density of 1.1 to convert volume to wet weight, and the carbon content was assumed to be 10 % of the wet weight (Reiss and Schmid-Araya, 2010, 2008). Taking all species found into account, the body mass range was ~ 10 orders of magnitude (see Table 1).

We computed the community size spectrum (CSS after White et al., 2007) for each borehole and time point by logarithmic binning of individual body masses, *M*, (White et al., 2007). The total range of $\log_{10}$ (*M*) values was divided up into eight bins of equal width and the total abundance, *N*, of all individuals within each size class were regressed against the bin centers (cf Reuman et al., 2008). The intercept of the relationships were fixed at the smallest size class (after Yvon-Durocher et al., 2011b) which reduces the correlation between the slope and the intercept of linear relationships and makes the intercept of the model, $\log N(M_{min})$, equivalent to the abundance of the smallest size class (here bacteria) as opposed to an infinite small mass. Size spectra slopes and intercepts were determined from simple linear regression analysis.

2.6. Statistical analysis

We used generalised additive mixed modelling (GAMM) via the *gamm* function in the mgcv package implemented in R (R Development Core Team, 2017) to investigate the general response of physical, chemical and biological variables in the open water and organism abundances through time. This approach allowed us to model the potential non-linear (smoothing) relationships between response variables and time (sampling week), accounting for the fact that each borehole is its own individual system (with unique baseline levels) and temporal correlations in residuals in the time series data (Zuur et al., 2009). We evaluated the significance of the smooth terms of the selected model based upon the extent to which their 95% CI intervals contained zero. A maximum number of knots of six was chosen as further required choices in model implementation: changing this value had no substantive effects on the general results. A temporal auto-correlation term was added to the models of the form corAR1(form =~1|site) and the significance of the term was determined via a likelihood ratio tests of nested models with and without the auto-correlation term (Zuur et al., 2009).

For responses where relationships through time were linear, or where no significant smooth temporal trends were evident, we performed generalised least square (GLM) modelling to test our hypothesis that variation in the responses through time would be related to borehole depth. To do so we fitted Time (sampling week) and Borehole depth as continuous variables including an interaction term (Time × Borehole depth). We fitted the same temporal auto-correlation structure as with the GAMM modelling and assessed the significance of this term as described above. We plotted all responses as histograms, as well as the residuals of the models, to identify non-normally distributed responses. Responses that were not normally distributed (concentrations, abundances and biomass density) were $\log_{10}$-transformed prior to model fitting. A simple linear regression analysis was performed for the

respiration data (nutrient levels explaining microbial activity). All statistical analysis was performed in R, version 3.4.1 (R Development Core Team, 2017).

3. Results

3.1. Open water measurements: waterlevels, DOC and bacteria

Our study captured the tail of groundwater flooding in the sampled aquifer induced by heavy rains in the winter of 2013 (Fig. 1). Groundwater flood response varied spatially, with levels reaching the surface at two locations (WB and NF in Dorset) and three locations were less affected by flooding (CF, GF and BB in Berkshire, which are also the deepest boreholes, i.e. here we sampled the deeper aquifer; Table A.1). The water table in all boreholes was unusually high at the start of the sampling regime because the groundwater was still closer to the surface compared to its normal levels (at least 2 meters closer to the surface in all boreholes; Fig. 1; Table A.1).

Waterlevel, DOC, bacteria biomass and bacterial functional richness all changed over time (Fig. 2 shows the GAMM models that describe the patterns over time best, but see Fig. B.1 for raw data). The model inference procedure clearly selected the most complex GAMM models unambiguously rejecting both the null model (Table 2) and models without a temporal auto-correlation term (Appendix B, Table B.1) for all four responses. Examination of the P-values of the individual smooth terms (Table 2) and their confidence intervals (Fig. 2) provided further support for nonlinear temporal trends through time.

Over the 25 weeks study period, waterlevel decreased in an almost linear fashion (Fig. 2a), back to baseline levels (Fig. 1). DOC concentrations were highest at the beginning of the sampling regime and decreased until week 10 before gently rising towards week 16 and then dropping again (Fig. 2b); i.e. we observed the highest DOC values when waterlevel was high and the lowest DOC values when water level was low, but whilst waterlevel and DOC appear related, the pattern is not a simple, linear one (Fig. 2). Bacterial biomass in the open water increased over the sampling period, as waterlevel and nutrients were declining (Fig. 2c, Table

B.1), but peaked around week 16 before declining. Thus, DOC pulses (Fig. 2b) were not immediately tracked by the open water bacteria (Fig. 2c). Bacterial functional richness was generally high with 45 to 100% of carbon substrates utilised. Functional richness was initially high at the beginning of the sampling regime and decreased until week 5 before rising towards week 10 and dropping after week 20 (Fig. 2d).

Groundwater samples from May 2014 showed microbial metabolic activity above control conditions, indicating recharge driven nutrient fertilisation of groundwater microbial communities. The analysis of Raz/Rru conversion rates as indicators of microbial metabolic activity revealed that increased DOC levels correlated with high microbial metabolic activity and respiration ($R^2 = 0.66$, Fig. B.2).

3.2. Sediment biota: community composition and size structure

In the sediment, we identified 67 different protist taxa, 7 microscopic metazoan taxa and 9 macrofaunal taxa (Table B.2). Very small ciliates and flagellates were the most abundant component of the protists (Table B.2), while copepods dominated the meiofauna in all boreholes (Table B.2), and *Niphargus kochianus* was the most frequently found and abundant stygobite within the macrofauna (in Calversley Farm the total counted exceeded 1000 individuals over the 25 weeks).

There was no general relationship between abundance and time for the four organism groups. This was the case irrespective of whether GLS or GAMM models were used (Tables B.3 and B.4), indicating that the responses of organism abundance through time were borehole specific and could not be captured using either non-linear or linear models or by the addition of borehole depth as an explanatory variable. Although bacteria and protists increased in abundance over time in most boreholes (Fig. B.3), there was considerable difference between boreholes regarding abundance peaks and (as a consequence) the

abundance patterns were unrelated to borehole depth (Table B.3) or any other variables we investigated. Bacterial counts exceeded 10^9 ind. dm^{-2} and protists showed an average abundance of ~70,000 ind. dm^{-2}. While bacteria and protists were present in all boreholes and on all sampling occasions, meiofauna and macrofauna were sometimes absent on particular sampling occasions (Fig. B.3).

Meiofauna were always present in BB, BL, CF and MB and macrofauna were always present in BB, CF and MB. In two of the boreholes, Bottom Barn and Calversley Farm, macrofaunal abundance increased steadily from under 10 to over 40 individuals per dm^2 (mainly *N. kochianus and P. cavaticus*) over the 25 weeks (Fig. B.3). Mean abundance of meiofauna and macrofauna in the boreholes was ~ 5 ind. dm^{-2} and ~ 7 ind. dm^{-2} respectively.

We used mass-abundance spectra to estimate the response of the entire community. As expected, over 10 orders of magnitude in body mass from bacteria to macrofauna (Figs. 3 and B.4), abundance declined linearly. The average slope of the size spectrum, across sites and sampling occasions was -1.12 (95% CI: -1.16 to -1.07, Fig. 3). Size spectra slopes did not vary systematically through time or with borehole depth (Table 3). However, a significant time × depth interaction was evident (Table 3) with slopes becoming steeper (more negative) through time for 'shallower' boreholes (Fig. 3). This pattern can be explained by the fact that, whilst not individually significant, bacteria and protozoan abundances tended to increase through time in the 'shallower' boreholes, whilst meiofauna and macrofauna abundances were relatively constant (Fig. B.3). This is also supported by the marginally significant p-value for the time × depth interaction for size spectra intercepts which were fixed at the minimum body size class (Table 3).

4. Discussion

This study was the first to analyse the impacts of groundwater flooding induced intensive recharge using an extensive analysis of both abiotic and biotic factors. We showed that nutrient concentrations increased in response to elevated groundwater levels, with more nutrients being available for groundwater organisms in consequence. Bacteria in the open water (i.e. bacteria not associated with sediment) became more abundant as groundwater levels and DOC concentrations decreased, indicating that they incorporated the additional energy. The measures of bacterial functional richness show that bacterial richness was high when DOC was high, demonstrating the link between nutrients and functional richness and bacterial activity. This finding was further supported by the respiration study that revealed a positive correlation between DOC concentrations and microbial metabolic activity in boreholes (see 4.1 Open water chemistry and biota). In the sediment, the change over time back to baseline levels was reflected in changes of the size distribution (measurements of individual groups were less conclusive) of the community towards steeper M-N slopes because, overall, small organisms (bacteria and protozoans) increased in abundance while the larger ones did not. This was however not the case in the deeper aquifer which was also much less affected by the flooding (see 4.2 Sediment biota).

4.1. Open water chemistry and biota

Groundwater that is enriched with nutrients (e.g. in catchments with intensive agriculture) has previously been shown to exhibit high bacterial biodiversity values (Stein et al., 2010), which is atypical for pristine groundwater of comparable systems (Griebler et al., 2010). In accordance with these studies, we found that temporally variable DOC levels can produce similar differences in bacterial richness over time (however, in our case measured as functional richness not as operational taxonomic units used by these authors). Intriguingly,

the DOC pattern observed over time shows a 'new pulse' of nutrients after some weeks of sampling. We can only speculate about the reasons behind this pattern but two scenarios are possible: the new pulse could be the result of a new input from the surface or be the result of food web responses: the pattern resembles nutrient dynamics in chemostats, where bacteria can first utilise the nutrients, then crash and subsequently recover (e.g. Behrends et al., 2014).

4.2. Sediment biota

Intriguingly, with exception of the macrofauna in two of the boreholes, the sediment biota (bacteria, protists, microscopic metazoans and macrofauna) did not show any statistically significant response to the changes in nutrient concentrations or water table (although trends of increasing abundance of bacteria and protists in the shallow aquifer were obvious). The non-significance is largely due to two methodological issues: firstly, boreholes were used as replicates, which 'worked' for very robust patterns such as water level decline, DOC levels and size spectra, but for abundance patterns of individual groups each borehole represents its own unique system (e.g. in some boreholes groups such as meiofauna were almost absent despite the fact that the borehole connects to the same aquifer as a near-by borehole with meiofauna; see Fig. B.3). Secondly, GAMM is a useful method to show trends but it is not possible to fit linear correlations (such as fitting borehole depth as a predictor).

For all boreholes, species richness of macrofauna was similar to that reported elsewhere in the UK (Robertson et al 2009) but lower than reported in Europe (Griebler et al., 2010). Protozoan species richness was comparable to other studies outside the UK (Loquay et al., 2009), possibly because protozoans can colonise 'extreme' habitats more easily than metazoans. Previous studies on 5 of the 8 boreholes found the same macrofauna species, and that, like this study, these species were more abundant than meiofaunal copepods (they represent the majority of the meiofauna) (Johns et al., 2015; Maurice et al., 2016). This

pattern seems to be unique for groundwater habitats, for example, in streams, and rivers, meiofauna are always more abundant than larger invertebrates (e.g. Stead et al. 2003) and indeed, we would expect higher abundances of small invertebrates compared to larger ones from metabolic theory (Brown et al., 2004).

We observed systematic changes in the size spectrum over the 25 weeks monitoring period, with slopes of the size abundance relationship (the spectra) becoming steeper (more negative) in the 'shallower' boreholes. This pattern is consistent with the notion that the community dynamics at the bottom of shallower boreholes are more closely coupled with conditions in the open water compared to deep boreholes. Shallow boreholes received increased carbon resources and exhibited an increase in microbial biomass and metabolism over the sampling period. In other words, compared to the shallow aquifer, the deeper aquifer receives much less nutrients and water load when groundwater is flooded. These findings also highlight that size spectra analysis can provide key insights into the responses of ecosystems to environmental variability (Petchey and Belgrano, 2010) and understanding energy flow through groundwater food webs.

The size spectra analysis also revealed that groundwater communities were strongly size-structured with the slope of the relationships ($N \sim M^{-1.1}$) similar to the negatively isometric pattern often reported for aquatic food webs (Kerr and Dickie, 2001). For any given size spectrum, the slope depends on the community-wide mean predator–prey mass ratio (PPMR: the mean size of predators relative to prey), and the trophic transfer efficiency (TE: the proportion of prey production converted to predator production) (Kerr and Dickie, 2001). The model of Brown and Gillooly (2003) incorporates general allometric scaling principles to predict the scaling of abundance, N, with body mass, M, as:

$N \propto M\lambda \times M\log(TE)/\log(PPMR)$, where λ is the M-N scaling coefficient within trophic levels (typically −3/4), the reciprocal of the mass dependence of metabolic rate for

many multicellular taxa (Brown et al., 2004). The trophic structure of groundwater food webs is largely unknown but assuming a TE range of between 10 and 20% (Jennings and Mackinson, 2003) suggests that the PPMR ratio in these systems is somewhere between 100:1 and 600:1, similar to other benthic freshwater ecosystems (Perkins et al.2018, Woodward and Warren 2007).

Abundances were generally high compared with other aquifer studies (Griebler et al., 2010), which is explained by the fact that we sampled boreholes (Hahn and Matzke, 2005; Sorensen et al., 2013) and did not pump the aquifer water (see references in Griebler and Lueders, 2009 and Korbel et al., 2017). Collecting groundwater from boreholes is the most common method for groundwater sampling because they provide the only suitable sampling 'window' into deeper aquifers. Previous studies have shown that boreholes have higher abundance but the same species composition compared to the rest of the aquifer (Hahn and Matzke, 2005, Sorensen et al., 2013). However, boreholes do still represent groundwater community patterns over time (our focus) and species composition in the aquifer.

4.3. The wider context and future directions

Ecological assessment of groundwater is a very recent discipline (see reviews by Danielopol et al., 2003; Gregory et al., 2014; Griebler et al., 2014; Steube et al., 2009). However, we now have methods, such as cytometry (Bayer et al., 2016), that help us to monitor stygobite populations and also to assess microscopically small organisms in groundwater (see literature for bacteria, protists and meiofauna reviewed in Griebler and Lueders (2009) and Novarino et al. (1997) for protists).

It will be crucial to determine if groundwater biota have the potential to buffer inputs of nutrients through increased secondary production. Although their environment is stable, the relatively low temperatures might inhibit the incorporation of these additional nutrients

into biomass. We believe a more rigorous analysis of the groundwater food web is needed to show how the composition and activity of bacteria changes when nutrient availability changes and whether the bacterial response is coupled with increased secondary production of eukaryotes. Further, it is vital to include hydrological knowledge in these studies because they determine how stable the habitat is. Menció et al. (2014) found that steadier groundwater head levels and lower nitrate concentrations promote a more diverse and abundant stygofauna community. Historical hydrological data from UK chalk aquifers, and our study, showed that that the deep aquifer is less affected by flooding and it could therefore represent a very stable environment for groundwater organisms. However, shallower aquifers (down to 100 m) seem to respond rapidly to flooding and we do not know to what extent the groundwater community is perturbed in these important underground layers that hold clean fresh water. As extreme rainfall events are predicted to become more frequent in the Northern hemisphere, it is likely that groundwater quality will ultimately be affected.

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Figure legends

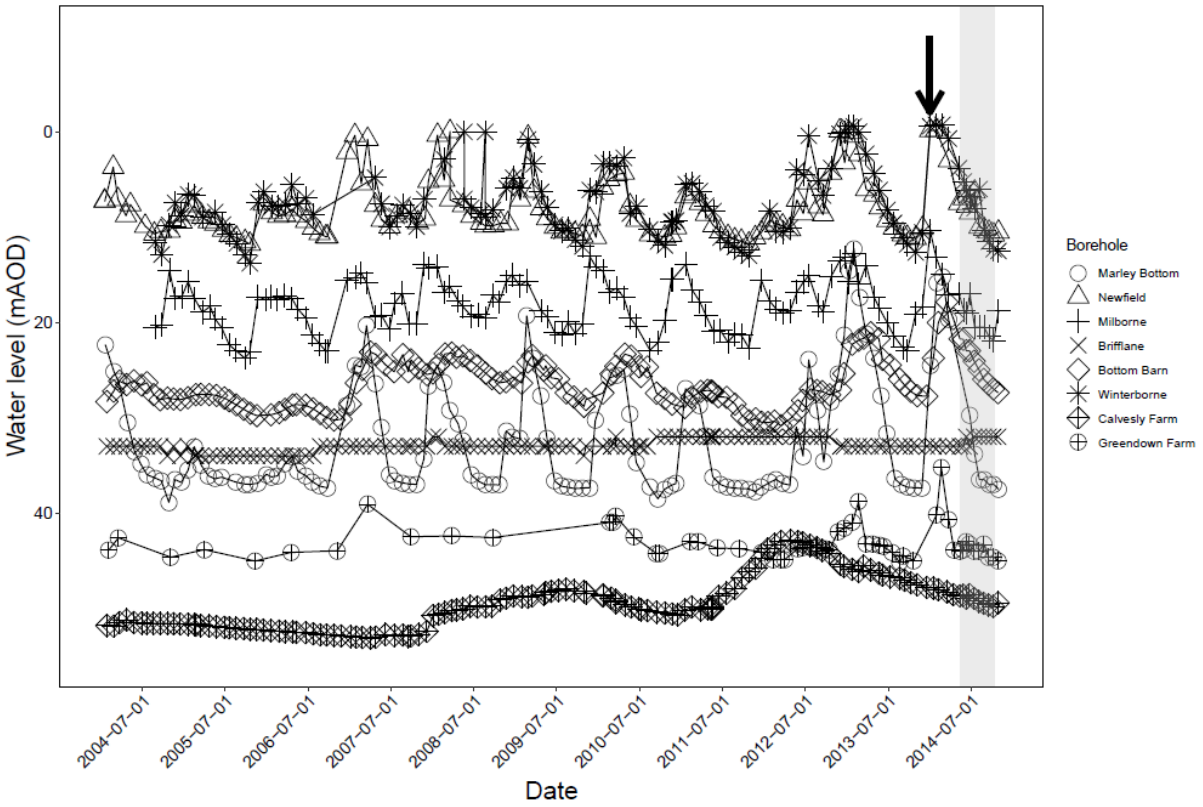

Fig. 1: 10 yrs time series of the groundwater table (measured as metres below the surface) for eight boreholes in the UK Chalk aquifer. The arrow shows the start of the flooding period (induced by heavy rains in Winter 2013) and the area shaded in grey indicates the sampling period (10 sampling occasions over 25 weeks). The water level on the y-axis is given as mAOD (Metres Above Ordnance Datum); a value of zero indicates that the waterlevel had reached the land surface.

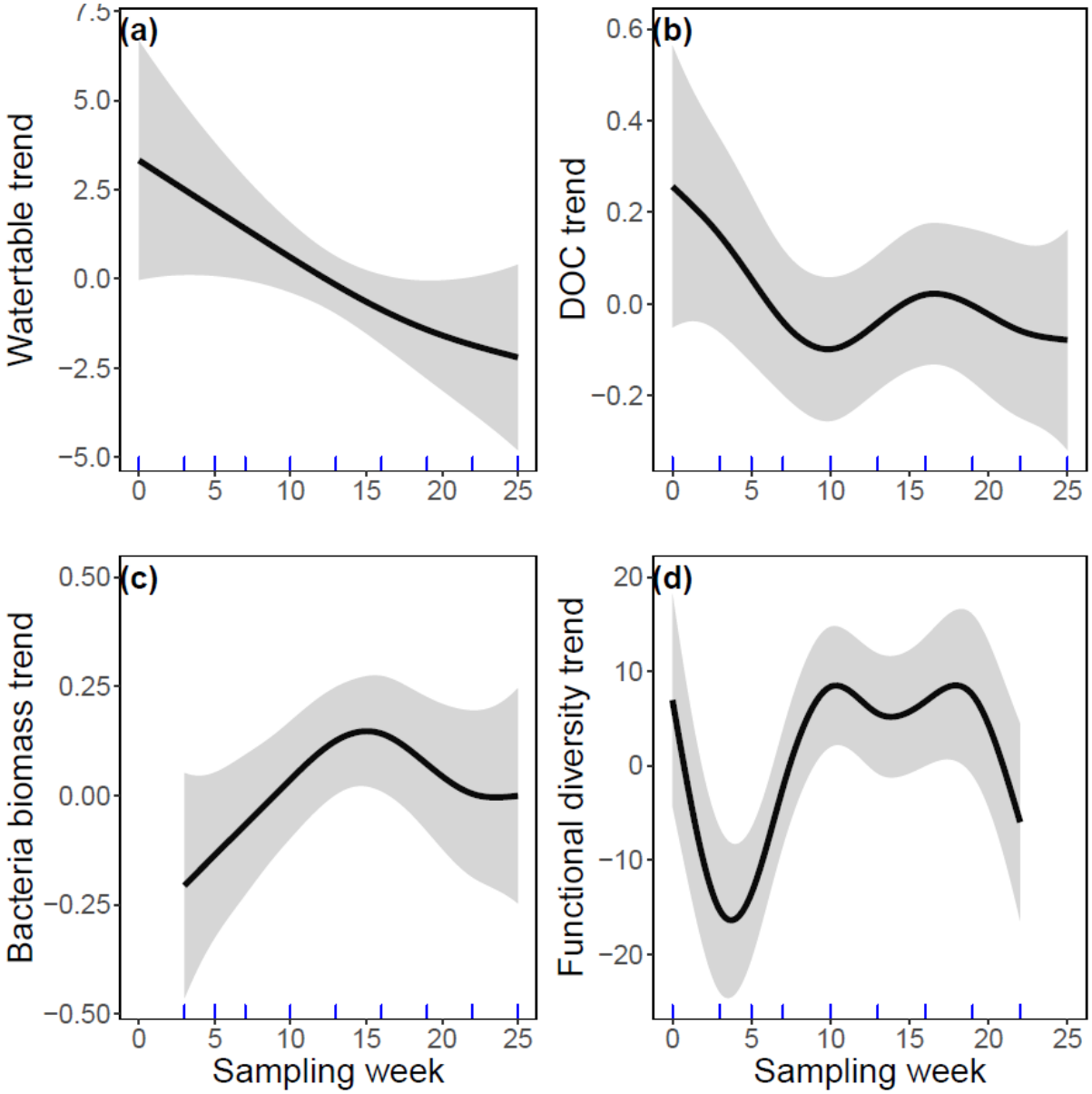

Fig. 2: Water levels (a), DOC dynamics (b), bacterial biomass (c) and bacterial functional richness (d) over a 25 weeks study period following the tail of groundwater flooding in a chalk aquifer (two locations [Berkshire and Dorset] and a total of 8 boreholes). The plots are the results of GLMM and the patterns over time are significant in all cases. Note that peaks and dips in DOC correspond to those observed for dissolved organic carbon and functional richness of bacteria.

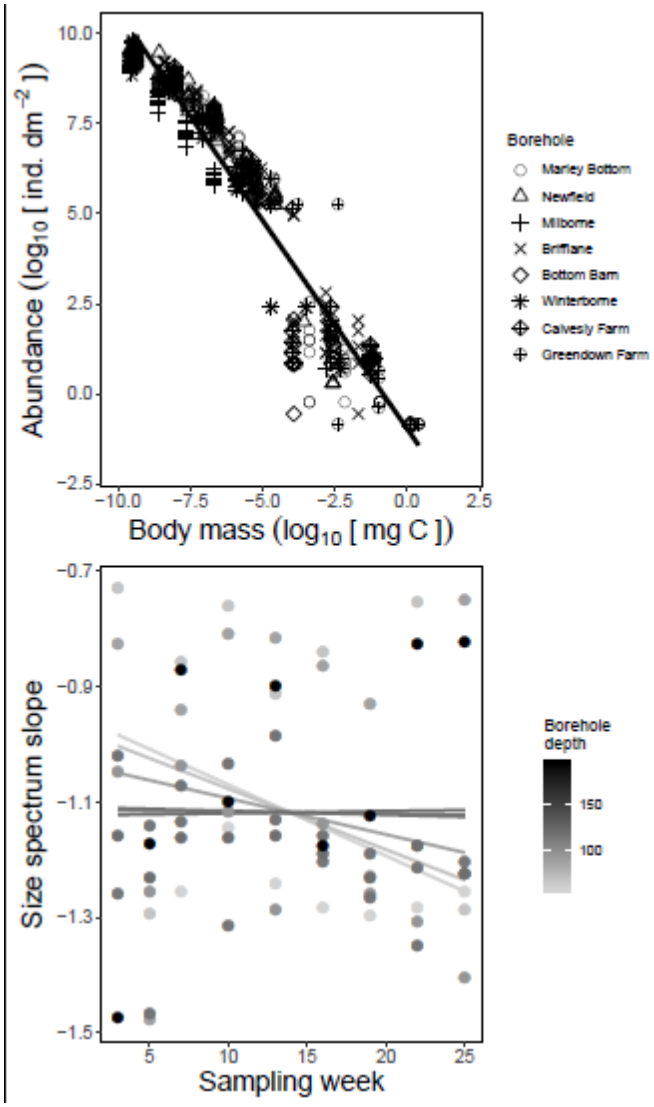

Fig. 3: Upper panel: Body mass and abundance scaling in groundwater communities. The average slope of the size spectrum, estimated from mixed-effects modelling, was - 1.12 (95% CI: -1.16 to -1.07) across boreholes and sampling occasions. Lower panel: Variation in size spectrum slopes in relation to sampling occasion and total borehole depth (here represented by shading of data points). The size spectrum slope steepened with each sampling occasion in the shallow boreholes (light grey data points).

Table 1

Overview of the biota found in eight boreholes showing sampling effort, body mass, number of individuals measured and the total of functional groups/taxa recorded.

Group	Total no of samples scanned	Min DWC in g	Max DWC in g	Total no of individuals measured and identified	Total functional groups/taxa
Open water bacteria	80	0.0000000001	0.000001	>4Mio	31 functional groups
Sediment bacteria	77	0.0000000001	0.000001	>4Mio	na
Protists	69	0.0000000428	0.000052	>2000	67
Meiofauna	77	0.0000321386	0.008484	>700	7
Macrofauna	77	0.0000681359	6.395189	>3000	9

Note: Across boreholes, this sampling resulted in 77 samples for open water bacteria (see S1), 69 sediment samples for protists, 77 samples for meiofauna and macrofauna (see S1); and 90 cotton strips were evaluated for fungal biomass (no ergosterol, i.e. fungal biomass, was detected). DWC= dry weight carbon.

Table 2

Summary output from generalised additive mixed modelling (GAMM) of open water parameters through time (sampling week).

Response	Smoother term	edf	F	p-value
Waterlevel	s(time, k = 6)	4.14	3.83	0.007
DOC (log10)	s(time, k = 6)	1.93	10.22	<.0001
Bacteria biomass (log10)	s(time, k = 6)	3.85	5.51	<.0001
Functional diversity	s(time, k = 6)	4.95	40.75	<.0001

Note: The results provide support for nonlinear temporal trends in response to flooding. Significant terms are highlighted in bold, k refers to the number of knots in the smoother term.

Table 3

Summary output from generalised least squares modelling (GLS) of size spectra parameters.

		Size-spectrum slope		Size-spectrum intercept	
Term	DF	F-value	p-value	F-value	p-value
Time	1,66	0.25	0.619	0.95	0.334
Depth	1,66	0	0.979	0.35	0.557
Time:Depth	1,66	4.98	**0.029**	2.89	0.094

Note: Significant terms are highlighted in bold.

APPENDIX A: APPENDICES TO THE METHODS

Table A.1: Overview of sampling locations, borehole characteristics and sampling effort for each borehole over a 25 week study period with 10 sampling occasions.

Location	Borehole name	Borehole diameter (dm)	Borehole area (dm2)	Borehole depth (m)	Waterlevel decrease during study period (m)	Baseline water level (m) average from 2004-2013	2014 Flooding levels (m)	(m) Flooding	Bacteria and macrofauna sampled on sampling occasion	Protists and meiofauna sampled on sampling occasion
Berkshire	Calversley Farm	3.05	7.31	120	75 to 68	70	75	7	1 to 10	2 to 10
Berkshire	Brifflane	3.05	7.31	95	65 to 62	62	65	3	1 to 10	2 to 10
Berkshire	Bottom Barn	3.05	7.31	114	93 to 87	87	97	10	1 to 10	2 to 10
Berkshire	Greendown Farm	3.05	7.31	197	155 to 152	153	155	3	1to 10	2 to 10
Dorset	Winterborne	0.50	0.20	116	113 to 103	108	116	10	1 to 10	2 to 10
Dorset	Newfield	0.80	0.50	66	58 to 55	56	66	10	1 to 10	2 to 10
Dorset	Milborne	0.50	0.20	87	70 to 68	68	76	8	1 to 10	2 to 10
Dorset	Marley Bottom	1.50	1.77	57	27 to 19	24	41	17	4 to 10	4 to 10

Table A.2: Overview of sampling occasion and sampling techniques for groundwater biota. The open water bailer sampled 270ml of water, the bottom bailer sampled 0.1134 dm^2 of sediment with biofilm and the net sampled the entire borehole area, which differed between boreholes.

Sampling occasion	Date	Week	DOC	Other chemistry	Open water bacteria	Sediment bacteria	Sediment protists	Sediment meiofauna	Sediment macrofauna
1	6/5/14	0	yes	yes	bailer	bottom bailer	na	na	net
2	28/5/14	3	yes	yes	bailer	bottom bailer	bottom bailer	net	net
3	10/6/14	5	yes	yes	bailer	bottom bailer	bottom bailer	net	net
4	24/6/14	7	yes	yes	bailer	bottom bailer	bottom bailer	net	net
5	15/7/14	10	yes	yes	bailer	bottom bailer	bottom bailer	net	net
6	5/8/14	13	yes	yes	bailer	bottom bailer	bottom bailer	net	net
7	26/8/14	16	yes	yes	bailer	bottom bailer	bottom bailer	net	net
8	16/9/14	19	yes	no	bailer	bottom bailer	bottom bailer	net	net
9	5/10/14	22	yes	no	bailer	bottom bailer	bottom bailer	net	net
10	28/10/14	25	yes	no	bailer	bottom bailer	bottom bailer	net	net

Table A.3: Overview of some borehole parameters: borehole depth, average oxygen saturation (in %, averaged for 10 sampling occasions for the top water layer, the medium water layer and the bottom of the borehole) and average abundance of invertebrates (macrofauna, meiofauna and protozoans).

Location	Borehole name	Borehole depth	O_2 top	O_2 middle	O_2 bottom	Macro-fauna	Meio-fauna	Protozoa
Berkshire	Bottom Barn	114	81	64	54	18	1	105049
	Brifflane	95	8	6	11		5	94964
	Calversley Farm	120	81	80	80	23	5	76183
	Greendown Farm	197	94	89	85	2		45498
Dorset	Marley Bottom	57	74	74	75	7	2	52376
	Milborne	87	81	82	79	5		60312
	Newfield	66	55	47	33	2	2	71422
	Winterborne	116	94	93	92	8	10	73009

Additional methods

Fungi

Cotton strips were frozen and used for the determination of fungal biomass via the ergosterol method (Gessner and Chauvet, 1993; Gonçalves et al., 2013). Because the ergosterol extraction did not give a positive result for fungi for any of the 90 samples tested, we do not report any results on fungi in the results section. It is possible that in these boreholes, no fungal mycelia were established on the cotton strips. Other studies have found fungi in groundwater when exposing autoclaved leaf litter (Chauvet et al., 2016).

Nutrients

On seven sampling occasions, we also measured dissolved organic nitrogen (DON), nitrate (NO_3^-), chlorine (Cl), sodium (Na^+), Potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}) and sulphate (SO_4). These data are not reported here.

Microbial Metabolic Activity

On the second sampling occasion only, we took samples to characterise the metabolic activity in the boreholes (except for MB) and relate these measurements to DOC levels at that time. Microbial metabolic activity and ecosystem respiration was measured by applying the "smart tracer" Resazuring/Resorufin (Raz/Rru) system that has been first used in ecohydrological applications by (Haggerty et al., 2008). It has since been applied in reach-scale field experiments, flume-scale (Haggerty et al., 2014) and been introduced for microcosm incubation studies by (Baranov et al., 2016). Raz, a weakly-fluorescent substance transforms under mildly reducing conditions irreversibly to the highly-fluorescent Rru, thus,

representing an indicator of microbial metabolic activity and being correlated to aerobic respiration (Haggerty et al., 2008).

Raz to Rru conversion was measured in this study by fluorometry using excitation and emission wavelengths of 570 nm and 585 nm, respectively. Therefore, groundwater samples collected between 28 May 2014 and 30 May 2014 were cooled and transferred to the laboratory without filtering to preserve the microbial community that could be present. For incubations, 250 ml of sampled groundwater was incubated in 1000 ml microcosms with a concentration of 100 µg/l Raz for 24 hrs. Each sample was incubated in duplicate. Two microcosms were prepared with only deionised water to act as control treatments. Incubations started on 9 June 2014.

The Raz to Rru conversion ratio was quantified at t=0, 2, 4, 6, 8 and 24 hours of incubation by measuring both compounds for filtered samples using Albilla GGUN-FL 30 (Albillia, Neuchatel, CH) bench top fluorimeters (Baranov et al., 2016; Lemke et al., 2013). Samples were discarded after measurement. Microcosms were incubated in an incubation fridge, shielded from light, at 12±0.12°C. Temperature was monitored using a Tinytag Aquatic 2 temperature logger (Gemini Data Loggers Ltd, Chichester, United Kingdom). The raz-to-rru conversion rate was calculated as:

$$respiration_t = ln\left(\frac{rru_t}{raz_t} + C\right) \qquad \text{Eq. 1}$$

with $C = 1$, which is a constant value for amount of tracer recovered. Respiration at time t was calculated for each time step, through which a simple linear regression line was fitted. The slope of this line was then compared between all samples, where a higher value correlates with higher microbial metabolic activity.

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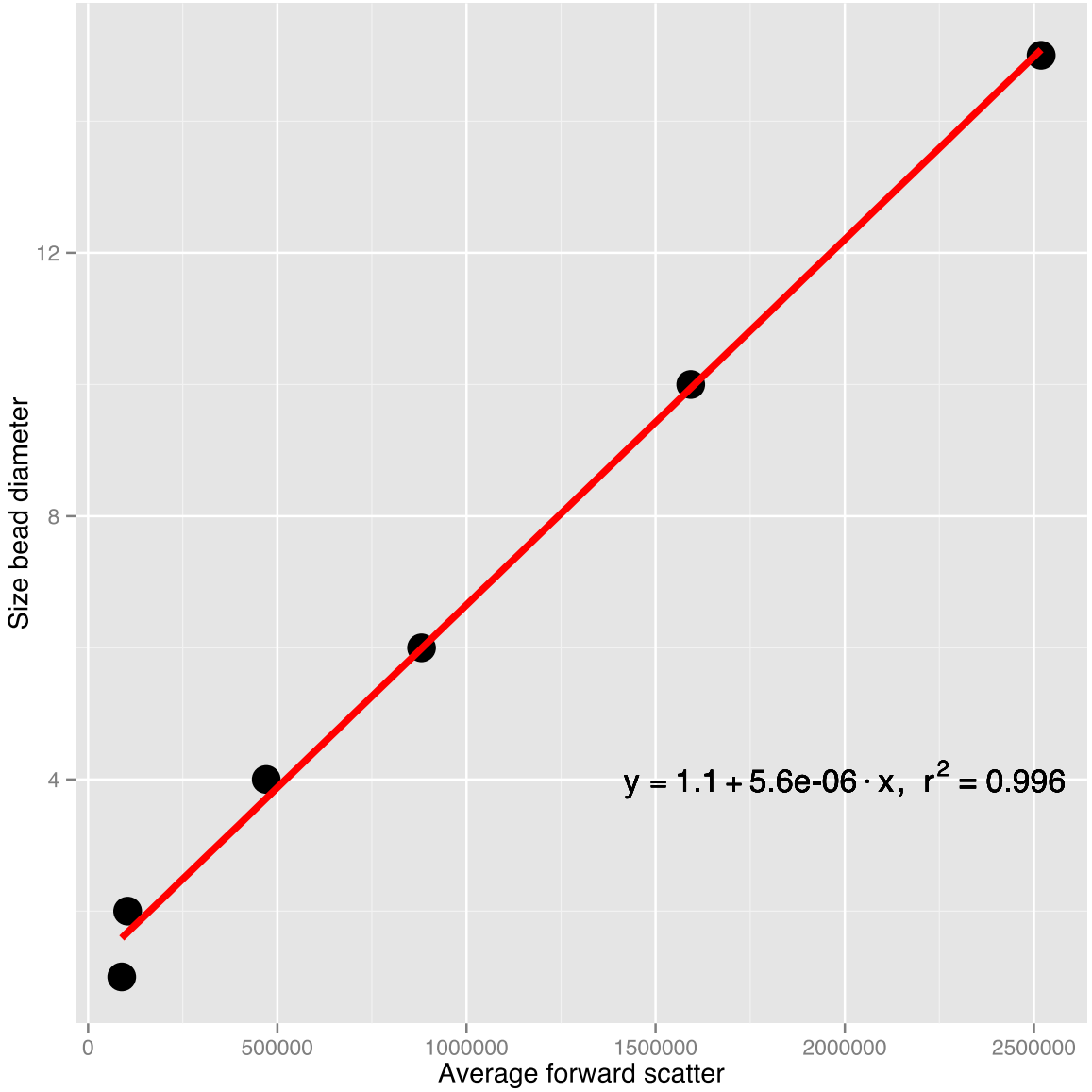

Fig. A.1: Cell size calibration from flow cytometry data. Cell size (µm) was estimated from the relationship between calibration beads of known size and forward scatter values returned by flow cytometer.

APPENDIX B: APPENDICES TO THE RESULTS

Table B.1: Output of GAMM modelling testing for the significance of the temporal autocorrelation term. The significance of the temporal autocorrelation term was determined by comparing nested models with (M_{cor}) and without the autocorrelation term (M) via a likelihood ratio test. The value ρ is generally high and indicates that residuals separated by one week have a correlation of >0.70.

Response	Term	ρ	F	p-value
Waterlevel	Anova (M_{cor}, M)	1.00	425.51	<.0001
DOC (log_{10})	Anova (M_{cor}, M)	0.74	51.62	<.0001
Bacteria biomass (log_{10})	Anova (M_{cor}, M)	0.95	1451.06	<.0001
Functional diversity	Anova (M_{cor}, M)	0.93	1282.06	<.0001

Table B.2: List of taxa found within the macrofauna, meiofauna and protozoans. The number after the taxon name indicates its relative abundance within that group. The most frequent and abundant taxa are in bold.

Macrofauna		
1	Crangonyx subterraneus	1.76
2	Microniphargus leruthi	1.95
3	Niphargus fontanus	0.72
4	Niphargus glenniei	0.03
5	**Niphargus kochianus**	**91.43**
6	Niphargus spec.	2.23
7	Planaria	0.03
8	Proasellus cavaticus	1.82
9	Turbellarian	0.03
Metazoan meiofauna		
1	**Copepoda**	**94.25**
2	Nematoda	2.24
3	Paracyclops	0.14
4	Rotifera	0.70
5	Stenostomum sp.	0.70
6	Testudinella sp.	0.14
7	Turbellaria	0.42
Protists		
1	Amoeba	0.04
2	Amphileptus sp.	1.23
3	Arcella sp.	0.51
4	Aspidisca sp.	0.47
5	Astylozoon sp.	0.25
6	Bursaria sp.	0.34
7	Chanea stricta	3.61
8	Chilodonella sp.	1.19
9	Chilondontopsis depressa	0.04
10	Ciliate noID 1	0.08
11	Ciliate noID 2	0.13
12	Colpoda sp.	0.89
13	Enchelymorpha vermicularis	0.08
14	Euglena sp.	0.25
15	Euglypha sp.	0.04
16	Euplotes sp.	0.04
17	Euplotes moebiusi	0.04
18	Flagellata	0.38
19	Flagellate colony	0.08
20	Frontonia sp.	0.08
21	Glaucoma sp.	0.42
22	Glaucoma scintillans	1.87
23	Gymnostom	0.04
24	Heliozoan	0.21

Protists continued		
25	Holophrya sp.	0.04
26	Holosticha sp.	1.27
27	Hymenostomata	0.04
28	Hypotrich	0.63
29	Kahlilembus attenuatus	0.08
30	Lacrymaria sp.	0.21
31	Linostoma vorticella	0.08
32	Litonotus sp.	0.85
33	Loxodes sp.	0.04
34	Metopus sp.	0.08
35	Nassula sp.	0.08
36	Oligotrich ciliate	0.17
37	Oxytricha sp.	0.08
38	Parameciumsp.	3.10
39	Parapodophyra conf	0.04
40	Peritrich cilaite	0.04
41	Phascolodon sp.	0.04
42	Phialina sp.	0.89
43	Placus luciae	0.04
44	Protist species	0.04
45	Pseudochilodonopsis sp.	0.04
46	Pseudomicrothorax sp.	0.13
47	round flagellate	0.08
48	small ciliate	4.29
49	small ciliate noID	0.64
50	small ciliatenoID	0.47
51	small ciliatenoIDBL1	1.36
52	small flagellate	0.42
53	small flagellate noID	0.13
54	Strombidium sp.	0.42
55	Stylonychia sp.	0.04
56	Suctoria swarmer	1.23
57	Tachysoma sp.	0.72
58	Tetrahymena sp.	1.36
59	thecamoeba	0.21
60	Thigmogaster sp.	0.04
61	**tiny ciliate**	**23.02**
62	**tiny flagellate**	**27.49**
63	Trochilia sp.	1.61
64	Uronema sp.	0.17
65	Urotricha sp.	1.06
66	**Vampyrella sp.**	**14.66**
67	Vorticella sp.	0.13

Table B.3: Statistics for sediment biota and time. Summary output from generalised least squares modelling (GLS) of organism groups sampled from sediments.

	Bacteria			Protists			Meiofauna			Macrofauna		
Term	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value
Time	1,66	0.46	0.501	1,64	1.91	0.172	1,68	0.37	0.547	1,68	0.43	0.515
Depth	1,66	0.11	0.745	1,64	2.89	0.094	1,68	0.06	0.805	1,68	0.01	0.912
Time:Depth	1,66	0.71	0.402	1,64	1.16	0.286	1,68	0.04	0.838	1,68	0.40	0.531

Table B.4: Statistics for sediment biota and time. Summary output from generalised additive mixed modelling (GAMM) of abundances of organism groups sampled from borehole sediments through time (sampling week). No significant patterns were evident highlighting system specific responses to recovery from ground water flooding. k refers to the number of knots in the smoother term.

Response	Smoother term	edf	F	p-value
Bacteria	s(time, k = 6)	1.00	0.46	0.500
Protists	s(time, k = 6)	1.00	1.85	0.178
Meiofauna	s(time, k = 6)	1.00	0.50	0.48
Macrofauna	s(time, k = 6)	1.00	0.51	0.477

Fig. B.1: Variation in (a) waterlevel (b) DOC (c) bacterial biomass and (d) bacterial functional richness with sampling week for each borehole. Smoother lines are added to aid the visualisation of the data only.

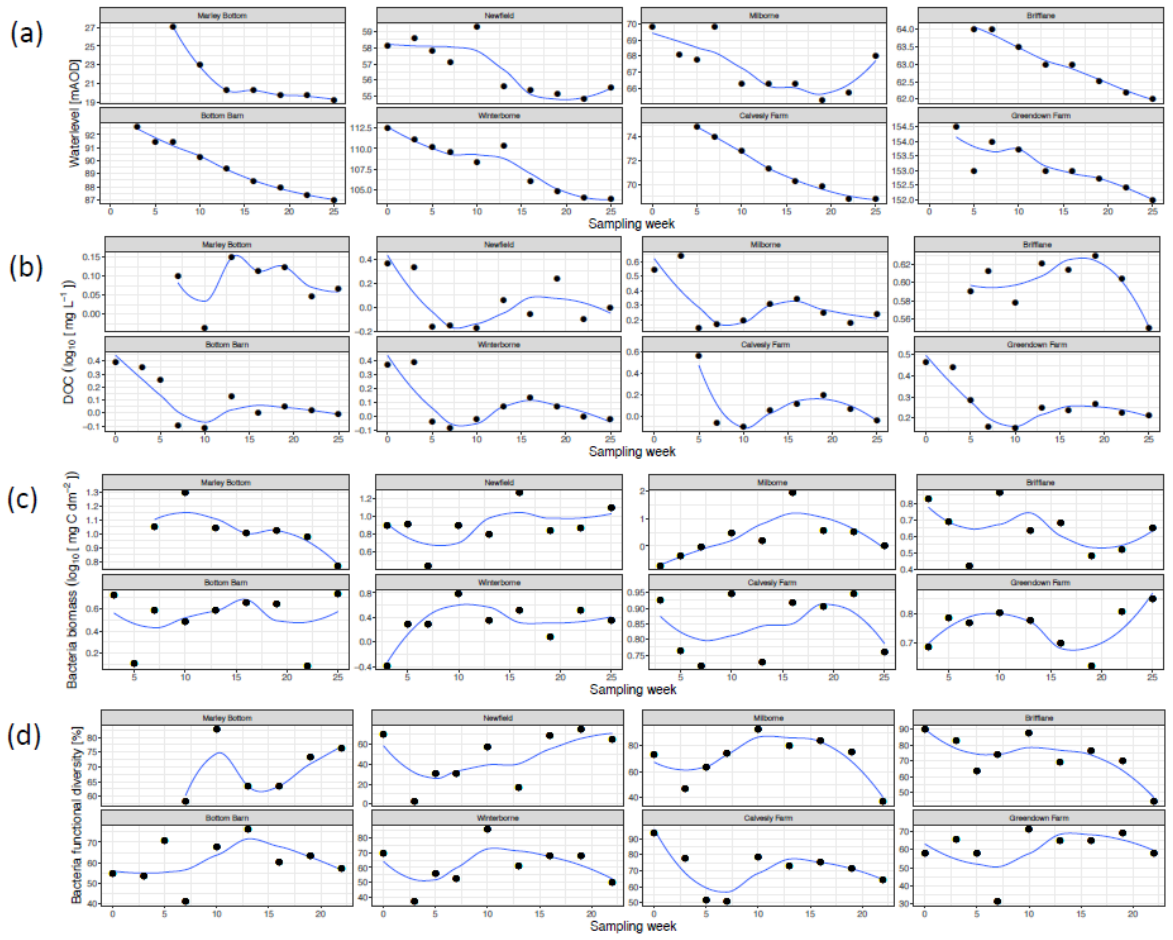

Fig. B.2: Relationship between DOC and microbial metabolic activity (top) and between nitrate and microbial metabolic activity (bottom) for all analysed boreholes

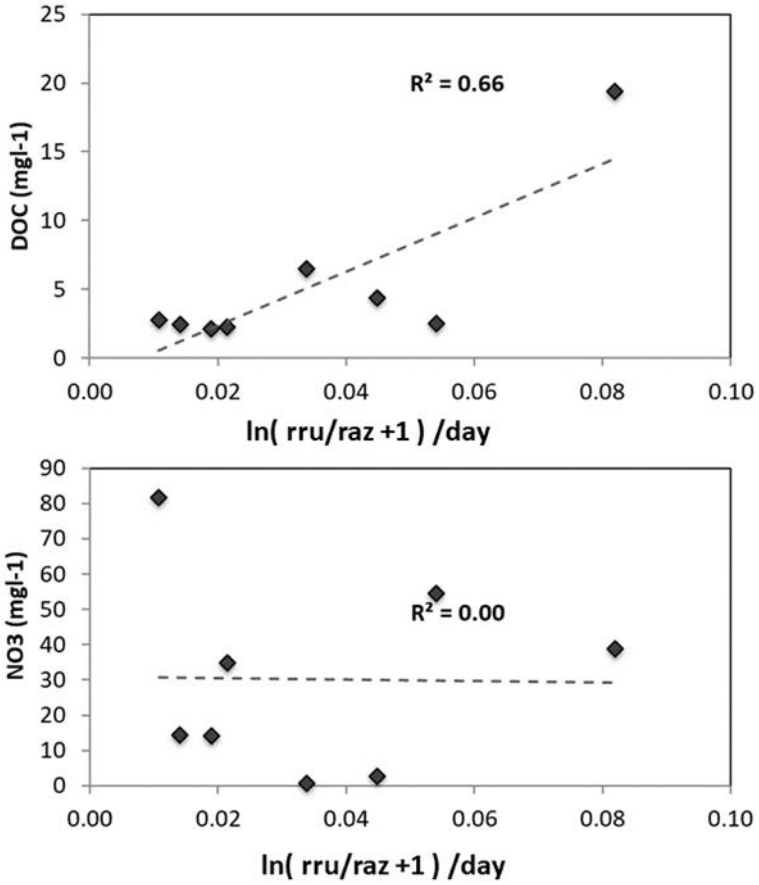

Fig. B.3: Variation in the abundance of (a) bacteria (b) protists (c) meiofauna and (d) macrofauna with sampling week for each borehole. Smoother lines are added to aid the visualisation of the data only.

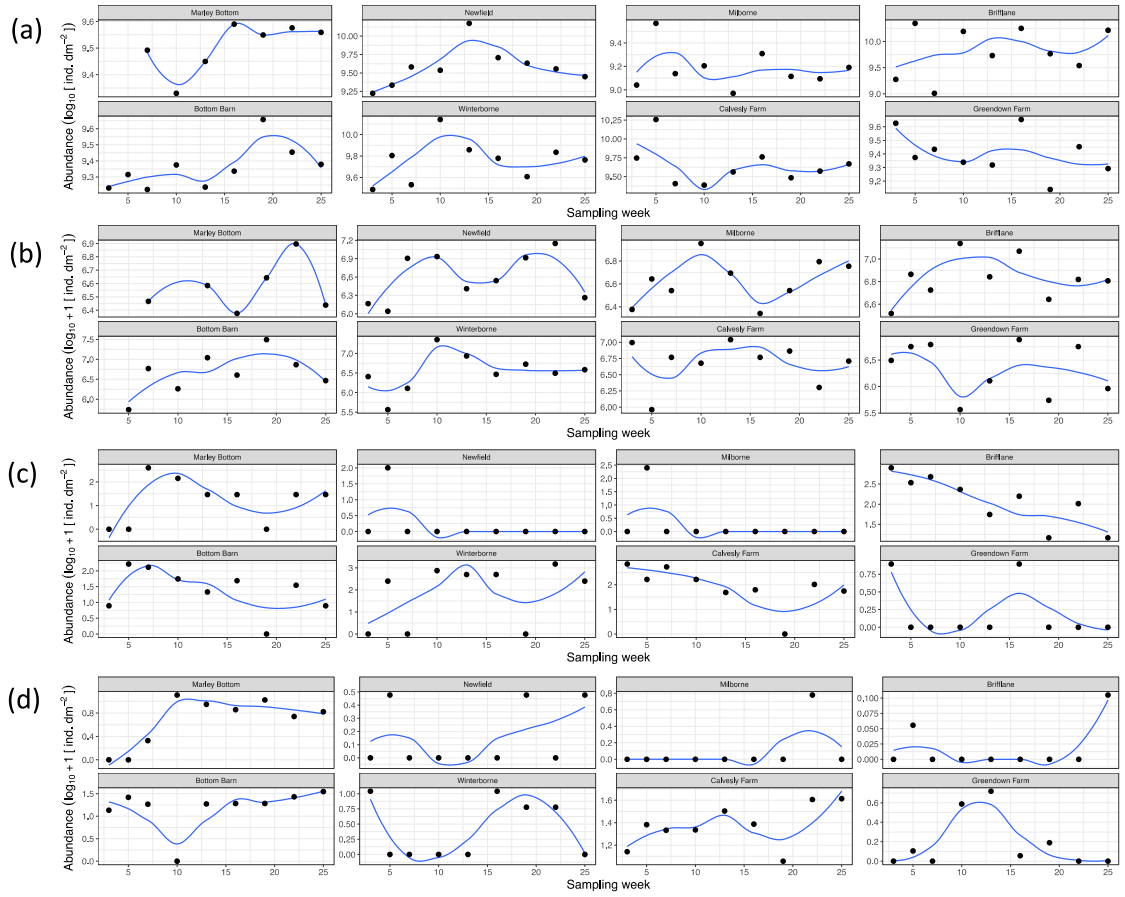

Figure B.4: Body mass - abundance (M-N) relationships constructed for each borehole and sampling occasion. Each data points represent the total abundance of organisms within that (logarithmic) body mass class.

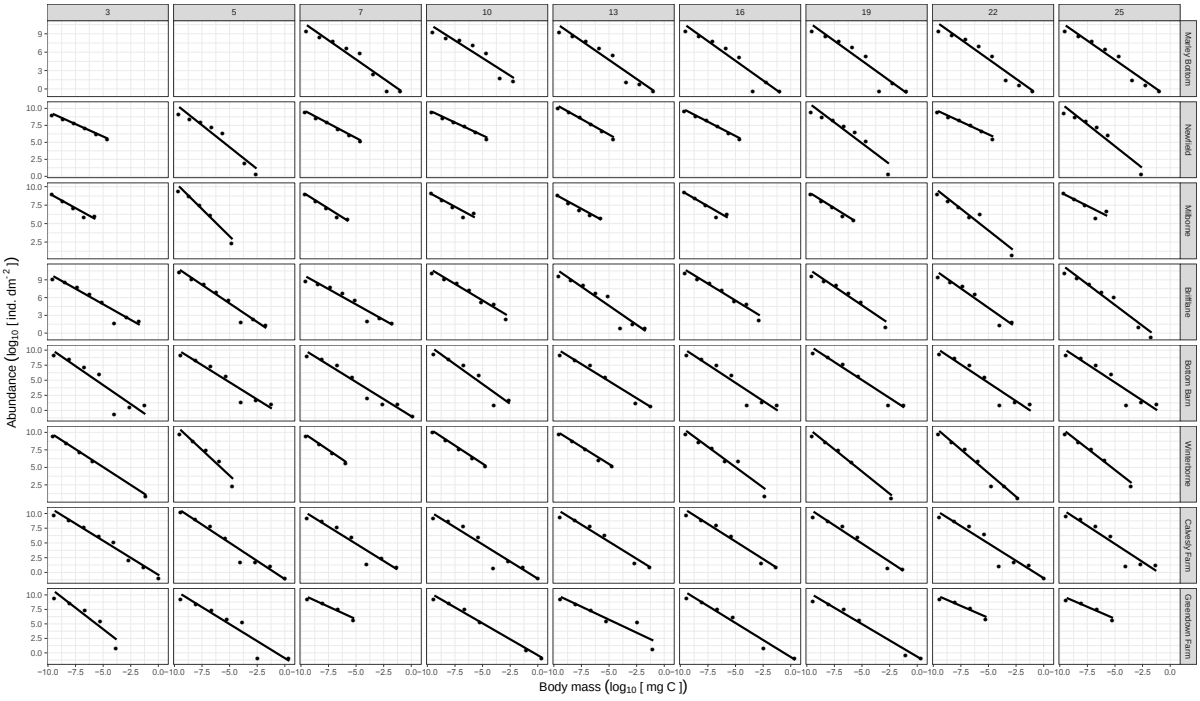

Fig. B.5: Variation in (a) slope and (b) intercept of size spectra relationships with sampling week for each borehole. Smoother lines are added to aid the visualisation of the data only.