Impacts of flow alteration on stream ecosystems

Raquel Arias Font

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ABSTRACT

Worldwide, more than two-thirds of rivers are affected by channelization, damming, hydropower and/or water abstraction creating highly modified river flow regimes that potentially have profound impacts on their ecological integrity. Climate change is expected to further exacerbate these effects as extreme events, such as drought, increases in frequency and duration. Our knowledge of flow-ecology relationships is increasing but our current understanding of flow regulation is often confounded by additional stressors generated by dams, regional factors and human activities and thus experiments are required to disentangle these drivers. The research presented in the thesis used manipulative experiments in mesocosms mimicking headwater streams to investigate how flow regulation generated by irrigation (reversal of flow seasonality), flow homogenisation (no flow variability) and drought (reduced flow magnitude and dewatering) affect stream ecosystem structure and functioning (water quality, biofilm and macroinvertebrates). The different flow regimes had markedly different effects on benthic communities and their function. Flow homogenisation generated a stable and persistent flow with ecosystem functioning similar to the natural control, but promoted the occurrence of lentic macroinvertebrate communities. By contrast, reversal of the timing of high and low flows (flow 'reversal') increased stochasticity in biofilm growth, which generated two alternative states, and altered the taxonomic and functional trait composition of the macroinvertebrate assemblage. Specifically promoting survival strategies such as drift resistance, promoting big body size and reducing macroinvertebrates abundances. Drought reduced the functioning of the biofilm by constraining its growth and processing rates, but resilience and resistant strategies were present in the biofilm (temporal adaptation to wet and dry phases) and benthic macroinvertebrate assemblages (resource partitioning and persistence on refugia). A second mesocosm experiment investigated how biofilm functioning in streams was modified as a consequence of future climate change (low flow and heatwaves). Findings revealed that under low flow conditions, heatwaves increased heterotrophic processes more than autotrophic ones, with decomposition rates of recalcitrant fraction especially affected. New environmental flow strategies to mitigate the effects of flow regulation on stream ecosystems are need to preserve freshwater biodiversity and ecosystem services. Further research is required to identify the impacts on higher trophic levels and meta-community dynamics; and the potential for interactive effects with additional stressors.

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Y si no lo sé, me lo invento

[usando la lógica y todo el conocimiento a mi alcance]

And if I do not know it, I will make it up

[using logic and all the knowledge I possess]

-Childhood sentence-

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and categorized following Cohen (1988). All p-values were calculated using Chi-squared likelihood ratio tests and were adjusted to control for type 1 errors (see methods). Bold text indicates statistically significant results and underlined text, biologically relevant results (i.e. d category >trivial and p<0.20). 207

Table 4.4. Results of the three-way ANOVA to test the effect of the three sequential heatwaves events on metabolism dynamics. For each of the metabolism measurements (GPP, ER, NEP), 8-days cumulative values for each heatwave-flume were calculated and the effect of the heatwaves treatment (HW), low flow treatment (LF) and the interaction of the two (HW&LF) was analysed. To assess changes of this effects between heatwave events, we assess the effects of heatwave event (HWE) and its interactions with the treatments (HW:HWE, LF:HWE, HW:LF:HWE). Standardized effect size (Cohen's f) is presented for each factor and categorized following Cohen (1988), describing the proportion of the variability accounted for each treatment. All p-values were calculated using Chi-squared likelihood ratio tests and were adjusted to control for type 1 errors (see methods). Bold text indicates statistically significant results and underlined text, biologically relevant results (i.e. d category >trivial and p<0.20). 210

1. Introduction

1.1. Introduction

1.1.1. The natural flow regime: a key concept in river ecology

Rivers, freshwater bodies and deltas are among the most biodiverse ecosystems on Earth, but also they are among the most threatened (Dudgeon *et al.*, 2006; Reid *et al.*, 2019). Flow regimes (i.e. long term patterns including inter and intra annual flow variability) are essential to determine integrity of those ecosystems (Poff *et al.*, 1997; Olden and Naiman, 2010). Poff *et al.* (1997) characterised the main components of rivers flow regimes: magnitude, frequency, duration, timing (or predictability), and rate of change (or flashiness). These five components describe the characteristic patterns of river flow quantity, timing and variability, and are essential for determining water quality, energy sources (i.e. resources), physical habitat and controlling biotic interactions (Poff *et al.*, 1997). Furthermore, these components are pivotal for shaping species life-story strategies and assessing community responses to flow events (Poff and Ward, 1989; Poff, 2018; Palmer and Ruhi, 2019). Bunn and Arthington (2002) stressed the importance of the natural flow regime in determining the integrity of freshwater ecosystems and generated four principles that govern biodiversity patterns and ecosystem responses (Fig. 1.1):

(1) High flows are essential for physical habitat creation and variability;
(2) Extreme events (flood and drought) are key in shaping species traits by selecting traits able to cope with the given flow extremes (functional trait selection and evolution);
(3) High flows promote lateral and longitudinal connectivity; and
(4) As species are adapted to the local flow regime, species invasions are naturally resisted.

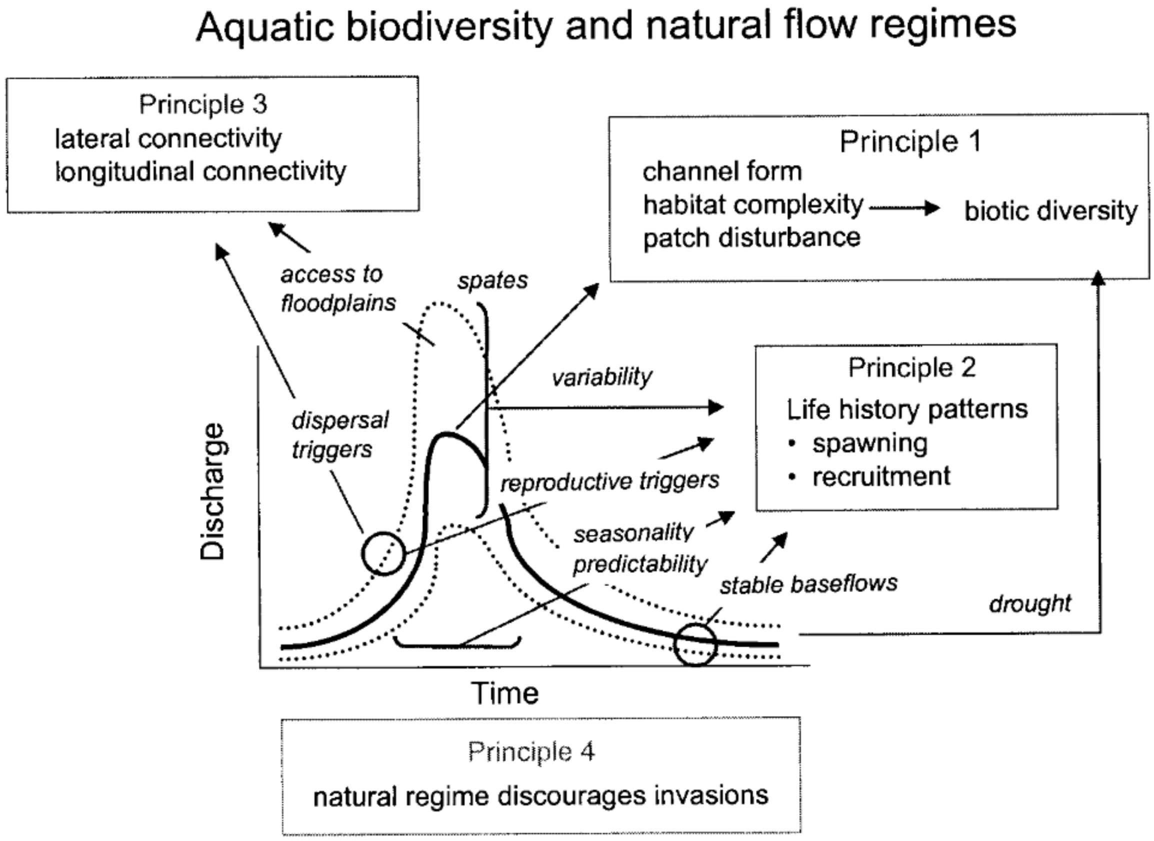


Fig. 1.1. Influences of the natural flow regime on river biota. The four principles underpinning flow-ecology relationships (after Bunn & Arthington, 2002) are: high flows are the major determinant of physical habitat creation and variability (principle 1); species life-history traits have adapted to cope with the extreme events (floods and drought) (principle 2); high flows promote lateral and longitudinal connectivity (principle 3); and adaptations to the local flow regime discourage species invasion (principle 4). The figure shows a simplified hydrograph with the black line representing mean discharge and the dashed lines scenarios of interannual variability (i.e. dry year vs wet year).

The natural flow regime is typically characterised at sub-catchment/river level and depends on the climate (i.e. snowfall, snow melting, rainfall and temperature), groundwater influences (i.e. water table depth and aquifer persistence) and geology/topography of the catchment (i.e. slope, ground permeability, soil and landcover)(Poff *et al.*, 1997). These characteristics determine the run-off and the groundwater inputs in the river, which together yield discharge and generate variability spatially (within river reach and between sub-catchments) and temporally (inter- and intra-annual variability). This sub-catchment variability generates a regional scale variability in flow regimes. In turn, this has important implications for biodiversity and community persistence, as it maintains a diverse pool of functional strategies at the regional level with enhanced recolonization potential following disturbance (i.e., regional-scale dynamic equilibrium, Deangelis and Waterhouse, 1987).

1.1.2. Rivers in the Anthropocene

Historically, rivers played an eminent role in the success of ancient cultures. Many proliferated around river valleys and their deltas, for instance, the Mesopotamia civilization grew around the Tigris and Euphrates, Egypt developed along the Nile, the Indus civilization grew in the alluvial plain of the Indus river and along the Ghaggar-Hakra river; and the Yangtze and Huanghe civilizations expanded along the Yangtze and the yellow river, respectively (Bianchi, 2016). In the wake of these civilizations, irrigation from rivers was essential to early agricultural development, as was the accessibility of transportation of people and goods along the course of rivers (Solomon, 2010). Fulfilling these needs promptly led to the modification and control of rivers with a view to increase productivity and efficiency of crop production and transportation (Ripl, 2003). Thus, for millennia, the exploitation of water resources has shaped the fate of human societies just as the fate of human societies has shaped our river courses and deltas.

Worldwide, over 500,000 km of canals, and more than 2.8 million dams and water diversion schemes, have been designed and built to provide water supply, irrigation, flood control, navigation and power generation for human use (Grill *et al.*, 2019). Currently, less than a quarter of rivers worldwide flow uninterrupted to the sea (Grill *et al.*, 2019, Fig. 1.2). These modifications affect all aspects of river connectivity: longitudinal (from the source to the river mouth), lateral (connection to floodplains and riparian areas), vertical (connection to the hyporheic zone and groundwater) and temporal (seasonal dynamics) (Vannote *et al.*, 1980; Ward and Stanford, 1995; Grill *et al.*, 2019). River fragmentation and flow regulation are the dominant alterations affecting connectivity worldwide, (Fig. 1.3) and are often interlinked and caused by impoundments, over abstraction and/or poor water management (i.e. flow regulation). The global extent of dams is thus already extensive, and expansion continues unabated, fed by a rising societal concern about water scarcity in the future (Solomon, 2010; Zarfl *et al.*, 2015; Hermoso, 2017).

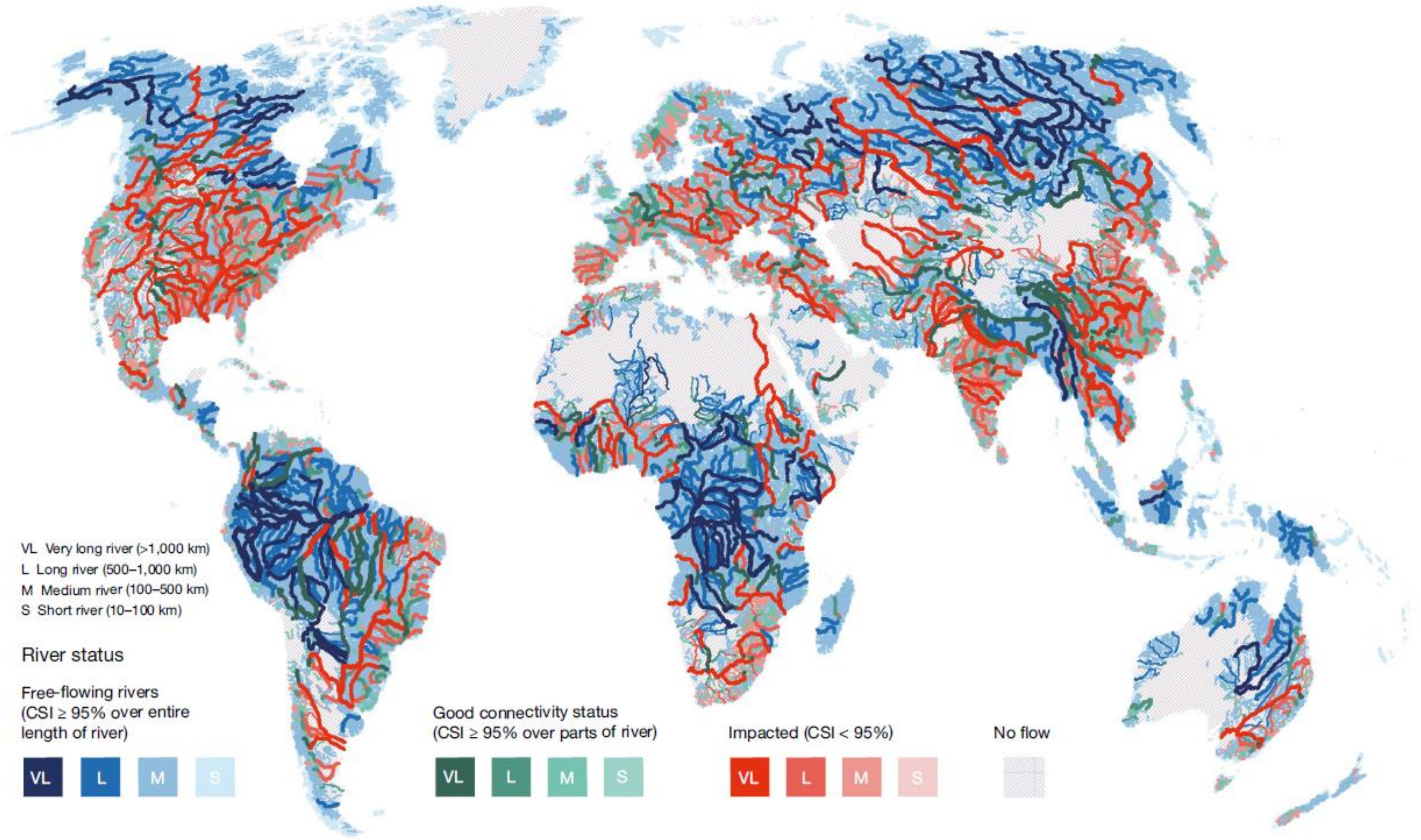


Fig. 1.2. Map of the distribution of free-flowing rivers (blue) and those impacted to a lesser (green) or greater (red) extent by disconnectivity. The connectivity status index (CSI) is defined by the authors as the spatial extent of stressors affecting any of the components of river connectivity (longitudinal, lateral, vertical and temporal). Drivers of disconnectivity include river fragmentation, flow regulation, sediment trapping, water consumption and infrastructure development in riparian areas and flood plains. Source: Grill *et al.*, 2019.

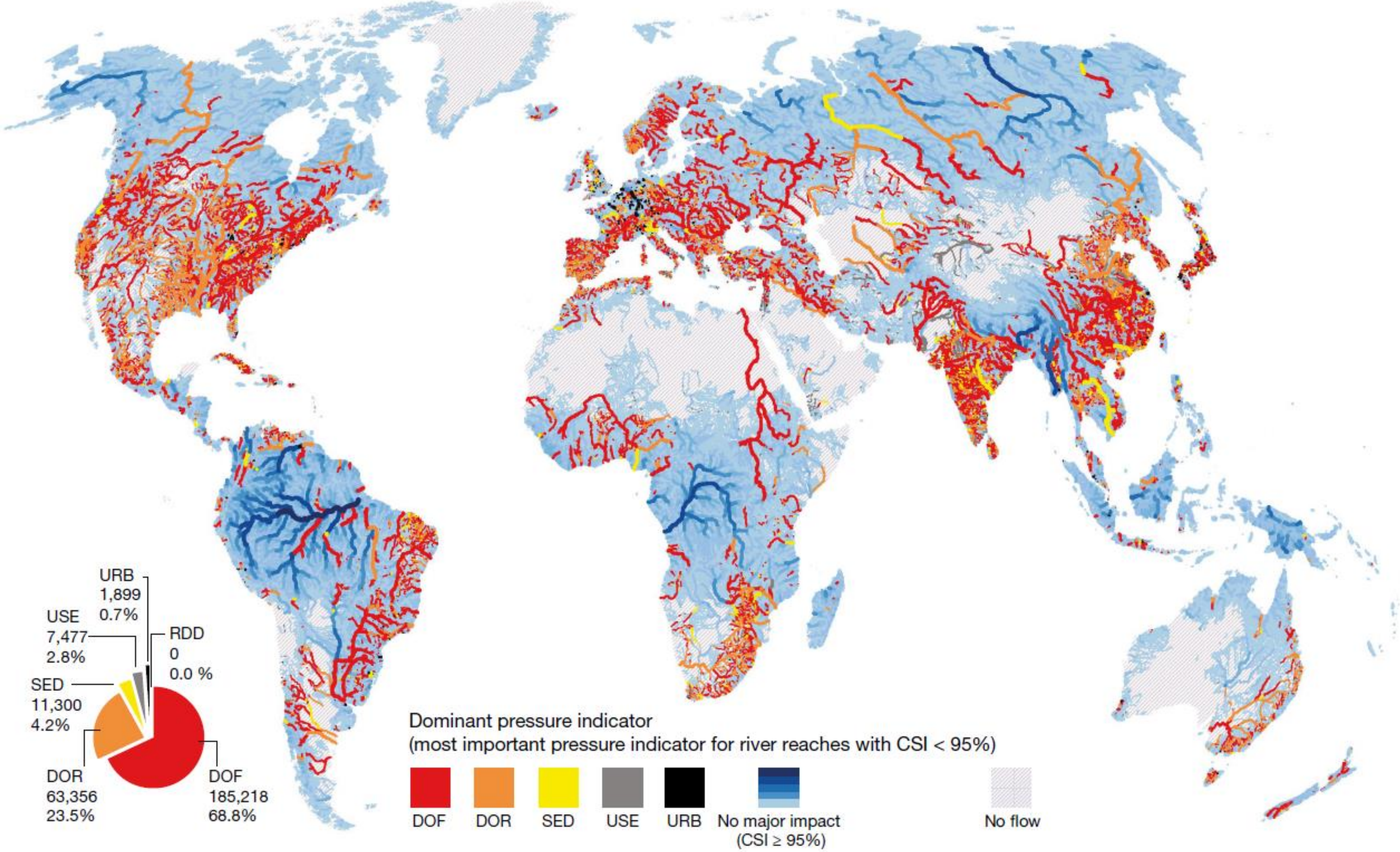


Fig. 1.3. Dominant pressure indicators for non-free flowing rivers worldwide. These are: degree of fragmentations (DOF, red), degree of flow regulation (DOR, orange), sediment trapping (SED, yellow), water consumption (USE, grey), urbanization and construction on the riparian areas (URB, black) and no major impact (shades of blue). Source: Grill *et al.*, 2019

1.1.3. Dams

Dams not only affect river connectivity but also modify the physicochemical quality of water downstream (temperature, nutrient inputs and sediments). How conditions change downstream depends on whether water is released from the hypolimnion or epilimnion and the residence time of the dam (McManamay *et al.*, 2013; Poff, 2018). Due to reservoir stratification, hypolimnetic releases generate colder water downstream with higher nutrient loads and lower oxygen concentration, while epilimnetic releases generate warmer conditions downstream (Olden and Naiman, 2010; Salmi, Malin and Salonen, 2014). Dam residence time determines the propensity for nutrients to be buried in reservoirs behind the dam as well as the amount of POC and DOC reaching downstream (Maavara *et al.*, 2015, 2017). Sediment depletion and changes in sediment size are also common below dams (Yarnell *et al.*, 2015; Poff, 2018). The overall effect of damming on water quality can vary from site to site and regionally depending on factors such as climate, land use and reservoir size (Maavara *et al.*, 2017). Furthermore, dams tend to enhance hydrological stability downstream, reducing streambed mobility and geomorphological activity (Poff *et al.*, 2007; Ponsatí *et al.*, 2015; Lobera *et al.*, 2017). While it is widely recognised that the effects of dams go beyond river fragmentation and flow regulation, flow still plays an essential role and rehabilitation of the other factors alone, such as increased habitat complexity (meandering, logs and boulders introduction) or improvement of water quality, have rarely provided any ecological improvement (Standish *et al.*, 2014; White *et al.*, 2017; Bauer *et al.*, 2018).

1.1.4. Flow regulation by dams

River impoundments have led to regional and temporal homogenisation of flow dynamics, leading to a simplification and deterioration of riverine ecosystems (Poff, 2018; Ruhi *et al.*, 2018). Historically, dam management has been based on water quality and minimum flow maintenance, completely neglecting the temporal and spatial dynamism of river systems (Poff *et al.*, 1997). Dam baseline releases (i.e. flow when there is no water demand) are often based on traditionally reference-based approaches such as a proscribed percentage of mean annual unimpacted flow (Tennant, 1976) or monthly mean flow (Richter *et al.*, 2012). Such management reduces temporal variability (intra- and inter-annual variability) and often decreases the magnitude of stream flows (at least for wet months). While the exact nature of flow alteration in any given locale depends the purpose of the impoundment, the magnitude of high and low flows are almost always modified — typically resulting in lower high flows and higher low flows (Poff *et al.*, 2007). Modification of the timing of high and low flows is commonly observed to cater to specific water supply and/or irrigation requirements (Poff *et al.*, 2007). These flow alterations by dams can be further exacerbated downstream as water is abstracted along its course, further reducing stream flow (Acreman *et al.*, 2008). Thus, ultimately, a wide range of flow regimes can be generated downstream of dams, depending on the specific role of the structure, with contingent effects on the abiotic and biotic components.

Flood protection dams often create hydrologically stable flow regimes characterized by an overall reduced discharge with no annual variation; such changes to flow regimes are known as ‘flow homogenisation’ (Fig. 1.4) (Ebrahimnezhad and Harper, 1997; Poff *et al.*, 2007). Homogenisation often results in a system with low sheer stress and little or no bed mobilization (Sabater *et al.*, 2008; Ponsatí *et al.*, 2015; Lobera *et al.*, 2017; Shangguan *et al.*, 2017), consistent with the discharge and abiotic conditions typical of higher stream orders. Similar hydrological conditions are found in some navigation canals which are controlled by weirs and variability and discharge maintained at minimum (Ebrahimnezhad and Harper, 1997).

In dams used for irrigation, the degree of the flow alteration downstream depends on the climate and the water resource requirements associated with downstream users. Often, these types of dams generate high summer flows to ensure agricultural demand and low winter flows as water is being used to fill reservoirs (Fig. 1.5, Poff *et al.*, 2007). These dam uses not only affect the timing of flows, but often alter the magnitude of low and high flows. Similarly to water supply dams, high flows are reduced in terms of magnitude and frequency and low flows are increased (Ponsatí *et al.*, 2015). The lack of low flows is especially relevant for arid regions and snow and ice dependant systems (Sabater and Tockner, 2009; Costigan *et al.*, 2017). These conditions generate a flow regime unsynchronised with species life-cycles (Poff *et al.*, 2010; Acreman *et al.*, 2014), climatic factors (i.e. light, temperature) and catchment dynamics (i.e. remobilization of nutrients and DOC during rain-fall season) (Bowes, Leach and House, 2005).

Reduction of flow magnitude generated by dams (Fig. 1.5) can cause, or exacerbate the impact of, drought downstream, with streams often becoming intermittent in their driest months (Boix *et al.*, 2010; Ledger and Milner, 2015; Van Loon, 2015). Flow intermittency and dewatering events reduce river wetted area and increase temperature during the hottest months. Predictability of these events determines whether species are adapted to the hydrological instability generated by drought conditions and its associated water quality changes (Lytle and Poff, 2004; Cid *et al.*, 2017) and determine ecosystem resilience, resistance and recovery. Given climate change and anthropogenic pressure, the magnitude, frequency and duration of these events is increasing globally and river communities and ecosystems are unable to adapt at the required rates (Ledger and Milner, 2015; Woodward *et al.*, 2016).

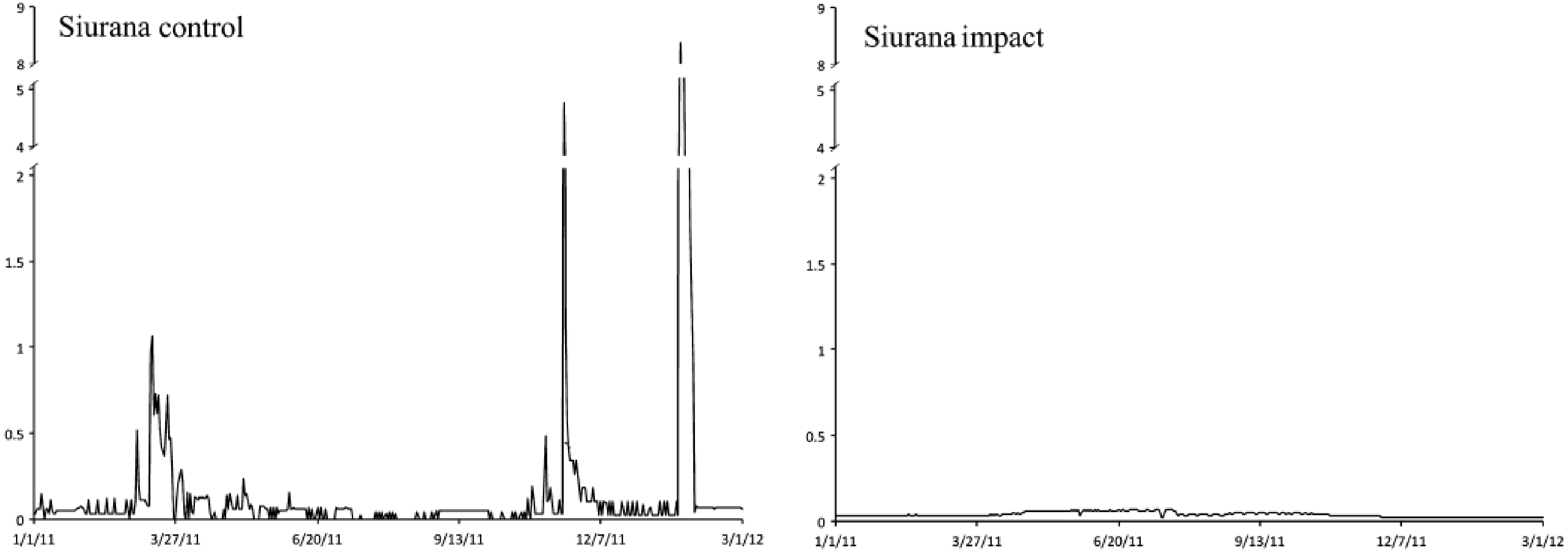
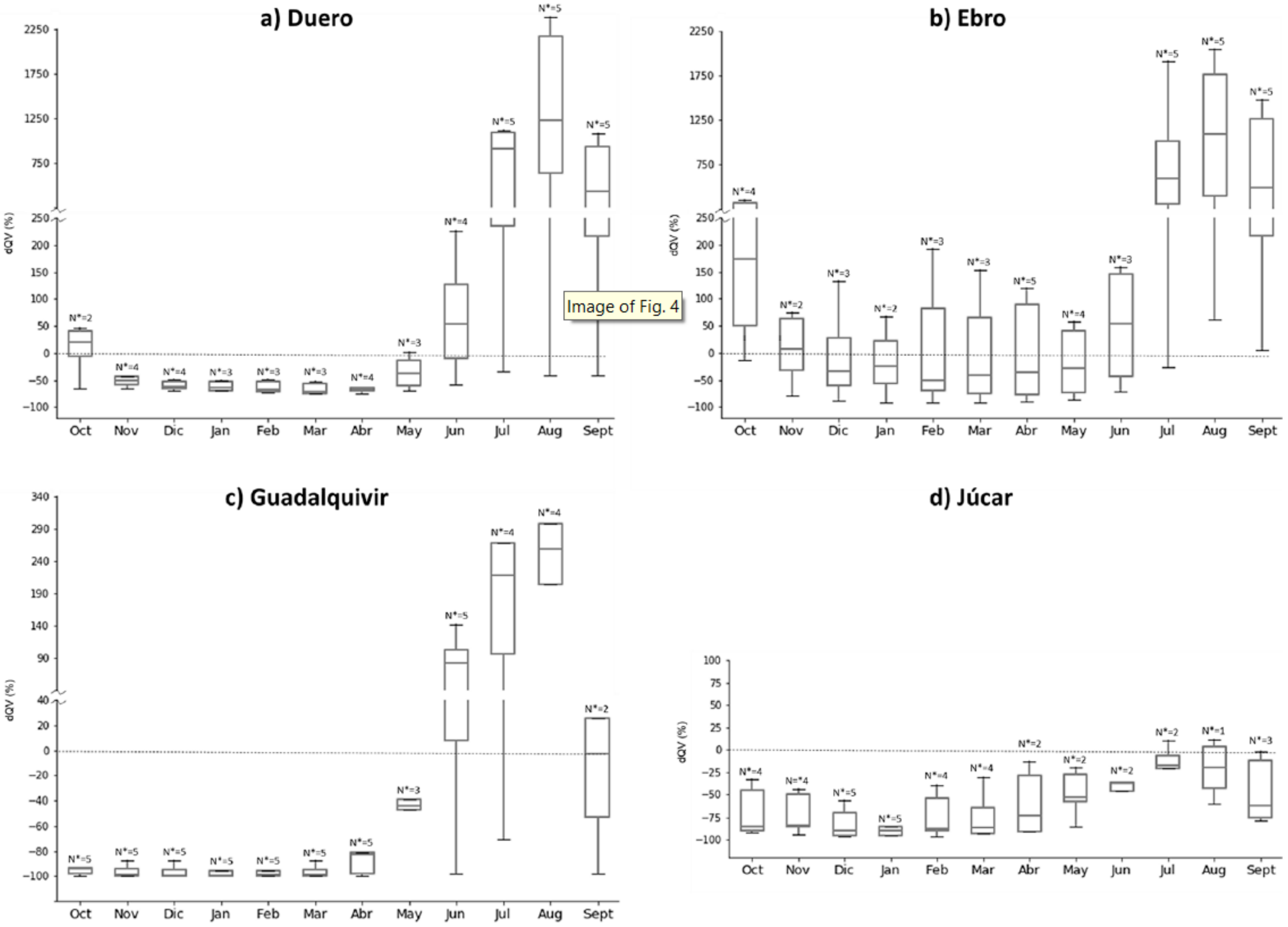


Fig. 1.4. Example of flow homogenisation due to reservoir flow regulation in the north-east of Spain (Siurana dam, in the Ebro catchment). The figure shows the discharge ($m^3 s^{-1}$) upstream (left panel) and below (right panel) the dam. The dam has set "environmental flows" defined as 10% of the seasonal flow. Nevertheless, flows below the dam are higher in spring and summer to accommodate water supply or/and recreational activities downstream (i.e. rafting or kayaking). Source: Aristi *et al.*, 2014.



a) Duero

b) Ebro

c) Guadalquivir

d) Júcar

Fig. 1.5. Example of seasonal flow inversion (a, b, c, flow 'reversal') and overall flow magnitude reduction (d) in four river basins in Spain. The figure shows the degree flow of variation (dQV, %) before dam construction (un-regulated flow, natural flow) and after the dam construction (regulated flows) for each month. For both periods, before and after dam construction, a minimum of 15 years of daily flow were used to calculate monthly medians discharge. Posteriorly, degree flow of variation was calculated as relative deviation of the magnitude of the medians. The main use of the dam was irrigation for 21 or the 23 dams studied. Box plots present median and percentile values and numbers above bars indicate the number of rivers where change in monthly flow were statistically significant. Source: Mezger, González del Tánago and De Stefano, 2021.

1.1.5 Ecological impacts of flow alterations

Rivers are among the most dynamic ecosystems in the world, with their temporal and spatial dynamism being determined by the natural flow regime (Poff *et al.*, 1997; Poff, 2018). In impounded rivers, flow regimes are mostly determined by human requirements leading to the synchronization of flow regimes on the regional or catchment scale. This results in a spatial and temporal homogenisation of the beta-diversity (i.e. large-scale biodiversity), which reduces ecosystem functionality and recolonization potential (Poff, 2018; Ruhi *et al.*, 2018). Below dams, the lack of high flows generated by dams often results in a lack of geomorphologic activity (Elosegi and Sabater, 2013; Lobera *et al.*, 2017). This further reduces biodiversity as a result of fewer habitats available (i.e. reduced structural complexity of the ecosystem) and the lack of colonization patches (i.e. reduced successful arrival of new species). Furthermore, fine sediments often accumulate below dams due to the high fine particulate matter content of the dam's release and the lack of flashing flows (Wohl *et al.*, 2015; Consoli *et al.*, 2022). This fine sediment often clogged the hyporheic zone generating adverse conditions for fish and macroinvertebrates spawning (Sear, 1993; Poff, 2018; Kukuła and Bylak, 2020). Fine sediment and nutrients below dams reduce the importance of autotrophic organisms (i.e. increased turbidity) while often enhancing the heterotrophic compartment (i.e. priming effect) (Acuña *et al.*, 2015). Furthermore, the absence of high flows also generates lateral disconnection from the riparian ecosystems and floodplains (Tockner and Stanford, 2002; Tonkin *et al.*, 2018, 2021). Thus, disconnecting the breeding area for some species (Tennant, 1976) and modifying the carbon inputs in the river system (Poff *et al.*, 1997).

The increased hydrological stability downstream promotes biomass accumulation, but decreases species diversity and efficiency (Lobera *et al.*, 2017; Truchy *et al.*, 2022). Often, below dams, biofilms tend to be thicker, but less efficient (Sabater *et al.*, 2002; Ponsatí *et al.*, 2015) and macroinvertebrates present higher total abundance, but with fewer species than non-regulated rivers (Lobera *et al.*, 2017; Truchy *et al.*, 2022). As a result of this hydrological stability, lentic areas are generated downstream which led to communities being dominated by species typical of higher-order streams (i.e. lentic areas). This results in epilithic biofilm being replaced by free-floating planktonic algae and bacteria (Proia *et al.*, 2016; Shangguan *et al.*, 2017; Goldenberg-Vilar *et al.*, 2022); macroinvertebrates' functional strategies shifting from aerial-dominated life cycles (i.e. aerial adults) to fully aquatic life cycles (Lobera *et al.*, 2017); and/or macroinvertebrates feeding strategies shifting from the dominance of shredders, filter-feeders and predators, to scrapers (Menéndez *et al.*, 2012; Mbaka and Mwaniki, 2017; White *et al.*, 2017; Sarremejane *et al.*, 2021). Overall, the dam's disturbances generate a disruption of the river continuum concept (Vannote *et al.*, 1980; Ellis and Jones, 2013; Proia *et al.*, 2016). Furthermore, dam mismanagement can generate artificial drought downstream which leads to communities being drought resistant with lower diversity and richness (Ledger and Milner, 2015; Stubbington *et al.*, 2022). Habitat contraction generated by artificial drought creates an overall reduction of ecosystem functionality, due to reduced niche availability and changes in water quality (increased DOC, reduced O2) with more severe effects for organisms depending on surface habitats (Arroita *et al.*, 2017; Arias-Real *et al.*, 2020).

1.1.6 Current flow management scenarios:

Over the last three decades, river restoration projects and river management strategies have increased exponentially to mitigate the current degradation of freshwater systems and to protect river ecosystem services (Bernhardt and Palmer, 2007; Palmer *et al.*, 2008; Schinegger *et al.*, 2012). However, the focus on improving structural complexity of river habitats (i.e. meandering, insertion of logs) and the lack of recognition of river dynamism has yielded limited improvement in ecological integrity (Poff *et al.*, 2003; Bauer *et al.*, 2018). Currently, restoration projects and management are shifting to more functional and dynamic approaches (i.e. process-based and non-stationary) (Beechie *et al.*, 2010; Darch and Jones, 2012; Poff, 2018).

The concept of environmental flows (e-flows) has been developed to partially re-establish the river natural dynamism by ensuring flow levels that sustain biodiversity and ecosystem services (Declaration, 2007; Poff *et al.*, 2007). E-flows define the magnitude, timing, frequency and water quality needed to preserve freshwater ecosystems (Acreman, 2001; Acreman and Dunbar, 2004; Acreman and Ferguson, 2010). There are currently two paradigms in the e-flow approaches; designer flows and natural flows. Designer flows aim to reproduce certain moments of the natural flow regime (i.e. reproducing flow magnitude and variability for a given duration); while natural flows aim to mimic the annual natural flow regime by releasing a proportion of it (McManamay *et al.*, 2013; Chen and Olden, 2017). Most of these designer e-flows typically consist of high magnitude flow events of short duration, often biased to target certain biota (Acreman *et al.*, 2009; Wilby *et al.*, 2010; Tonkin *et al.*, 2021) or high energy flushing flows (Robinson, Siebers and Ortlepp, 2018; Consoli *et al.*, 2022). In other cases, simplistic natural e-flow approaches are based on seasonal means of river discharge (i.e. long time series discharge) (Aristi *et al.*, 2014). These simplified e-flows overlook the high and low flows of rivers and neglect intra-seasonal and inter-annual variability. And often, when water demand exceeds the e-flow magnitude, water demand discharge is prioritized leading to the seasonality of e-flows being reversed or non-existent (see Fig. 1.4).

Effective implementation of e-flows depends on the comprehension of flow-ecology relationships (flow-biota-ecosystems process), the science of which is still in early stages (Palmer and Ruhi, 2019). Understanding how flow regimes alterations affect ecosystem structure and the implications for ecosystem functioning is essential for the development of this applied science, but few studies have looked at both structural and functional components together and interactions between trophic levels (Truchy *et al.*, 2022). Furthermore, much of our current knowledge is biased toward the stressors generated by hydropower and water supply (Palmer and Ruhi, 2019; Wang *et al.*, 2020). This has led to most management interventions being focused on the alteration of frequency of flashy high flow (hydropower) and reducing flow extremes (i.e. lower high flows and higher low flows, water supply). While the reduction of flow extremes is persistent in water supply dams, the timing, frequency and magnitude of medium flows depend on the climate and the tourism (Gössling *et al.*, 2012). Thus, the flow regimes resulting from water supply are quite diverse and the range of flow-ecology relationships are not well constrained (Wang *et al.*, 2020).

This study aims to establish flow-biota-process relationships by looking into the ecological impacts of the most extreme flow regime generated by flow regulations. To date, flow irrigation alterations have been largely overlooked despite being the main water use globally (Fig. 1.6) (Poff *et al.*, 2007; Doll, Fiedler and Zhang, 2009), and potentially resulting in major changes to species functioning and life-story traits (Poff and Ward, 1989; Bernhardt *et al.*, 2022). On the other hand, increased flow stability is a wide spread effect of dams (i.e. water supply and flood protection) (Poff *et al.*, 2007) but the mechanistic relations governing these environments are often confounded by other factors. Field studies make flow-ecology relationships hard to extrapolate to other scenarios, due to dam characteristics and the impossibility of control over flow regulations. Mesocosm experiments provide an ideal set up to understand mechanistic flow-ecology relationships by removing dam's variability and increasing control over the flow regimes (Petersen and Englund, 2005; Ledger, R. M. L. Harris, *et al.*, 2009; Brown *et al.*, 2011; Stewart *et al.*, 2013). Mesocosms approaches allow higher control and replicability of experiments, while being able to mimic ecosystem functionality and structure for benthic river communities (biofilm and macroinvertebrates) (Ledger, Harris, *et al.*, 2009; Ledger *et al.*, 2013).

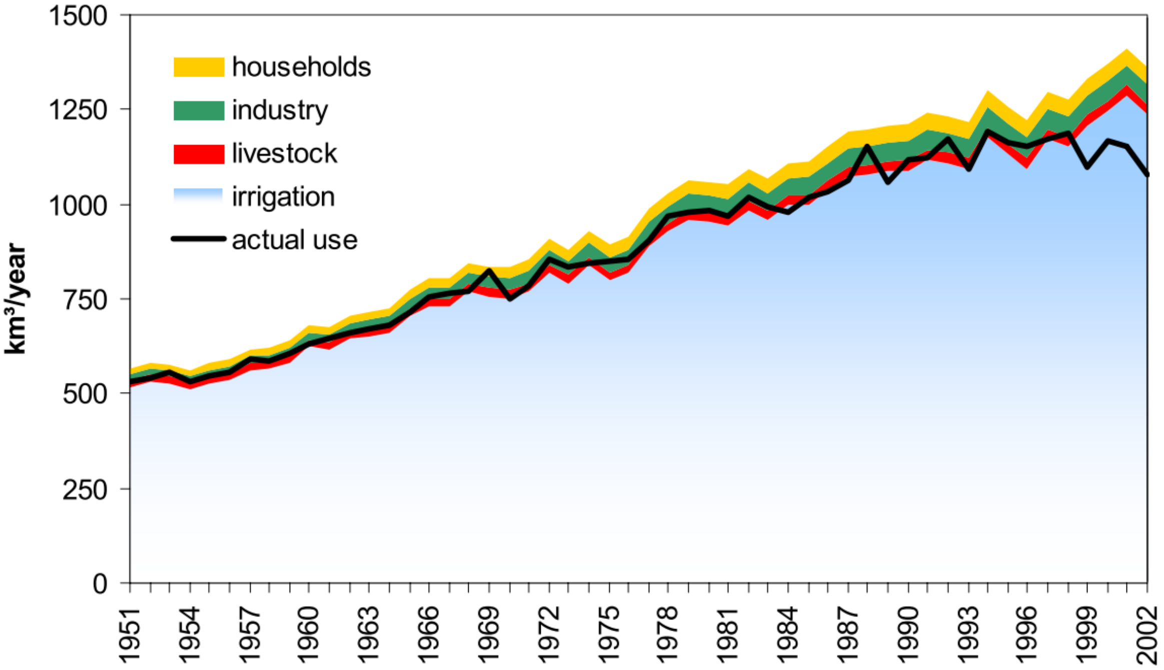


Fig. 1.6. Global water consumption simulated by the WaterGAP model classified by their respective uses (households, industry, livestock and irrigation) and the actual water use. WaterGAP simulates river discharge time series and their alterations due to water withdrawals and reservoirs/dams. Notice that this graph only presents consumptive water use which means water that is not returned to the rivers or not completely; in contrast to non-consumptive water uses as hydropower production. Source: Doll, Fiedler and Zhang, 2009.

The extent of biodiversity loss in running waters highlights the degree of deterioration of conditions our river systems (Vörösmarty *et al.*, 2010; Hooper *et al.*, 2012). Currently, the most prominent drivers of the biodiversity crises are land use change, pollution and exploitation of natural resources, however, the relevance of climate change is expected to increase (Dudgeon, 2019; Jaureguiberry *et al.*, 2022). Changes in precipitation patterns due to climate change coupled with increasing water demand are already intensifying the occurrence of supra-seasonal drought (Fig. 1.7) (Fowler, Kilsby and Stunell, 2007; Jin *et al.*, 2012; Elkouk *et al.*, 2022; Satoh *et al.*, 2022). Furthermore, there is an urgent need to understand how river systems would respond to extreme events such as heatwaves and how that may interact with flow reduction (Ledger and Milner, 2015).

Fig. 1.7. Satellite view of the UK during the drought experienced in summer 2022 (10th of August 2022). Image provided by: James Cheshire.

1.2. Thesis aims and objectives

In line with the research priorities outlined in the previous sections, this study used a mesocosm approach to simulate regulated flows in a control and replicable environment. The two primarily aims of this thesis are to improve our understanding of: 1) how flow regulation modifies the structure and function of river communities, and 2) how flow magnitude reduction interacts with extreme events (i.e. drought or heatwaves). My specific objectives, addressed in the successive chapters are:

1) Identify water quality and biofilm functional changes generated by regulated flow regimes and understand how alterations of the hydrological stability and predictability of flow regimes affect biofilm functioning (Chapter 2)
2) Assess the changes in macroinvertebrate community structure and functioning caused by alterations of the hydrological stability and predictability of flow regimes and detect interactions between trophic levels (biofilm, macrophytes) influencing community changes (Chapter 3)
3) Elucidate the effect of heatwaves on biofilm functioning and the possible interactions with flow magnitude reduction (Chapter 4)
4) Integrate the findings from previous sections to define implications and management strategies (Chapter 5)

To address these objectives, this study used two experimental designs tailored to the required spatial and temporal scale (Fig. 1.8). Each chapter is presented in a journal paper format with their own introduction and methods sections. In chapter 5, I discuss the main implications of the work and the management and conservation advice.

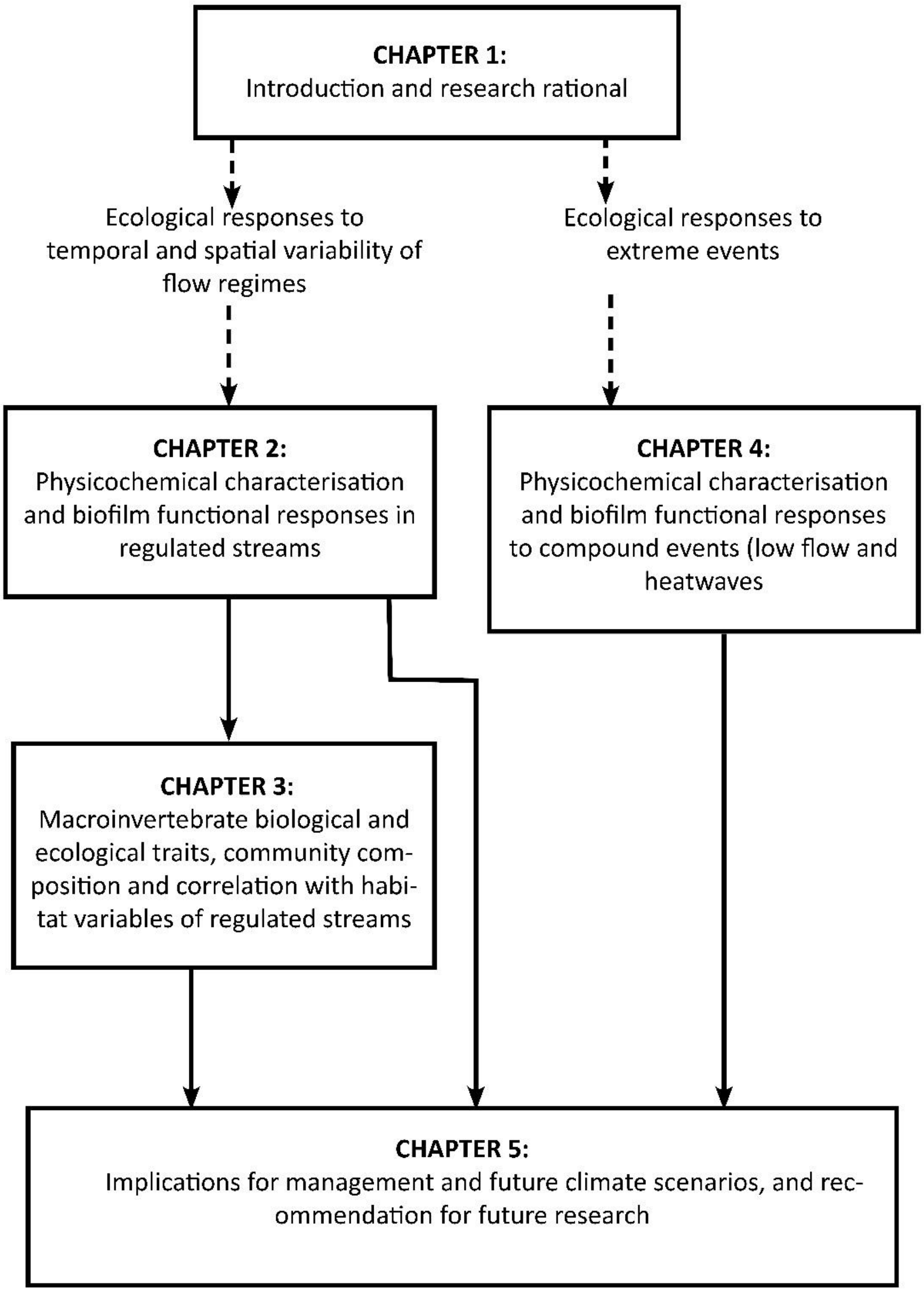


Fig. 1.8. Thesis structure diagram which shows the different experimental approaches used in this study (dashed lines) and the links between chapters (solid lines).

1.3. Summary and synthesis

There is an urgent need to establish flow-ecology relationships, which could inform the implementation of environmental flows to re-establish the natural hydrology of rivers. Whilst there is a broad consensus that dam flow regulation leads to a simplification of ecosystem structure and function worldwide, less is known about the relative impact of contrasting flow regimes downstream of dams, or the mechanistic basis and interaction among drivers of ecological responses. To address these urgent research gaps, this study aims to take a novel experimental approach, using stream mesocosms to mimic flow regimes in regulated rivers and streams, and assess their impacts on ecosystem structure and function. Given the prospective future climate change scenarios, this study also aims to understand how flow regulation effects may be influenced by extreme climate events.

1.4. References

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2. Effects of flow alteration on microbial communities in streams

2.1. Introduction

Over the centuries, human dependency on surface waters has led to the development of infrastructure and management strategies that modify and regulate rivers and streams worldwide. Currently, more than two-thirds of rivers are affected by channelization, damming, hydropower and/or water abstraction (Vörösmarty *et al.*, 2010; Palmer and Febria, 2012; Schinegger *et al.*, 2012; Grill *et al.*, 2019). These alterations often yield highly modified river flow regimes in terms of magnitude, frequency, timing (or predictability), rate of change of flow conditions and duration of flow events (Poff *et al.*, 1997, 2007; Doll, Fiedler and Zhang, 2009; Pokhrel *et al.*, 2018). These flow alterations can degrade river ecosystems through geomorphic alterations (Elosegi and Sabater, 2013), fragmentation and lateral disconnection from the riparian ecosystems (Tonkin *et al.*, 2018) and floodplains (Tockner and Stanford, 2002). Flow modification can further exacerbate the effects of eutrophication (Smith and Schindler, 2009; Woodward *et al.*, 2012), and other pollutants (Pereira *et al.*, 2017; Hannah *et al.*, 2022).

Rapid biodiversity loss in freshwaters highlights the extent of the ecological degradation of rivers and surface waters (Dudgeon *et al.*, 2006; Hooper *et al.*, 2012; Reid *et al.*, 2019), with major implications for ecosystem services (Hoekstra and Mekonnen, 2012; Dudgeon, 2019). Uncertainties about the future of freshwater systems arise with predicted stressors from climate change and human population increase, which further endangers the provision of those services (Laizé, Acreman and Overton, 2017; Petra Doll *et al.*, 2018). The need for management strategies and restoration measures is compelling but often constrained by the limited understanding of the relations between abiotic conditions, biodiversity and ecosystem functioning (Cortina *et al.*, 2006; Palmer, Menninger and Bernhardt, 2010). These limitations have been further exacerbated by river management and restoration approaches which often fail to recognise the temporal and spatial dynamism of the river ecosystems (Poff *et al.*, 1997; Weber and Peter, 2011).

Flow regimes influence the life cycles and adaptations of freshwater species (Tennant, 1976), govern lateral and longitudinal connectivity, and modulate the input of allochthonous organic matter and nutrients to the river (Poff *et al.*, 1997). Dams can profoundly alter stream flow regimes by altering the magnitude and the timing of low and high flows (Poff *et al.*, 2007). There are a variety of river management approaches depending on the structure and purpose of dam infrastructure that can generate different flow regimes downstream with potentially contrasting impacts on ecosystem processes (Poff, 2018; Palmer and Ruhi, 2019). For example, flood protection dams generate flow homogenization (hydrological stability) by eliminating high and low flow events and increasing water depth (Magilligan and Nislow, 2005; Ponsatí *et al.*, 2015; Proia *et al.*, 2016). Dams used for water supply and irrigation can 'reverse' natural flow regimes, creating winter low and summer high flows, by storing water in winter and periodically releasing water in summer (Magilligan and Nislow, 2005). Thus, generating hydrologically unstable flow regimes unsynchronized with species life-history adaptations (Lytle and Poff, 2004). Dam baseline releases often decreases the magnitude of stream flows and these reductions can be further exacerbated by the impact of meteorological drought, with streams often becoming intermittent in the driest months (Ledger and Milner, 2015).

These flow alterations generated by dams will also influence structure and functioning of stream communities. Depending on dam characteristics (release type and residence time), downstream sections have modified water quality (nutrients, water temperature, DOC and

POC) (Maavara *et al.*, 2015, 2017; Proia *et al.*, 2016; Poff, 2018). Inversion of the flow regime and increased hydrological stability downstream of dams reduce geomorphological activity (Poff *et al.*, 2007; Ponsatí *et al.*, 2015; Lobera *et al.*, 2017). Thus, promoting an increase in macroinvertebrates abundance and biomass but decreasing diversity (Lobera *et al.*, 2017; Truchy *et al.*, 2022). Increased hydrological stability below dams often yields to macroinvertebrates' functional strategies shifting from aerial-dominated life cycles (aerial adults) to fully aquatic life cycles (Lobera *et al.*, 2017). Greater hydrological stability downstream can yield thicker biofilm which tends to be more metabolically active, but less efficient (i.e. less processing of organic matter per unit of biomass) (Sabater *et al.*, 2002; Ponsatí *et al.*, 2015). In some cases, epilithic biofilm is replaced by free-floating planktonic algae and bacteria as a result of lentic areas being generated downstream (Proia *et al.*, 2016; Shangguan *et al.*, 2017; Goldenberg-Vilar *et al.*, 2022). On the other hand, artificial drought often leads to taxa within communities being drought resistant with lower diversity and richness (Ledger and Milner, 2015; Stubbington *et al.*, 2022). As habitat contracts, overall ecosystem functionality is reduced due to reduced niche availability and changes in water quality (increased DOC, reduced O_2) which affects species persistence in the system and physiological performance (Arroita *et al.*, 2017). Intermittency of surface water flow induces a strong reduction in the autotrophic biofilm as the streambed desiccates (Acuña *et al.*, 2015), but heterotrophy is often maintained in the hyporheic zone (Burrows *et al.*, 2017; Arias-Real *et al.*, 2020).

While our understanding of flow alteration is growing, most of the current knowledge comes from observational studies below dams which are strongly influenced by covarying local conditions, especially water quality and specific dam characteristics (i.e. residence time and release type). Consequently, disentangling the direct and indirect effects of flow modification on river ecology can be challenging (Maavara *et al.*, 2015, 2017; Proia *et al.*, 2016; Poff, 2018). Furthermore, given field limitations, most studies to date have focused on the community structure of key biota (Lobera *et al.*, 2017; Shangguan *et al.*, 2017; Jansen, O'Dowd and Bouma-Gregson, 2020; Goldenberg-Vilar *et al.*, 2022). The few studies that have focused on functional responses have centred on a limited range of response variable such as extracellular enzyme activity, nutrient uptake and/or sediment respiration using chambers (Timoner *et al.*, 2014; Ponsatí *et al.*, 2015; Proia *et al.*, 2016).

Mesocosm experiments capture some of the realism of field studies and provide an ideal framework to study the effects of flow alterations with results which are more generalisable and move beyond the traditional approach of individual case studies (Yvon-Durocher *et al.*, 2010; Stewart *et al.*, 2013; Piggott *et al.*, 2015; Gossiaux *et al.*, 2020). Given their small size and short life cycles, microbial communities (bacteria, attached algae and fungi or biofilms) are particularly amenable to experimentation at the mesoscale (Petersen and Englund, 2005; Sabater *et al.*, 2007; Stewart *et al.*, 2013; Cheng *et al.*, 2017; Freixa *et al.*, 2017). In lotic ecosystems, microbial communities constitute the majority of biomass and activity within the aquatic community and play a crucial role as bottom-up regulators of the food-webs (Fischer and Pusch, 2001; Craft, Stanford and Pusch, 2002). For instance, alterations to the functioning of microbial communities might shift the basal resources in the stream ecosystem by altering the nutrient cycle, dissolved organic carbon composition, biofilm primary productivity and decomposition (Allan and Castillo, 2007).

Despite the importance of microbial communities for maintaining ecosystem function, knowledge of the relation to flow regime components remains limited, especially in relation to the biofilm's ability to adapt to altered flow. Addressing such gaps in knowledge would aid the development of informed river management practices designed to minimize the ecological impacts of river impoundments. Hence, the main aim of research in this chapter is to determine attached microbial communities' (biofilm) responses to different degrees of hydrological stability and alterations to the magnitude and timing of high and low flows. Viewed through the lens of the harsh-benign theory, hydrologically stable regimes should generate benign abiotic conditions with biotic interactions controlling community structure (Peckarsky, 1983; Poff and Ward, 1989). Hydrologically unstable flows, by contrast, should generate harsh abiotic conditions where community structure and functioning are controlled largely by physical processes (dewatering, scouring and/or changes in water quality). Nevertheless, community's life-history strategies should be shaped by long-term flow patterns (natural flow regime) (Lytle and Poff, 2004). Therefore, flow predictability (timing of high and low flows) could determine community capacity to adapt to the altered flow (Lytle and Poff, 2004).

In the following chapter I investigated the effects of flow alteration on stream microbial community functioning in stream mesocosms. Four key flow regime types were reproduced in mesocosms, mimicking headwater streams with contrasting hydrological stability and alterations to the magnitude and timing of high and low flows. The four regimes were: (1) 'natural' flow (controls with summer low and winter high flows), (2) flow 'reversal' (i.e. summer high and winter low flows), (3) homogenised flow (stable flow with no highs or lows) and (4) drought (with 80% flow reduction and dewatering). Given the greater hydrological stability (benign abiotic factors) in the homogenised flow treatment, I expected that it would result in biofilm dynamics being driven by biotic relationships (e.g. competition, and predation and resource partitioning) (Poff and Ward, 1989; Townsend and Hildrew, 1994; Clausen and Biggs, 1997). By contrast, hydrologically unstable flows generated in the drought and the flow reversal treatments should result in dynamics driven by abiotic factors with biofilm functioning mostly affected during extreme flow events (low and high flows). These harsh abiotic conditions might lead to biofilm presenting convergent functionality depending on the pressure this abiotic factor exert on the communities (ecological filter). I expected that the highly unstable hydrology in the drought treatment would lead to an overall reduction of ecosystem functioning, especially during dry periods as habitat contracts (Timoner *et al.*, 2012; Arroita *et al.*, 2017). Nevertheless, ecosystem functioning during the wet period would be sustain by fast growing and resistance strategies. Flow reversal should generate a hydrologically unstable flow regime marked by altered timing of high and low flow. Thus, unpredictable high flows would generate a harsh scouring environment that would reduce biofilm growth as communities are not adapted (Thomen *et al.*, 2017). The hypotheses to be tested are:

- Relative to controls, flow homogenisation should result in:
    - H_1: Similar biofilm growth and decomposition processing rates, despite higher stability (i.e. less scouring), due to increased competition for resources;
    - H_2: Processing of a more diverse range of carbon substrates due to resource partitioning
- Relative to the natural control, drought should result in:
    - H_3: Lower biofilm growth and decomposition processing rates which will be most pronounced during dry periods due to habitat contraction and instability;
    - H_4: A higher proportion of diatoms due to this group's higher resistance to desiccation.
    - H_5: Processing a less diverse range of carbon substrates but at higher rates due to biofilm fast growing strategies.

- Relative to the natural control, flow reversal will result in:
    - H_6. Reduced biofilm growth and process rates during high-flow periods due to reduced colonization success (i.e. scouring);
    - H_7. Limited range of carbon substrates processed and low processing rates due to harsh abiotic factors forcing convergent functionality (i.e. strong abiotic filtering).

2.2. Methods

2.2.1. Experimental facility and monitoring

The research was conducted over two years (October 2018 - October 2020) in 16 outdoor stream mesocosms at the University of Birmingham Environmental Change Outdoor Laboratory (EcoLaboratory), U.K. (52.45° N, 1.93° W). Mesocosms were stainless steel channels (each 12 m length × 0.5 m width × 0.5 m height, Fig. 2.1) fed by partially recirculated groundwater pumped from a borehole (tapping a Permo-Triassic Sandstone aquifer) via a 2000 L header tank (Fig. 2.1). Groundwater was of moderate nutrient status (total nitrogen, 2.6 mg L^{-1}, phosphate 0.03 mg L^{-1}) and was distributed independently to each mesocosm flume via underground pipes with inputs controlled using programmed solenoid valves (Fig. 2.1). Water delivered to each flume was then independently recirculated to generate specific flow rates. Water was piped to the inlet of each flume from a sump (245 L) collecting water from the flume outlet by a sump pump (Lowara DOMO submersible pump, Whisper Pumps Ltd, UK). The water in each flume turned over every 7 days as new borehole water displaced water in each sump to waste via a sump drain. Mesocosm channels were filled with 760 kg of commercial gravel (particle size 50% 10 mm, 50% 20 mm). Three riffle-pool sequences were created in each flume and an additional short riffle was added at the end of the flume to slow the water flux into the outlets (Fig. 2.2). Each riffle or pool was 1.5 m long, with gravel depths of 0.2 m in riffles and 0.05 m in pools.

Fig. 2.9. Environmental Change Outdoor Laboratory (EcoLaboratory) at University of Birmingham. The EcoLaboratory is equipped with a range of semi-recirculating flumes (top). This study used 16 stainless-steel flumes (top right), which are groundwater-fed by a system of taps (bottom middle). Discharge and water depth are controlled by an outlet system (bottom right) and the number of pumps connected (bottom left and middle).

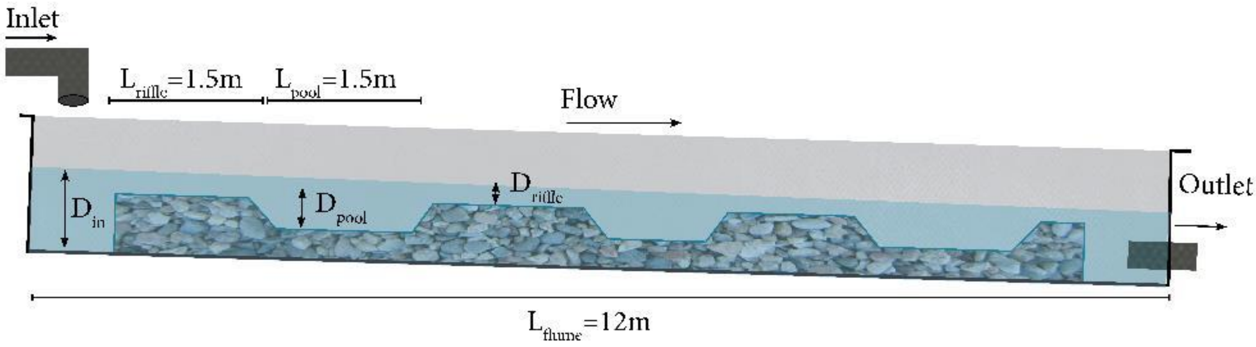

Fig. 2.10. Schematic representation of the structure within the flumes. Water enters the flume from a water inlet pipe, flows down the three riffle-pool sequences and exits via an outlet delivering water to a sump at the end of each flume.

For each flume, the flow was determined using continuous water level monitoring with a pressure transducer (IMSL - Submersible Level Transmitter). The water level was used to calculate the depth and a rating curve was used to transform continuous depth measurement to instant discharge (Q). A rating curve was done for each flume by measuring flow velocity with a flowmeter (MFP51, Geopacks, Devon, UK) at the inlet pipe(s) for a range of different discharge magnitudes (i.e. 0 L s^{-1} to maximum Q on a given flume). For low flows an adapter was used to ensure full pipe flow before measurements. After, the diameter of the pipe(s) was used to calculate the discharge by multiplying the area by the velocity of a given pipe, if a flume had multiple inlet pipes the discharge for each was summed. Continuous monitoring of DO (RDO PRO-X), water temperature (107 temperature probe, Campbell Scientific) and conductivity (CS547A-L water conductivity probe) were undertaken in each mesocosm. Measurements were recorded from July 2019 – a week before the experiment began – until December 2021. To ensure pre-experimental equal conditions, water level measurements started 4 months before the experiment began. Measurements were 15 min means based on scans every 5 minutes. Water quality parameters (pH, electrical conductivity, water temperature and dissolved oxygen) were also measured monthly in each flume using a handheld meter (Thermo Scientific Orion Star A329, Thermo Fisher Scientific, Waltham, USA).

2.2.2. Community establishment and experimental design

Before the experiment was initiated, there was an eight-month colonisation and community stabilisation period, which ended when the treatments were applied in June 2019. In February 2019, the flumes were seeded with macroinvertebrates and microbial communities attached to stones and organic particulate matter from two local streams (Bourne brook and River Rea). For each flume, invertebrates from two 3-minute kick samples from each stream were added to each mesocosm flume. Flumes were also likely colonized aerially from nearby water bodies. In February 2019, 50 L of commercial compost was added to each flume (peat and nutrient-free, John Innes No.1 Compost, Wyevale Garden Centres Ltd., London, UK) to provide standard quality and quantity of organic matter to enable community establishment and mimic sediment deposition in pools. More coarse organic matter was also added to the flumes, using 8g of decaying alder (*Alnus glutinosa*) leaves and 15 g of wood material from a local riparian woodland (Edgbaston Pool SSSI). In November 2019 an additional 50 g of recently fallen alder leaves were added to each flume to maintain the organic matter supply and prevent unrealistic decomposition measurements. In April 2019, three Spiked Milfoil (*Myriophyllum spicatum*) plants were rooted in each pool and two Brooklime (*Veronica beccabunga*) plants were added to each riffle to increase habitat complexity (niche and resource availability). Each plant species was selected based on their habitat preference. Flumes were then left to establish until June 2019 (Fig. 2.3).

Fig. 2.11. Initial establishment in the mesocosms. Brooklime (top-left) were planted and established in each flume's riffles. Macroinvertebrates communities from the kick-sampling and aerial colonizers were established in the flumes (top-right, Chironomidae, Simuliidae). Spiked Milfoil was established in the pools as well as biofilm communities (bottom).

During acclimation, all 16 flumes were of uniform discharge (mean ± SD, 6.85 ± 0.99 L s^{-1}) and water depth (0.2 ± 0.02 m). At the end of the establishment period, flumes were assigned to flow treatments and ANOVA was performed to test for any differences in flow and depth among these treatment groups of flumes before any treatments were applied. No statistically significant differences were identified (ANOVA Q; $F_{13,3}=0.72$, $P=0.974$; ANOVA depth, $F_{13,3}=0.335$, $P=0.8$). Flumes were well-oxygenated (99.1 ± 9.69 % saturation) with no significant differences evident (ANOVA, $F_{13,3}=0.222$, $P=0.879$). The mean water temperature was 18.51 ± 4.26 °C with no significant difference between the assigned treatments (ANOVA, $F_{13,3}=0.397$, $P=0.758$). Conductivity was relatively high (332.4 ± 32.45 µS cm^{-1}) given the groundwater geology, but was not significantly different among the assigned treatments (ANOVA, $F_{13,3}$=0.954, P=0.446).

Following acclimation, we initiated a one-factor experiment with a replicated (n=4) randomised design. There was one fixed factor with four levels (natural control, drought, flow reversal and homogenised flow). For each treatment, a standardised flow regime was applied across the replicate flumes (Fig. 2.4; n = 4). For each regime depth, discharge and flow velocity were varied by altering: i) the number of submersible pumps used for recirculation; ii) pump valve settings to ratchet flow; iii) the position of a U outlet to determine the water depth, and; iv) outlet tap settings to regulate water exiting the flume (Fig. 2.1). The natural control (N) aimed to mimic a natural flow regime with relatively low flows in summer which progressively increase in autumn and reached a peak during mid-spring (Fig. 2.4). Flow 'reversal' treatment (R) to natural flow is aimed to mimic a water supply management scenario where low flows occur during late autumn, winter and spring and high compensation flows occur during summer to supply irrigation downstream. The homogenised flow treatment (H) mimicked flood protection dam management in which discharge is based on a percentage of the annual inflow with a depth increased (e.g. lentic flow constant all year). The drought treatment (D) mimics a river where flow magnitude is strongly reduced generating artificial drought over the driest months (e.g. summer and early autumn). At the onset of the experiment (June 2019), flow management scenarios were applied for each treatment.

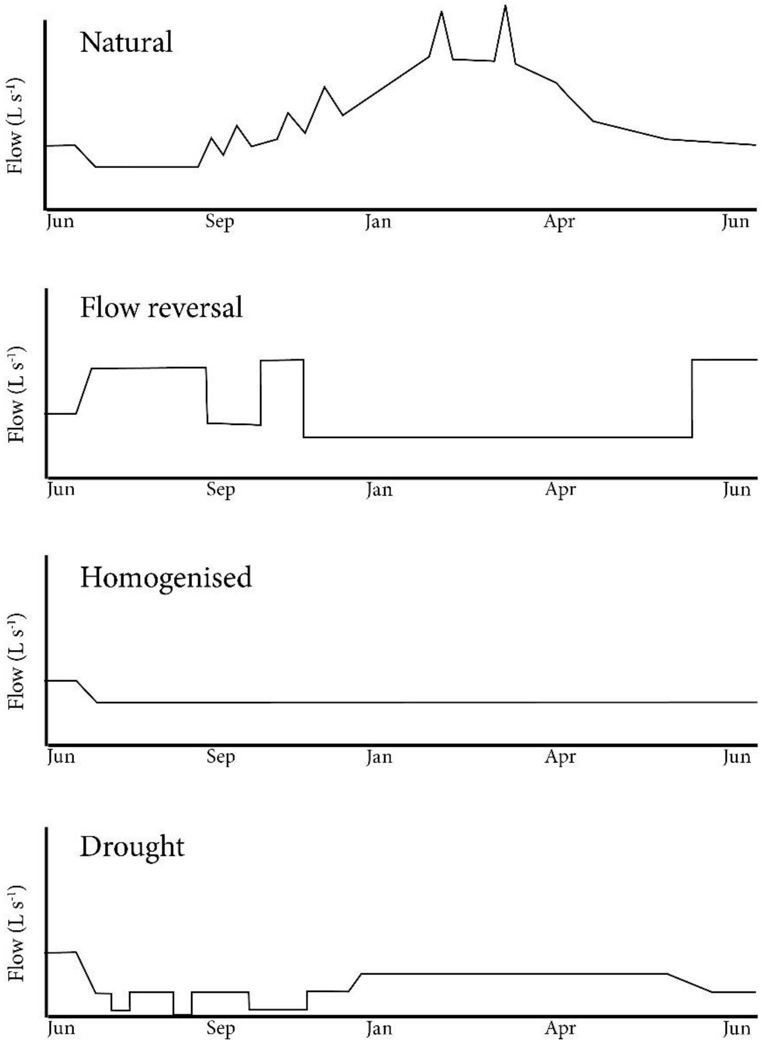

Fig. 2.12. Proposed hydrology for the natural control and the three flow treatments (flow reversal, homogenised and drought).

2.2.3. Sampling periods

After an initial year of treatment acclimation, I measured a set of variables to determine the effects of flow alteration in physiochemistry metrics and epilithic biofilm functioning and functional diversity. Sampling took place in summer and autumn (beginning of July 2020, Sum; and beginning of November 2020, Aut). For both sampling periods, 45 mL of water samples were collected from each flume, filtered at 0.45µm (Whatman GD/X disposable filter device) and frozen for subsequent analysis of dissolved organic matter (DOM) and macronutrients (See section 2.2.4). Benthic biofilm growth and microbial breakdown of organic matter were measured in the riffles in summer and autumn (See section 2.2.5.1). At the end sampling point (i.e. autumn sampling), an integrated sample for each riffle (i.e. 3 cobbles were collected in each section[n=9]) was collected, stored in Ringer solution and immediately transferred to the laboratory for assessment of biofilm functional diversity (See section 2.2.6.2).

2.2.4. Macronutrients and dissolved organic matter

Macronutrient concentrations (total inorganic and organic nitrogen, phosphorus) and dissolved organic carbon quantity (DOC) and fluorescence dissolved organic matter (fDOM) quality (i.e. degree of humification, freshness, terrestrial vs microbial sources, tryptophan-like, tyrosine-like and aromaticity) were determined from a water sample (50mL) collected from each flume on two occasions (summer and autumn).

Dissolved Organic Carbon (DOC) and total dissolved nitrogen (N). The quantity of DOC and N was determined using a Shimadzu TOC-L TOC-L CPH+ASI-L total organic carbon and total nitrogen analyser (Kyoto, Japan). For each sample, readings of N and DOC were undertaken in duplicate or triplicate and ≤2% coefficient of variation was observed. Nitrate (NO_{3-}, mg L^{-1}), nitrite (NO_{2-}, mg L^{-1}), ammonia (NH_{4-}, mg L^{-1})) and phosphate (PO_4^{3-}, mg L^{-1}) concentrations in the flumes were determined using a continuous flow analyser (Skalar San ++, Skalar Analytical B.V., Breda, The Netherlands) following (Baird, Eaton and Rice, 2017). When nutrient concentrations were below the flow analyser detection limit, values were set to the limit (i.e. PO_4^{3-}, 0.02 mg L^{-1}). Total organic nitrogen (DON, mg L^{-1}) was obtained by subtracting dissolved organic nitrogen (DIN; NO_{3-}, NO_2 and NH_{4-}) from N. To assess flow alteration effects on stoichiometry, the C:N ratio was calculated as molar weights of C from DOC concentrations and of N from N concentrations. Given the low values of P (PO_4^{3-}) in the flumes (i.e. mostly being below detection levels), the calculation of C:P and N:P ratios was not possible.

Fluorescence dissolved organic matter (fDOM). Filtered water samples were analysed to determine seasonal changes in fDOM quality. Absorbance spectra (200–850 nm) were measured using a Jenway 6800 dual-beam spectrophotometer (Stone, UK; cuvette path length 10 mm) and quartz cuvettes that were triple rinsed with sample water. A Cary Eclipse Spectrofluorometer (Varian Inc., Palo Alto, USA) was used to measure fluorescence following methods outlined in Khamis et al. (2015). At the start of each instrument run, a Raman blank was measured to calibrate fluorescence intensity (Lawaetz and Stedmon, 2009). For each sample and a blank (ultrapure water), excitation Emission Matrices (EEMs) were determined with an overexcitation range of 200–400 nm (5 nm slit width) and emission range of 280–500 nm (2 nm slit width). EEMs were corrected by absorbance baseline correction, blank subtraction, inner-filter effect correction and Raman normalization (Zepp, Sheldon and Moran, 2004). Coble peaks and their ratios, Humification index (HIX), Fluorescence Index (FI), Biological Index and SUV_{254} (L mg C^{-1} m^{-1}) were calculated following Hansen et al. (2016) and described in Table 2.1. When Coble peaks were too low, a minimum value of 0.0001 was assigned to avoid realistic cobble ratios (i.e. infinite). All EEMs were processed and indices were calculated using the *staRdom* package (Pucher et al., 2019).

Table 2.1. Fluorescence Dissolved Organic Matter (fDOM) index calculations and interpretation. Abbreviations used in the table: Abs= absorbance; DOC= DOC concentration; C=carbon, ex=excitation, em=emission.

Index	Calculation	Purpose
$SUVA_{254}$	Abs. 254 divided by DOC	Indicator or DOC aromaticity
PeakA	Maximum fluorescence at ex260 in the em region 380:460 divided by DOC	UVA humic-like (resembles fulvic acid)
PeakB	Fluorescence at ex275/em310	Tyrosine-like
PeakC	Maximum fluorescence at ex350 in the em region 420:480 divided by DOC	UVA humic-like (high molecular weight and aromaticity)
PeakM	Maximum fluorescence at ex312 in the em region 380:420 divided by DOC	Microbial humic-like
PeakT	Fluorescence at ex275/em340 divided by DOC	Tryptophan-like
A:T	Ratio of peak A to peak T	Indication of humic-like (recalcitrant) vs fresh-like (labile)
C:A	Ratio of peak C to peak A	Indication of humic-like vs fulvic-like
C:M	Ratio of peak C to peak M	Indication of diagenetically altered (blue shifted)
C:T	Ratio of peak C to peak T	Indication of humic-like (recalcitrant) vs fresh-like (labile)
Fluorescence Index (FI)	Ratio of em at 470nm and 520nm obtained at ex. at 370nm	Relative contribution of terrestrial (1.3-1.4) and microbial (1.7-2) sources to DOM
Humification Index (HIX)	Peak area em 435-480nm divided by peak area 300-345nm plus 435-480nm at ex 254nm	Indication of humic substance content or humification. Higher values indicated a higher degree of humification
Biological Index (BIX)	Ratio of em at 280nm divided by 430 at ex 310	Indication of autotrophic productivity. Higher values (>1) suggest recently produced autochthonous DOM

2.2.5. Indicators of microbial community functionality

2.2.5.1. Microbial growth and processing rates

The effects of flow alteration on microbial processes were assessed as the rate of accrual of epilithic biofilm (autotrophic and total accrual), the relative contribution of each chlorophyll type to epilithic biofilm accrual, and the decomposition of labile and recalcitrant detritus on the stream bed. Each determinant was measured in three riffles per flume in summer and autumn 2020.

Biofilm accrual. Growth of epilithic biofilm was assessed as the accrual of total chlorophyll (chl_{Tot}) and ash-free dry mass (AFDM) on artificial substrates (small [4 cm^2] unglazed tiles). In each riffle, six tiles were previously incubated for a month (Fig. 2.5). After the incubation time, tiles in each riffle were sampled in triplicate and pooled, yielding three integrative samples per flume for both chlorophyll and AFDM analysis. The biofilm was removed from tiles using an electric toothbrush and washed with deionized water. Afterwards, the slurry was filtered through a combusted and weighted 0.7 µm glass fibre filter (Whatman GF/F 47mm) and the filtrate was frozen. The concentrations of chlorophyll *a, b* and *c* were determined spectrophotometrically as outlined in ASTM D3731 (ASTM International, 2020) and summed to yield the total chlorophyll biomass per unit area of substratum (mg cm^{-2}). Total biofilm biomass was estimated as AFDM (Hauer and Lamberti, 2017). Defrosted filters were oven-dried for 1h at 105 ℃ then weighed to the nearest 0.001 g, then combusted in a muffle furnace at 500℃ for 1h hour and reweighed at room temperature (e.g. Ledger and Hildrew, 1998). Accrual rates (mg cm^{-2} d^{-1}) were calculated as the mass of material sampled divided by the number of incubation days (~30 days). The relative contribution of each chlorophyll type to the Chl_{Tot} was estimated as a proxy for autotrophic community composition: $Comp_{chl\text{-}a}$,

$Comp_{chl\text{-}b}$, $Comp_{chl\text{-}c}$. This was calculated by dividing each chlorophyll type by Chl_{Tot}. The autotrophic index (AI) was calculated as the ratio of biofilm accrual biomass to algal accrual, with lower values indicating higher levels of autotrophy (Hauer and Lamberti, 2017)

Microbial breakdown of organic matter. Commercial tea bags (Twinnings green tea) and wooden craft sticks were incubated in triplicate in each riffle for a month before each sampling occasion (Fig. 2.5). Collected substrates were oven-dried at 65 °C for 48 h and weighed to the nearest 0.01g. Decomposition rates (k_{wood} and k_{leaf}, d^{-1}) were calculated based on a single exponential decay model (e.g. Wieder and Lang, 1982). Given the diversity of leaf structural constituents (cellulose, hemicellulose and lignin) and their lability, single decay models tend to underestimate decomposition rates of labile material as they assume a constant and trending to 0 decay coefficient. Thus, neglecting the slow decomposing process and non-decomposable fractions (Wieder and Lang, 1982). To assess the relative contributions of slow decomposition rates (i.e. recalcitrant organic matter with a high lignin content) to the detrital decomposition rates, the ratio of k_{wood}: $k_{leaf.}$ was calculated. To enable comparisons of process efficiency, we determine the decomposition rates excluding the temperature effect by calculating decomposition per degree-day (i.e. decomposition efficiency, $k_{dd\text{-}wood}$ and $k_{dd\text{-}leaf}$) following Chauvet *et al.* (2016).

Fig. 2.13. Riffle within a homogenised flow flume showing incubating tiles, wooden sticks and tea bags. Wooden sticks were half-buried in the gravel (inset).

2.2.5.2. Biofilm functional diversity

The effects of flow alteration on microbial functional diversity were assessed as processing rates of epilithic biofilm using the functional biodiversity index (richness, diversity and evenness) and process rates of different carbon sources (31 different carbon sources differing in complexity and nutrient content). Each determinant was measured in an integrative sample of the three riffles per flume in autumn 2020.

Functional diversity. Biolog Ecoplates (Biolog Inc. Hayward, California, USA) were used to assess biofilm organic substrate utilization potential at the end of the experiment (autumn, November 2020). Each Biolog Ecoplate contains a water blank and 31 different carbon sources with a redox dye (Table 2.2). We collected an integrative sample of three cobbles per riffle from each flume (i.e. 3 in each riffle, 9 in each flume). Biofilm was removed from the surface of each cobble using an electric toothbrush and washed into sterile 50 mL pots with 30mL of autoclaved Ringer solution. Under sterile conditions, we inoculated the Ecoplates with the biofilm solution (100µL in each well). Plates were then incubated at 20 °C for six days in dark conditions (Romaní *et al.*, 2014). Optical density (OD) was read on day 6 at 590 nm using a microplate reader (FLUOstar OMEGA, BMG LABTECH Ltd., Freiburg, Germany). OD values < 0.3 were set to 0 following Freixa (2016). For each substrate, OD was calculated by subtracting the blank OD (i.e. OD well with water + sample solution) from the raw OD of each well. Since only one replicate was done per flume, single reading OD was used to assessed the processing rates of each individual substrate. Ecoplates data was used to assess microbial community's functional diversity using biodiversity standardised index as richness, Shannon and evenness index (Freixa, 2016). Following Freixa (2016), functional richness (S) for each flume was then calculated as the number of positive colour development wells (i.e. OD>0.3). Following Shannon and Weaver (1963), biofilm functional diversity was calculated as Shannon index (H):

$$H = -\sum p_i(\ln p_i)$$

where p_i is the ratio of the OD value of each substrate to the sum of all the substrates' OD measured in each flume, and *i* is each individual substrate. Evenness (Ev) was calculated as the division of H to the log(S). Diversity indexes were calculated using the vegan package (Oksanen, Blanchet, Kindt, 2022). The individual substrates present within the plate were classified into six functional groups: Carboxylic acids (CAc), Polymers (Pol), Carbohydrates (CH), Phenolic Compounds (PhC), Amino acids (aa) and Amines (A) (Insam, 1997; Christian and Lind, 2006). For each functional group, Average Well Colour Development (AWCD) was calculated as the mean OD of the group.

Table 2.2. List of substrates present in the Ecoplates, classification of functional groups and nutrient content of the substrates (Nut). Acronyms used for substrates (Subs ID) and functional groups (Func ID) are presented.

Substrate	Subs ID	Functional group	Func ID	Nut
Water	water	water	water	0
Pyruvic Acid Methyl Ester	pyr	Carboxylic acids	CAc	C
Tween 40	t40	Polymers	Pol	C
Tween 80	t80	Polymers	Pol	C
α-Cyclodextrin	aCy	Polymers	Pol	C
Glycogen	Gly	Polymers	Pol	C
D-Cellobiose	Dcel	Carbohydrates	CH	C
α-D-Lactose	aDlact	Carbohydrates	CH	C
β -Methyl-D-Glucoside	bDGlu	Carbohydrates	CH	C
D-Xylose	Dxyl	Carbohydrates	CH	C
i-Erythritol	Ery	Carbohydrates	CH	C
D-Mannitol	Man	Carbohydrates	CH	C
N-Acetyl-D-Glucosamine	DgluA	Carbohydrates	CH	CN
D-Glucosaminic Acid	DgluAAc	Carboxylic acids	CAc	CN
Glucose-1-Phosphate	GluP	Carbohydrates	CH	CP
D, L-α-Glycerol-Phosphate	DLaglyP	Carbohydrates	CH	CP
D-Galactonic Acid γ-Lactone	DgalAcLac	Carbohydrates	CH	C
D-Galacturonic Acid	DgalaAc	Carboxylic acids	CAc	C
2-Hydroxy-Benzoic Acid	Ben2Ac	Phenolic compounds	PhC	C
4-Hydroxy-Benzoic Acid	Ben4Ac	Phenolic compounds	PhC	C
γ-Amino-Butyric Acid	aaButAc	Carboxylic acids	CAc	C
Itaconic Acid	itaAc	Carboxylic acids	CAc	C
A-Keto-Butyric Acid	aKeButAc	Carboxylic acids	CAc	C
D-Malic Acid	MalAc	Carboxylic acids	CAc	C
L-Arginine	Larg	Amino acids	aa	CN
L-Asparagine	Lasp	Amino acids	aa	CN
L-Phenylalanine	Lphe	Amino acids	aa	CN
L-Serine	Lser	Amino acids	aa	CN
L-Threonine	Lthr	Amino acids	aa	CN
Glycyl-L-glutamic Acid	GlyL_glu	Amino acids	aa	CN
Phenylethyl amine	PheA	Amines	A	CN
Putrescine	Putr	Amines	A	CN

2.2.6. Statistical analysis

A range of metrics was calculated to characterize hydrological conditions in each flow treatment. For discharge, annual means were calculated for each flume for the whole duration of the experiment (Q_{Annual}) and seasonal means for the summer and winter periods (Q_{Summer} and Q_{Winter}). Mean annual depth was calculated as well for each flume (D_{Annual}). Annual values were calculated for the duration of the experiment, while summer values were calculated from the beginning of July to the end of September (summer 2019 and 2020), and winter values were calculated from the beginning of January to the end of March (winter 2020). For each flume, these estimates were obtained using continuous data for depth and discharge. To assess Q variation in the flumes, I calculated the coefficient of variation (Q_{CV}) and the 95th and 5th quantiles as an indicator of high and low flows (respectively) that occurred in the flumes (Q_{95} and Q_5). A fixed effect model and one-way ANOVA type II test was used to test the effect of the flow treatments on each of the seven hydrological metrics (Q_{Annual}, Q_{Summer}, Q_{Winter}, D_{Annual}, Q_{9CV}, Q_{95} and Q_5). For each test, p-values were adjusted to control for Type 1 error following Benjamini and Hochberg (1995). Statistical significance was determined at 0.05. Posteriorly, a t-test pairwise comparison was used to assess differences between the control and each of the three flow treatments. The effect of seasonality on hydrology was assessed by analysing the effect of season (summer and winter) on mean Q. Given the time dependency of our experimental design, flume was used as a random effect which acknowledges that flume’s measurements for each season are not independent (i.e. pseudoreplication and flume specific variation). Given the sample size, flume dependency effect was accounted allowing a different intercept for each flume. I used a mixed effect model with flume as a random effect and flow, season and its interaction as fixed. A repeated measures (RM) two-way ANOVA type II test with adjusted p-values was conducted to check the significance of the effects. Posteriorly, marginal mean (MM) and marginal mean differences (MMD) were calculated and a Tukey pairwise comparison on MMD was carried out to assess the differences between seasons within treatments (e.g. N-Summer vs N-Winter).

To assess seasonal variation of macronutrients and DOM between the flow treatments, we determined at flume level: DOC quantity (DOC, mg L^{-1}), $SUVA_{254}$(L mg C^{-1} m^{-1}), fluorescence peaks (Coble peaks: peakA, peakB, peakC, peakM and peakT), peak ratios (A:T, C:A, C:M, C:T), fluorescence indices (FI, BIX and HIX), total nitrogen (N, DIN, DON, NO_{3-}, NO_2 and NH_3, mg L^{-1}), the relative contribution of dissolved organic and inorganic nitrogen (DON:DIN), inorganic phosphorous (P, PO_4^{3-}, mg L^{-1}), CN molar ratios (C:N) and conductivity (Cond, µS cm^{-1}). To assess organic matter differences among treatments an NDMS (Bray-Curtis similarity) was used to visualize the spatial distribution of macronutrients and fDOM in the flumes and the ellipses of the different seasons and treatments. Given the small samples size, ellipses were calculated using 50% confidence intervals. Highly correlated variables (R^2>0.8) in the NDMS space were deleted to avoid the NDMS axis being influenced by the collinearity (Björklund, 2019). C:T ratio was excluded given its correlation with A:T and NO_3 was excluded given its correlation to DIN. To test the significance differences between treatments and seasons, a PERMANOVA was performed and a pairwise PERMANOVA was used to test differences between groups.

To assess seasonal effects of flow treatments on biofilm growth and processing rates, we determine at each riffle level: total and autotrophic biofilm growth rates (AFDM and chl_{Tot}, mg m^{-2} d^{-1}), autotrophic index (AI), the contribution of chlorophyll type ($Comp_{chl-a}$, $Comp_{chl-b}$, and Comp$_{chl\text{-}c}$.), decomposition of labile and recalcitrant substrates (k$_{leaf}$, k$_{wood}$, d^{-1}), ratio k$_{wood}$:k$_{leaf}$ and decomposition efficiency of labile and recalcitrant substrates (k$_{dd\text{-}leaf}$, k$_{dd\text{-}wood}$, d^{-1} °C^{-1}). To assess the overall differences of the treatments considering all the biofilm variables, I performed a PERMANOVA test restricted by flume. Since the within-flume samples were not independent between seasons and within flumes, a restricted permutation was used with flume as a plot (sample grouping) and season as block (higher sample grouping) to avoid permutations on those levels. Posteriorly, a pairwise PERMANOVA was used to observe differences between groups and obtain R^2. Decomposition efficiency of labile and recalcitrant substrates (k$_{dd\text{-}leaf}$, k$_{dd\text{-}wood}$, d^{-1} °C^{-1}) were excluded from the PERMANOVA test to avoid highly correlated variables (Björklund, 2019). To assess the effects off individual biofilm responses, linear mixed-effect models were used. All samples were tested for normality and log+1 transformed when necessary (Zuur, Ieno and Elphick, 2010). Given the sample size of biofilm responses (i.e. greater than for hydrological and water quality measurements), time dependency of our experimental design (i.e. flume random effect) could be acknowledge by including only a random intercept for flume in the mixed effect model or by allowing as well a random slope for season. Following methods outlined by Zuur et al. (2005), the best mixed effect model structure was selected based on Akaike's Information Criteria (AIC) and graphical tools. Using graphical tools, I tested the distributions of fitted vs observed residuals and the equality of the variances of the residuals (i.e. homoscedasticity) for each of the model's effects (i.e. flow, season, flume). To meet these assumptions, some variables required further transformation (Order Quantile and Box-Cox transformation) due to heteroscedasticity (ADFM and chl$_{Tot}$). For each response variable, a global mixed effect model with random slope and intercept of the following form was fitted:

Response ~ flow * season + (season [random slope]|flume [random intercept])

Where flow treatment (Flow) and season are the fixed effects and their interaction and flume is a random effect with an independent intercept for flume and independent slope between seasons. A two-way RM-ANOVA type II test was performed for all final models and p-values adjusted to control for Type 1 error (Benjamini and Hochberg ,1995).

To assess flow effects on biofilm functioning at the riffle level, biology Ecoplates results were used to determine: biofilm functional richness (S, active substrate number) and diversity indexes (Shannon index, S; and Evenness, ev), carbon processing rates of different substrates and functional groups (OD of 31 substrates and AWCD of each functional group). To assess overall variability in substrate's use and C functional groups, a distance matrix was built based on Bray-Curtis similarity and then use for an NDMS for both, substrates and functional groups. For both matrices, a PERMANOVA test was performed and group differences were assessed using a pairwise PERMANOVA. To test the effects on the single biofilm functional responses, standard linear models were used. For each response variable, a fixed effect model with flow and season as fixed effects was fitted. Variables were tested for normality and homoscedasticity as described above and transformed when necessary. One-way ANOVA type II tests were performed for all final models and p-values adjusted as mentioned above. All the analyses were performed in R software version 4.2.1 with the package rstatix (Kassambara, 2021) for t-test pairwise comparisons of one-way ANOVAs, lme4 (Bates *et al.*, 2015) for mixed effect model ANOVAs and emmeans (Lenth, 2022) for MMD calculations and Tukey pairwise comparisons of mixed effect two way ANOVA, vegan (J Oksanen, FG Blanchet, R Kindt, 2022) to calculate the NMDS and the PERMANOVA and ecole (Robert Smith, 2021) was used for the pairwise PERMANOVA, *bestNormalize* (Peterson and Cavanaugh, 2020) for data transformation, and *sjstats* (Lüdecke, 2020) for extracting ANOVA values.

2.3. Results

2.3.1. Flow characterization: hydrological metrics

During the experimental period, the mean annual Q (Q_{Annual}) for the natural control was 6.12 L s^{-1} with lower Q in summer (Q_{Summer} = 5.74 ± 1.37 L s^{-1}), higher Q in winter (Q_{Winter} = 7.13 ± 0.88 L s^{-1}) and a moderate coefficient of variation (CV; Q_{cv} = 17.55 ± 3.62%). As expected, flow treatments significantly affected all hydrological metrics (one-way ANOVA, P<0.001 in all cases, Table S2.1). In the flow reversal treatment, annual hydrological metrics (Q_{Annual}, Q_{CV}, Q_{95}, Q_5 and D_{Annual}; Table 2.3) were similar to the natural control (i.e. pairwise comparison P>0.05, Table S2.2). Importantly however, the timing of high and low flows was reversed when compared with the control (Fig. 2.6). In flow reversal flumes, Q was higher in summer (Q_{Summer} = 7.25 ± 0.59 L s^{-1}) and lower in winter (Q_{Winter} = 5.74 ± 1.37 L s^{-1}) with metrics being significantly different to the control in both seasons (i.e. pairwise comparison: Q_{Summer}, P=0.31 and Q_{winter}, P<0.001). In the homogenised flow treatment Q_{Annual} was significantly lower, whereas D_{Annual} was higher and much less temporarily variable than the control (Q_{Annual}, 1.47 ± 0.29 L s^{-1}, P<0.001; D_{Annual}, 0.2 ± 0.01 L s^{-1}, P=0.015; and Q_{CV}, 7.26 ± 1.2%, P=0.01). In the drought treatment, Q (Q_{Annual} = 0.94 ± 0.19 L s^{-1}) was lower than any other treatment with an 81% reduction of baseflow relative to the natural (i.e. percentage of Q_{winter} from natural to drought). Drought flows were at minimum during the summer months (Q_{Summer} = 0.75 ± 0.17 L s-1) and highly temporally variable (Q_{CV} = 57.49 ± 5.18%) as a result of riffle dewatering and flow intermittency ($Q_5 = 0 \pm 0$). All of the drought hydrological metrics were significantly different from the natural control (i.e. pairwise comparisons $P<0.001$ in all cases, Table S2.2).

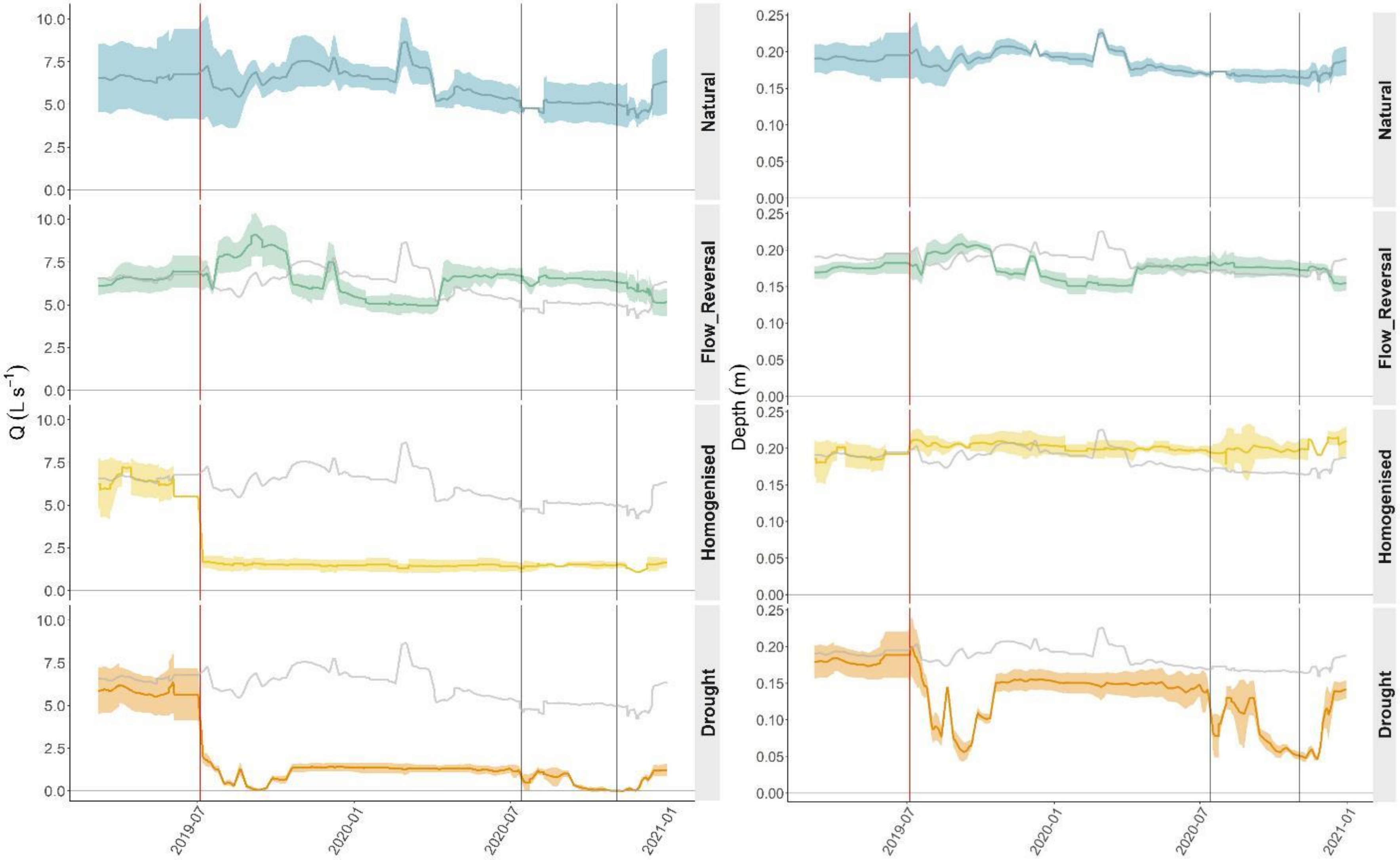

Fig. 2.14. Discharge (left panels) and pool depth (right panels) time series for each treatment: control (blue), flow reversal (orange), flow homogenization (yellow) and drought (red): data are presented as one-week rolling means (solid line) with associated standard deviations (shaded area). Flow treatments were first applied on July 4th 2019 (red vertical line) and are plotted with the mean discharge and depth of the control treatment (grey line) for comparison. Sampling for biofilm and water quality was done on the first week of July and November (Summer and Autumn, respectively, see solid black vertical lines).

Table 2.3. Hydrological characterization of each flow treatment: annual mean Q (Q_{Annual}), coefficient of variation (Q_{CV}), Q_{95}, Q_5, summer mean Q (Q_{Summer}), winter mean Q (Q_{Winter}) and annual mean depth (D_{Annual}). Hydrological indices were calculated in each flume in a given period, and then mean values and SD were calculated for each treatment (mean ± SD).

	Q_{Annual}	Q_{CV}	Q_{95}	Q_5	Q_{Summer}	Q_{Winter}	D_{Annual}
Natural	6.12±1.14	17.55±3.62	7.91±1.67	4.84±1.18	5.74±1.37	7.13±0.88	0.18±0.01
Flow Reversal	6.49±0.52	16.34±4.68	8.54±1.08	4.91±0.47	7.25±0.59	5.12±0.5	0.18±0.01
Homogenised	1.47±0.29	7.26±1.2	1.65±0.3	1.31±0.27	1.48±0.27	1.45±0.36	0.2±0.01
Drought	0.94±0.19	57.49±5.18	1.42±0.24	0±0	0.75±0.17	1.32±0.27	0.12±0.01

Seasonality had a significantly different effect in each flow treatment (repeated-measures ANOVA, Flow and season interaction, $F_{25,7} = 82.184$, $P<0.001$; Table S2.3). Seasonal contrasts in Q were greatest in the control and the flow reversal treatments (i.e. differences between summer and winter Q). Discharge in controls was higher in winter than summer (pairwise comparison Mean Marginal Differences (MMD), MMD = -1.39, $P<0.001$), whereas the converse was the case for the flow reversal treatment (pairwise comparison MMD, MMD = 2.13, $P<0.001$). In the drought treatment, Q were lower in summer but not significantly different (pairwise comparison MMD, MMD = -0.58, P = 0.09), whereas, in the homogenised flow treatment there was no significant difference in flow between seasons (MMD = 0.03, P=0.91).

2.3.2. Macronutrients and dissolved organic matter

Water quality metrics and nutrient concentrations varied among flow treatments and seasons (Table 2.4). In the controls, the water was relatively warm (especially in summer), well-oxygenated, and alkaline with high conductivity (Table 2.4). DOC was relatively low, and fDOM analysis suggested high contributions humic-like compounds (i.e. A:T and C:T). Nutrient concentrations were relatively low for both N and P (~3.8 mg L^{-1} and <0.02 mg L^{-1} respectively), and N was dominated by inorganic nitrogen forms (~20% was organic nitrogen). However, C:N ratios were small (range 3.9 to 7.9) indicating no N limitation in the flumes.

In the flow reversal treatment, temperature was higher in summer and lower autumn than in controls. N was higher in the flow reversal than in the natural control (Table 2.4). The relatively high proportion of organic nitrogen in the flow reversal treatment lowered the C:N ratio. During autumn, the fDOM in the flow reversal treatment was richer in humic-like compounds (i.e. HIX, C:T, A:T), with higher relative contributions from non-microbial sources (i.e. lower FI) and with lower tyrosine and tryptophan-like fluorescence (i.e. peak B and T, respectively) than the control. In the homogenised flow treatment, summer water quality was similar to the control, except for fDOM which was less humic-like (i.e. A:T and C:T) whereas in autumn, water quality was colder and less alkaline than controls (Table 2.4). Drought had the strongest effect on water physiochemistry with water being warmer and of lower conductivity in summer, and colder in autumn, relative to controls. Overall, fDOM under drought had a higher contribution from terrestrial sources or vascular plants (i.e. lower FI) and a higher degree of humification (i.e. HIX) than the control (Table 2.4). Nevertheless, C:T and A:T ratios indicate that fDOM was more labile in early summer, and was transformed to more recalcitrant forms in autumn. Overall, nitrogen concentrations were lower under drought than in the control in summer (i.e. DIN and DON) and the proportion of organic nitrogen was lower. This resulted in high C:N ratios in the drought treatment during summer (Table 2.4).

Table 2.4. Physicochemical variables, macronutrients and dissolved organic matter during the summer (Sum) and the autumn sampling (Aut) sampling (mean ± SD). Variables presented: conductivity (Cond), pH, dissolved oxygen concentration and saturation (DO), temperature (Temp), dissolved organic carbon (DOC), SUV254, fluorescence Index (FI), Humification Index (HIX), Biological Index (BIX), cobble peaks (peak A, B, C, M and T), peak ratios (A:T, C:T, C:M, C:T), Total dissolved nitrogen concentration (N) and phosphate (P), dissolved inorganic and organic nitrogen (DIN, DON), the ratio of DON:DIN, ammonia(NH3), nitrite (NO2-), nitrate (NO3-) and carbon to nitrogen ratio (C:N).

	Natural		Flow Reversal		Homogenised		Drought	
	Sum	Aut	Sum	Aut	Sum	Aut	Sum	Aut
Cond (µS cm^{-1})	404.3±19.5	419.7±10.3	403.4±25.1	421.2±7	405.2±14.8	404.7±16.9	358.4±18.9	411.5±10.4
pH	8.55±0.25	8.74±0.31	8.32±0.07	8.49±0.45	8.53±0.35	8.19±0.51	8.87±0.34	8.7±0.34
DO (mg L^{-1})	9.34±0.22	11.39±0.07	9.19±0.22	12.1±0.41	9.86±0.64	11.91±0.25	9.04±0.05	11.9±0.16
DO (%)	100.7±0.7	105.2±0.8	102.4±0.1	104±0.8	105.4±6.2	104±2.7	101.9±0.6	103.8±1
Temp (°C)	18±1.6	10.2±0.2	19.9±1.1	7.9±0.3	17.9±1.1	8.1±1	20.2±0.6	7.8±0.6
DOC (mg L^{-1})	2.91±0.62	2.37±0.27	2.89±0.3	2.67±0.22	2.65±0.51	2.48±0.12	3.72±0.34	2.39±0.1
SUVA$_{254}$ (L mg C^{-1} m^{-1})	0.013±0.003	0.008±0.003	0.015±0.005	0.013±0.003	0.013±0.001	0.009±0.001	0.014±0.003	0.01±0.002
FI	1.69±0.97	1.69±0.62	1.56±0.28	1.34±0.2	1.61±0.6	1.25±0.25	1.33±0.24	1.02±0.35
HIX	2.58±0.61	2.41±0.86	2.46±0.3	7.27±3.52	1.96±0.32	5.86±5	4.6±0.82	4.3±3.07
BIX	0.92±0.12	0.8±0.1	1.03±0.1	0.89±0.15	0.98±0.28	0.88±0.28	0.73±0.02	0.84±0.15
peakA	0.031±0.005	0.039±0.01	0.032±0.007	0.057±0.012	0.025±0.007	0.04±0.005	0.04±0.006	0.042±0.009
peakB	0.015±0.005	0.011±0.003	0.017±0.004	0±0.001	0.018±0.009	0.006±0.007	0.009±0.003	0±0
peakC	0.034±0.008	0.03±0.008	0.041±0.009	0.048±0.013	0.031±0.013	0.037±0.008	0.036±0.004	0.035±0.004
peakM	0.029±0.008	0.03±0.008	0.039±0.006	0.047±0.016	0.031±0.015	0.033±0.006	0.036±0.005	0.031±0.004
peakT	0.008±0.003	0.01±0.006	0.013±0.002	0±0	0.012±0.006	0.005±0.007	0.012±0.004	0±0
A:T	4.38±2.5	6.19±4.43	2.56±0.79	1506.37±298.32	2.4±0.68	447.02±513.4	3.49±1.16	996.59±224.93
C:A	1.09±0.12	0.77±0.07	1.29±0.07	0.84±0.07	1.22±0.2	0.92±0.16	0.88±0.04	0.85±0.11
C:M	1.17±0.09	1.01±0.12	1.05±0.09	1.02±0.08	1.05±0.19	1.11±0.07	1±0.06	1.13±0.15
C:T	5±3.39	4.67±3.25	3.3±1.02	1271.14±299.76	2.84±0.45	376.7±430.15	3.08±1.02	833.49±117.77
TN (mg L^{-1})	3.79±0.41	3.84±0.86	4.59±1.02	4.78±0.61	3.94±0.23	3.55±0.22	2.26±0.73	3.72±0.44
P (mg L^{-1})	0.02±0	0.02±0	0.036±0.027	0.028±0.015	0.04±0.041	0.029±0.019	0.023±0.005	0.022±0.003
DIN (mg L^{-1})	3.22±0.26	3.26±0.76	3.84±0.76	3.85±0.4	3.46±0.21	2.96±0.14	2.17±0.49	3.11±0.28
DON (mg L^{-1})	0.57±0.17	0.57±0.14	0.74±0.27	0.93±0.22	0.48±0.13	0.59±0.1	0.16±0.11	0.61±0.17
DON:DIN	0.17±0.05	0.18±0.03	0.19±0.03	0.24±0.03	0.14±0.04	0.2±0.03	0.07±0.04	0.2±0.04
NH3 (mg L^{-1})	0.043±0.019	0.017±0.005	0.045±0.013	0.023±0.007	0.041±0.006	0.025±0.011	0.041±0.011	0.023±0.014
NO2 (mg L^{-1})	0.027±0.002	0.029±0.002	0.028±0.005	0.029±0.004	0.04±0.026	0.036±0.011	0.03±0.003	0.03±0.001
NO3 (mg L^{-1})	3.153±0.266	3.215±0.757	3.772±0.764	3.801±0.399	3.383±0.203	2.894±0.125	2.101±0.489	3.058±0.296
C:N	6.931±4.262	5.125±1.901	4.946±1.731	3.474±0.71	6.897±2.535	5.011±1.075	22.455±6.639	4.899±1.737

There was a considerable overlap in the composition of dissolved organic matter and nutrient concentrations between homogenised flow flumes and controls, whereas drought and flow reversal treatments differed from controls, especially in autumn (Fig. 2.7). This differentiation was supported by the results of the PERMANOVA on fDOM and nutrients which revealed a significant difference between the treatments and seasons (PERMANOVA, Flow, R^2=0.20, P=0.009; Season, R^2= 0.45, P=0.002). Furthermore, season effects were flow regime dependent as evidenced by the significant interaction effect (PERMANOVA, R^2=0.21, P=0.016). In the NDMS plot, the first sample ordination index was significantly positively correlated with conductivity and humic-like Coble peak ratios (C:T and A:T). The second axis was negatively correlated with DOC and C:N and positively associated with all the nitrogen indicators, excepting NH_3. During autumn, the drought and flow reversal treatments separated from controls along the first axis in the NDMS plot, together with two samples of the homogenised flow treatment. This separation seemed to be firstly driven by C:T, A:T and HIX of these samples being higher than controls (Table 2.4). During summer, composition of organic matter and macronutrients of all flow treatments was strongly overlap with the control, except for the drought treatment which was clearly separated along the NDMS secondary axis (Fig. 2.7). Drought treatment's separation was the result of a negative correlation with the secondary axis driven by lower nitrogen, higher DOC and consequent higher C:N ratios in summer than the natural control (Table 2.4). The slight separation of homogenised samples in summer might be the result of lower humic-like fDOM (i.e. HIX, C:T and A:T).

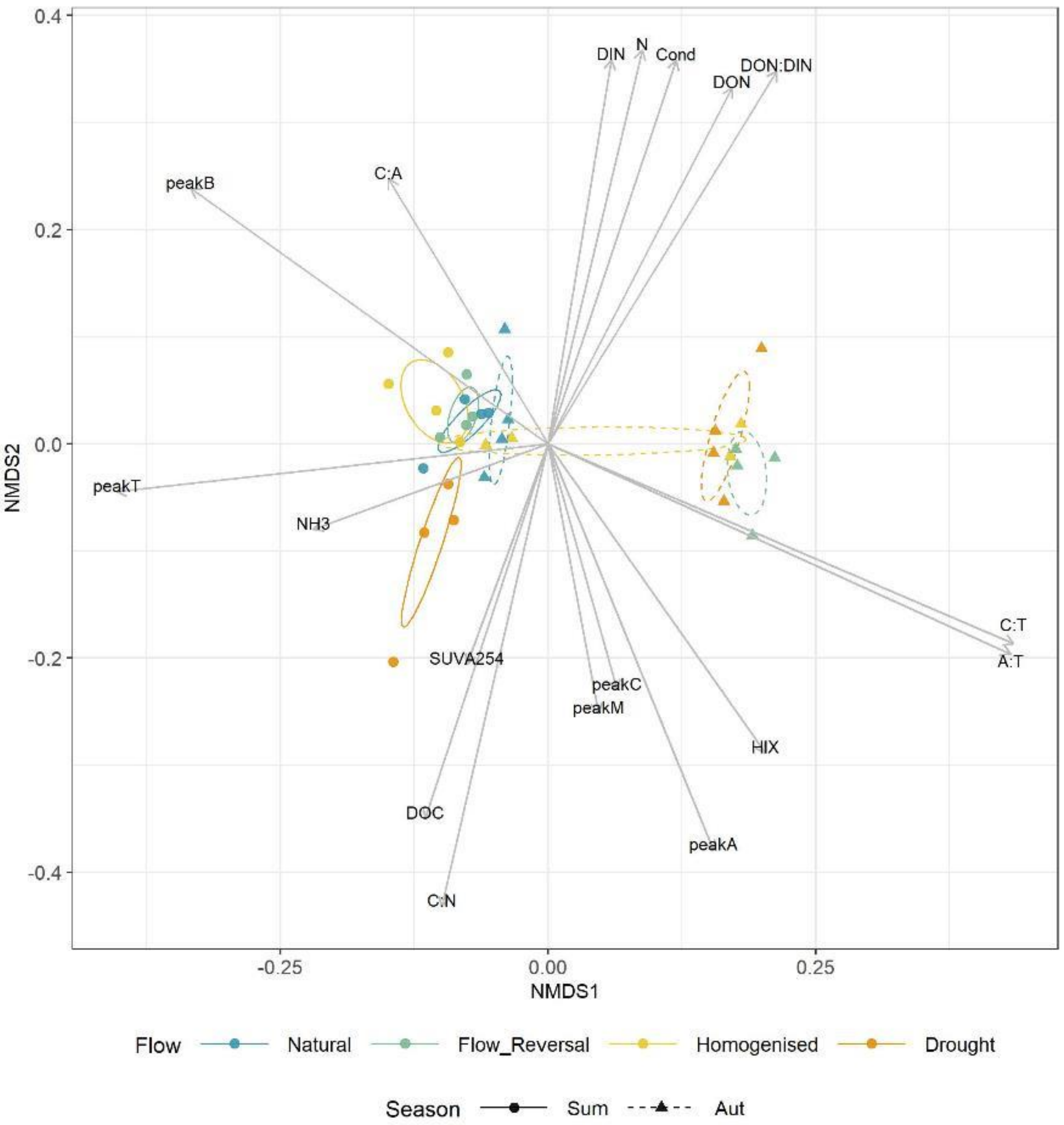

Fig. 2.15. NMDS plot of fDOM composition and macronutrients for summer (Sum) and autumn (Aut) for each of the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue). Ellipses for each season and treatment were drawn using 50% confidence intervals. Kruskal 2D stress was 0.09. Only significant variables are shown and their NDMS values were divided by 2 to improve the visualization of the spread in the flumes. From all the initial variables consider in the NMDS, only conductivity, HIX, FI, BIX, $SUVA_{254}$, peakA, peakB, peakT, peakC, peakM, A:T, C:A, C:T, C:M, DOC, C:N, NH3, DIN, N, DON and DON:DIN were kept.

2.3.3. Microbial growth and processing rates

A PERMANOVA test indicated that microbial growth and processing rates were significantly affected by the flow treatments (PERMANOVA, Flow, R^2=0.32, P=0.001). Microbial processing and growth rates for all the flow treatments were quite similar to the natural control, except for the drought treatment (Fig. 2.8, Table 2.5). Those differences were the result of significant effects on k_{leaf}, chl_{Tot}, $Comp_{chl\text{-}a}$, $Comp_{chl\text{-}c}$, AFDM and AI (RM-ANOVA, Table 2.6). Drought processing and growth rates (i.e. k_{leaf}, chl_{Tot}) were generally lower than the control and AI was higher indicating higher heterotrophy (Fig. 2.8). The composition of chlorophyll was also affected, with $Comp_{chl\text{-}a}$ being lower in favour of $Comp_{chl\text{-}c}$.

Table 2.5. Pairwise PERMANOVA comparison of microbial growth and processing rates between treatments and seasons. Results presented are dissimilarity R^2 with significance values in bold. Data assess differences between flow treatments (natural control, N; flow reversal, R; homogenised, H; and drought, D) within season (lighter grey shaded cells), seasonal differences within treatments (darker shaded cells) and differences between treatments and seasons (clear cells).

		Sum			Aut			
Season	Flow	R	H	D	N	R	H	D
Sum	N	0.132	0.008	0.121	**0.224**	0.136	**0.188**	**0.588**
	I		0.083	**0.266**	**0.231**	0.096	**0.205**	**0.547**
	H			**0.137**	**0.183**	0.095	0.149	**0.539**
	D				**0.449**	**0.334**	**0.433**	**0.445**
Aut	N					0.049	0.009	**0.764**
	I						0.037	**0.636**
	H							**0.769**

Furthermore, there was a clear seasonal effect (PERMANOVA, Season, R^2=0.04, P=0.006), which was treatment dependant (Interaction Flow:Season, R^2=0.11, P=0.001). Season had a significant effect on all decomposition parameters (k_{leaf}, k_{wood} and k_{leaf}:k_{wood}), $Comp_{chl-a}$ and AFDM as indicated by the RM-ANOVA results (Table 2.6). Decomposition rates of leaf detritus were higher in summer while recalcitrant detritus decomposition was higher in autumn. Thus, resulting in higher k_{leaf}:k_{wood} ratios in Autumn (Fig. 2.8). The composition of the autotrophic biofilm also responded to the change in season with lower $Comp_{chl-a}$ in Autumn.

Consistent with increased discharge variability, drought lead to the strongest seasonal variability (R^2=0.45, P=0.003) with more pronounced differences in autumn (i.e. higher R^2; Table 2.5) as result of riffle dewatering, while it was not different from the natural control in summer (Table 2.5). These differences being the result of significant effects for k_{leaf}, ratio kl_{eaf}-k_{wood} and $Comp_{chl-a}$ (Table 2.7). Contrary, the homogenised and flow reversal treatments had no clear seasonality effect (H-Sum vs H-Aut, R^2=0.15, P=0.05; R-Sum vs R-Aut, R^2=0.09, P=0.11), and the effect was lower in the flow reversal than in the homogenised (i.e. lower R^2). Only k_{wood} and ratio kl_{eaf}-k_{wood} were significantly different between seasons in the homogenised treatment, while k_{leaf}, k_{wood} and ratio kl_{eaf}-k_{wood} were significantly different for the flow reversal (Table 2.7). Furthermore, the homogenised treatment was veritably similar to the control for both seasons (PERMANOVA; H-Sum vs N-Sum, R^2=0.008, P=0.91; and H-Aut vs N-Aut, R^2=0.009, P=0.84).

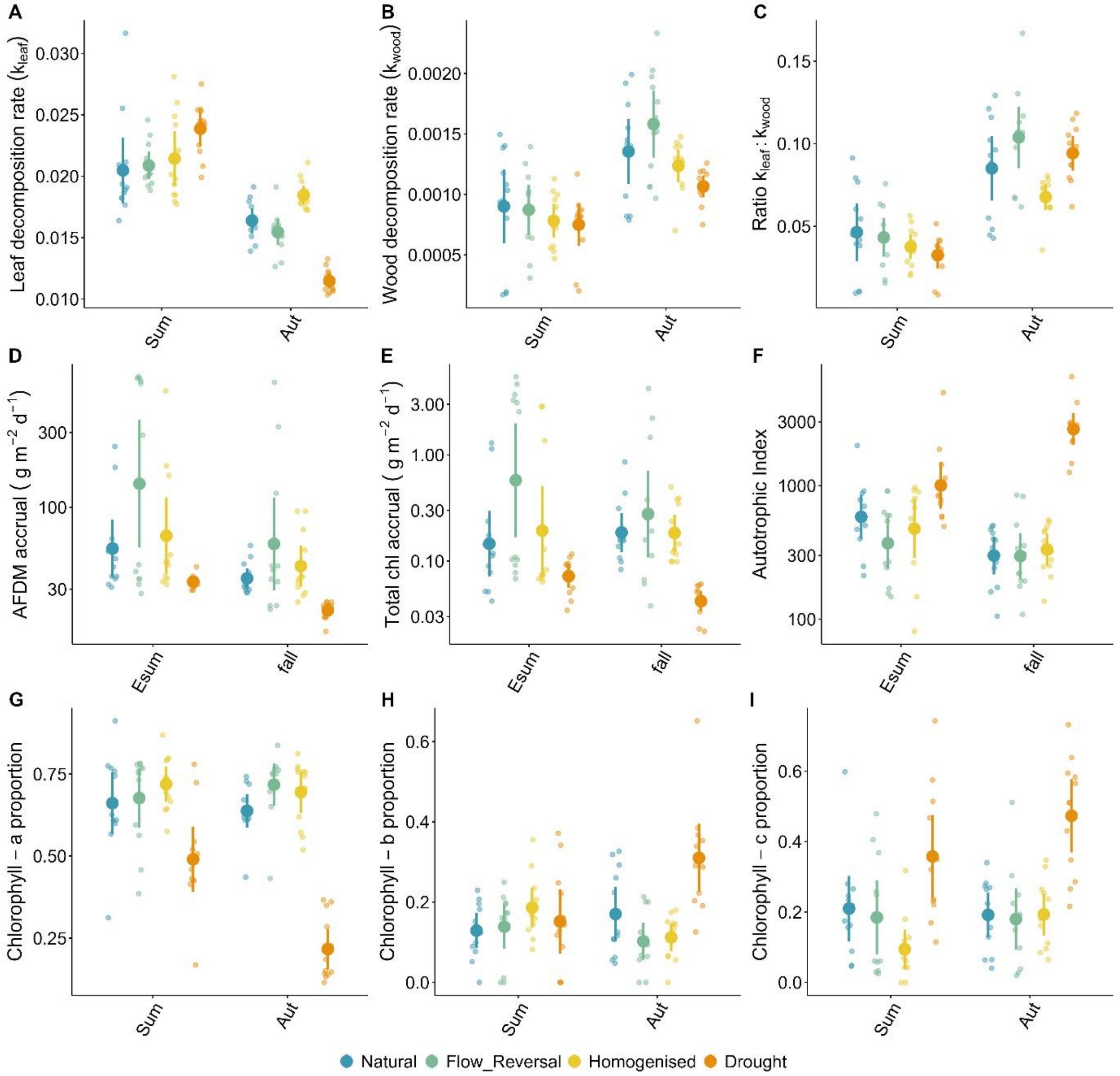

Fig. 2.16. Biofilm processing and growing rates: decomposition rate of leaf (A) and wood (B) substrates, ratio of k_{wood} and k_{leaf}(C), total biofilm accrual (AFDM, D) and autotrophic biofilm accrual (chl_{Tot},, E), autotrophic index (F), and relative proportion of each chlorophyll type to the total chl accrual rate ($Comp_{chl\text{-}a}$, G; $Comp_{chl\text{-}b}$, H; and $Comp_{chl\text{-}c}$, I). Means and CI_{95} are presented for the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue) during the summer (Sum) and autumn sampling (Aut) in 2020. Dots represent the values of each sample.

Table 2.6. Results of the RM-ANOVA with REML estimation of the biofilm variables. Note, all variables were log(x+1) transformed. Additionally, AFDM and chl_{Tot}, were box Cox transformed (Bc) and AFDM were orderNorm transformed (oN). The contribution of the different chlorophyll types is estimated by the ratio of each given chl (a, b, c) to the chl_{Tot}. Thus being: $Comp_{chl-a}$; $Comp_{chl-b}$ and $Comp_{chl-c}$.

variable	treat	Df	Chisq	p_adj	sig
k_{leaf}	**Flow**	**3**	**56.065**	**<0.001**	*******
	Season	**1**	**61.545**	**<0.001**	*******
	Flow:Season	**3**	**21.511**	**<0.001**	*******
k_{wood}	Flow	3	4.285	0.253	ns
	Season	**1**	**47.367**	**<0.001**	*******
	Flow:Season	3	4.075	0.253	ns
k_{leaf}:k_{wood}	Flow	3	2.217	0.529	ns
	Season	**1**	**84.969**	**<0.001**	*******
	Flow:Season	3	6.822	0.117	ns
$k_{dd-leaf}$	**Flow**	**3**	**64.798**	**<0.001**	*******
	Season	**1**	**22.296**	**<0.001**	*******
	Flow:Season	**3**	**22.987**	**<0.001**	*******
$k_{dd-wood}$	Flow	3	2.431	0.488	ns
	Season	**1**	**76.433**	**<0.001**	*******
	Flow:Season	3	2.707	0.488	ns
chl_{Tot}	**Flow**	**3**	**27.348**	**<0.001**	*******
	Season	1	0.156	0.693	ns
	Flow:Season	3	1.982	0.693	ns
$Comp_{chl-a}$	**Flow**	**3**	**224.983**	**<0.001**	*******
	Season	**1**	**5.321**	**0.021**	*****
	Flow:Season	**3**	**16.423**	**0.001**	******
$Comp_{chl-b}$	Flow	3	6.056	0.163	ns
	Season	1	0.532	0.466	ns
	Flow:Season	3	10.062	0.054	ns
$Comp_{chl-c}$	**Flow**	**3**	**26.604**	**<0.001**	*******
	Season	1	1.258	0.393	ns
	Flow:Season	3	1.733	0.63	ns
AFDM	**Flow**	**3**	**18.959**	**0.001**	*******
	Season	**1**	**8.277**	**0.006**	******
	Flow:Season	3	1.148	0.765	ns
AI	**Flow**	**3**	**101.648**	**<0.001**	*******
	Season	1	0.095	0.758	ns
	Flow:Season	3	7.616	0.082	ns

2.3.3.1. Microbial processing rates

In controls, the decomposition rate of wooden detritus accounted only 5 -9 % of the decomposition of the labile material (ratio k_{leaf}:k_{wood} Sum = 0.046 ± 0.03, Aut = 0.085 ± 0.03, Fig. 2.8). Decomposition for leaf detritus was significantly higher in summer than in autumn (MMD, N-Sum vs N-Aut = 0.0040, P=0.04), but the opposite pattern was found for wood (MMD pairwise comparison; N-Sum vs N-Aut; k_{wood}= -0.005, P=0.03). Accordingly, efficiency processing rates of labile substrates were equal between summer and winter (i.e. $k_{dd\text{-}leaf}$), but efficiency was higher for recalcitrant detritus in autumn (i.e. $k_{dd\text{-}wood}$, Table 2.6, Fig. S2.1). All treatments had processing rates of recalcitrant material comparable to the control and similar efficiencies, with main differences significantly explained by season (RM-ANOVA, Chiq=47.3, P<0.001, Table 2.6). In contrast, decomposition of labile material was significantly affected by all the treatments, with different responses depending on the flow treatment as well as the different seasons (i.e. significant interaction).

Decomposition rates of labile material were mostly affected by the drought treatment, with significantly different responses for both seasons (Fig. 8a, Table 2.7 , RM-ANOVA, interaction, Chi= 21,5, P<0.001). In summer, the drought treatment had higher, but not significant, k_{leaf} than the control (Aut, D-Sum vs N-Sum MM=0.0033, P>0.05, Table 2.7), while in autumn, rates were significantly lower (MMD; D k_{leaf}: D-Aut vs N-Aut, MMD=– 0.0048, P<0.001). Accordingly, decomposition processing efficiency in autumn was significantly lower than the control for labile substrates (i.e. $k_{dd\text{-}leaf}$; Fig. S2.1, Table 2.6). On the other hand, in summer, flow reversal and homogenised had similar k_{leaf} than the control, but differences in autumn (Fig. 8a). Contrasting to the other treatment responses, the homogenised treatment presented higher k_{leaf} than the control in autumn (MMD: H-Aut vs N-Aut, MMD=0.002, P=0.042), with decomposition efficiencies ($k_{dd\text{-}leaf}$) similar to summer ones (Fig. S2.1, MMD=-0.00001, P=0.9). Contrary, the flow reversal treatment had decomposition efficiencies of labile detritus lower than the control in autumn (Fig. S2.1, MMD=-0.00015, P=0.034).

2.3.3.2. Biofilm growth rates

In the controls, the autotrophic index of the biofilm was low for both seasons, although slightly higher in autumn (AI, Sum = 691.5 ± 468; Aut = 332.3 ± 139; MMD N-Sum vs N-Aut = -0.66). The composition of the different chlorophylls in the biofilm was similar between seasons, with chl-a accounting for ~65%, chl-b, ~15% and chl-c, ~20%. Drought reduced biofilm growth rates and chanced chlorophyll composition, with higher proportions of chl-c and lower chl-a. In contrast, neither the flow reversal nor the homogenised flow treatments affected these determinants. Nevertheless, high variability of biofilm growth rates in the flow reversal suggested that flow reversal partially affected growth rates.

In the drought treatment, algal accrual was lower than the natural control, with stronger effects in autumn (Fig. 2.8e; chl_{Tot}, MMD=–1.716, P=0.028), whereas AFDM remained unchanged (P>0.05, Table 2.7). Thus, resulting in lower autotrophic biofilm relative to controls (AI, MMD=2.17, P<0.001; Fig. 2.8f). The proportions of each chlorophyll type were impacted by drought. With significantly lower $Comp_{chl\text{-}a}$ and higher $Comp_{chl\text{-}c}$ (Fig. 2.8g, Fig. 2.8i), especially in autumn (MMD=–0.299, P=0.002; MMD=0.20, P=0.03; respectively). In summer, the same patterns for $Comp_{chl\text{-}a}$ and $Comp_{chl\text{-}c}$ were observed, but those were less marked and not significant when compared to the natural control. However, the lower proportion of $Comp_{chl\text{-}a}$ was significant if compared to the homogenised and flow reversal treatment and $Comp_{chl\text{-}b}$ was significantly higher than the homogenised (Table 2.7).

Flow reversal biofilm accrual was not significantly different to the control. Nevertheless, there was high dispersion on accrual between flow reversal flumes, especially marked in summer, which likely resulted in a lack of significance (chl_{tot}, AFDM; Fig. 2.8d-f). Despite this lack of significance, the PERMANOVA R^2 results indicated that the flow reversal was the most different treatment from the control in summer (pairwise comparison PERMANOVA; R-Sum – N-Sum, R^2=0.132). Thus, suggesting that these differences can be attributed to the differences in biofilm accrual in the flow reversal (chl_{tot}, AFDM). Furthermore, the significant seasonal effect and interaction of $Comp_{chl\text{-}a}$ were likely due to the flow reversal, unlike other treatment, presenting higher values in autumn (MMD: R-Sum vs R-Aut, MMD= 0.025, P=0.74; Table 2.7).

Table 2.7. Marginal mean differences between the Flow treatment and season. Values are the difference between the column names group to the row group (i.e. MM N-Sum – MM N-Aut). Bolded values are significant comparisons (P<0.05). Values are mean differences of the transformed data as marginal means depend on the mix effect model used.

			Sum			Aut			
variable	Season	Flow	R	H	D	N	R	H	D
k_{leaf}	Sum	N	0.0004	0.0009	0.0033	**-0.0040**	**-0.0049**	-0.0020	**-0.0089**
		R		0.0005	0.0029	**-0.0044**	**-0.0053**	-0.0024	**-0.0093**
		H			0.0024	**-0.0050**	**-0.0059**	-0.0029	**-0.0098**
		D				**-0.0073**	**-0.0083**	**-0.0053**	**-0.0122**
	Aut	N					-0.0009	**0.0020**	**-0.0048**
		R						**0.0030**	**-0.0039**
		H							**-0.0069**
k_{wood}	Sum	N	0.0000	-0.0001	-0.0002	**0.0005**	**0.0007**	0.0003	0.0002
		R		-0.0001	-0.0001	0.0005	**0.0007**	0.0004	0.0002
		H			0.0000	0.0006	**0.0008**	**0.0005**	0.0003
		D				0.0006	**0.0008**	0.0005	0.0003
	Aut	N					0.0002	-0.0001	-0.0003
		R						-0.0003	-0.0005
		H							-0.0002
Ratio k_{leaf}:k_{wood}	Sum	N	-0.0029	-0.0082	-0.0132	**0.0362**	**0.0534**	0.0204	**0.0450**
		R		-0.0054	-0.0103	**0.0391**	**0.0563**	0.0232	**0.0478**
		H			-0.0049	**0.0445**	**0.0616**	**0.0286**	**0.0532**
		D				**0.0494**	**0.0666**	**0.0335**	**0.0581**
	Aut	N					0.0172	-0.0159	0.0087
		R						-0.0331	-0.0085
		H							0.0246
AFDM	Sum	N	0.4764	0.1209	-0.6611	-0.6191	-0.2939	-0.4125	**-1.9684**
		R		-0.3556	-1.1375	-1.0955	-0.7704	-0.8889	**-2.4448**
		H			-0.7820	-0.7399	-0.4148	-0.5334	**-2.0893**
		D				0.0420	0.3672	0.2486	-1.3073
	Aut	N					0.3251	0.2066	-1.3493
		R						-0.1186	-1.6745
		H							-1.5559
Chl_{tot}	Sum	N	0.7410	0.1306	-0.5972	0.3543	0.4307	0.3519	-1.3623
		R		-0.6104	-1.3383	-0.3867	-0.3103	-0.3891	**-2.1033**
		H			-0.7278	0.2237	0.3001	0.2213	-1.4929
		D				0.9515	1.0280	0.9492	-0.7651
	Aut	N					0.0764	-0.0023	**-1.7166**
		R						-0.0788	**-1.7930**
		H							**-1.7142**
AI	Sum	N	-0.4526	-0.2022	0.5428	-0.6654	-0.6752	-0.5608	**1.5122**
		R		0.2504	0.9954	-0.2128	-0.2227	-0.1082	**1.9648**
		H			0.7450	-0.4632	-0.4731	-0.3586	**1.7144**
		D				**-1.2082**	**-1.2181**	**-1.1036**	0.9694
	Aut	N					-0.0099	0.1046	**2.1776**
		R						0.1145	**2.1875**
		H							**2.0730**
$Comp_{chl-a}$	Sum	N	0.0098	0.0372	-0.1097	-0.0113	0.0356	0.0223	**-0.3104**
		R		0.0275	**-0.1194**	-0.0211	0.0258	0.0126	**-0.3202**
		H			**-0.1469**	-0.0485	-0.0016	-0.0149	**-0.3477**
		D				**0.0984**	**0.1453**	**0.1320**	**-0.2008**
	Aut	N					0.0469	0.0337	**-0.2991**
		R						-0.0133	**-0.3460**
		H							**-0.3328**
$Comp_{chl-b}$	Sum	N	0.0075	0.0491	0.0163	0.0338	-0.0242	-0.0145	0.1459
		R		0.0416	0.0088	0.0263	-0.0317	-0.0220	0.1383
		H			-0.0328	-0.0154	-0.0733	-0.0636	0.0967
		D				0.0175	-0.0405	-0.0308	0.1296
	Aut	N					-0.0580	-0.0483	0.1121
		R						0.0097	0.1700
		H							0.1604
$Comp_{chl-c}$	Sum	N	-0.0235	-0.0967	0.1134	-0.0121	-0.0247	-0.0107	**0.1974**
		R		-0.0732	0.1369	0.0114	-0.0012	0.0128	**0.2209**
		H			**0.2101**	0.0846	0.0721	0.0860	**0.2942**
		D				-0.1255	-0.1381	-0.1241	0.0840
	Aut	N					-0.0126	0.0014	**0.2095**
		R						0.0140	**0.2221**
		H							**0.2081**

2.3.4. Biofilm functional diversity

Biofilm functional diversity showed a similarity among flow treatments, but a clear differentiation from the control. The natural control presented lower mean values for richness (S) and diversity (H) and higher dispersion (i.e. SD) than the rest of the treatments (Fig. 2.9, S= 24.75 ± 5.56; H= 3.09 ± 0.24, Table S2.4). Nevertheless, these differences were not significant given its high variability (ANOVA: S, $F_{3,16}$ =1.59, P=0.24; H, $F_{3,16}$ =2.51, P=0.10; and Ev, $F_{3,16}$ =0.66, P=0.58).

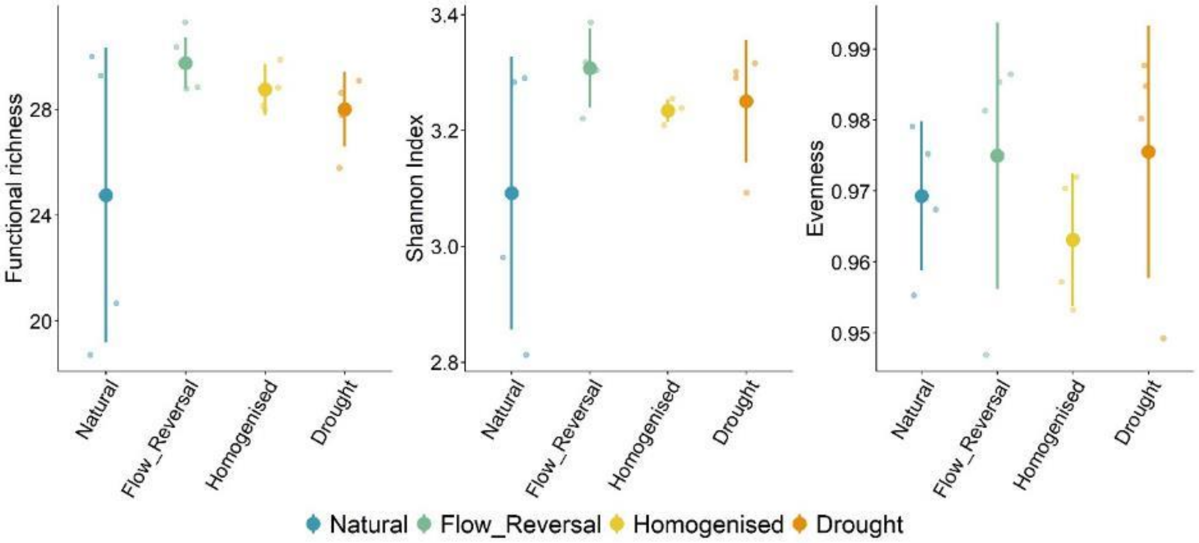

Fig. 2.17. Microbial functional diversity indexes for Ecoplates: functional richness (S), Shannon Index (H), and Evenness (Ev). Means and CI_{95} are presented for the three treatments (flow reversal, green; homogenised, yellow; and drought, orange) and the control (natural, blue) during autumn sampling (Aut) in 2020. Dots represent the values of each sample

Similar to the results observed for the richness and diversity index, NDMS on the individual substrates indicated that the control displayed lower processing rates for all the substrates (i.e. the substrates were negatively correlated with axis 1 but control replicates displayed positive scores, Fig. 2.10). Similarly, when considering substrates by functional groups these were negatively correlated to axis 1 with N control displaying positive scores for this axis (Fig. 2.11). Furthermore, the natural control presented higher dispersion than the flow treatments for both individual substrates and functional groups (ellipse area for substrates N=0.27, H=0.17, R=0.15 and D=0.02; and ellipse area for functional groups, N=0.23, H=0.20, R=0.14 and D=0.02).

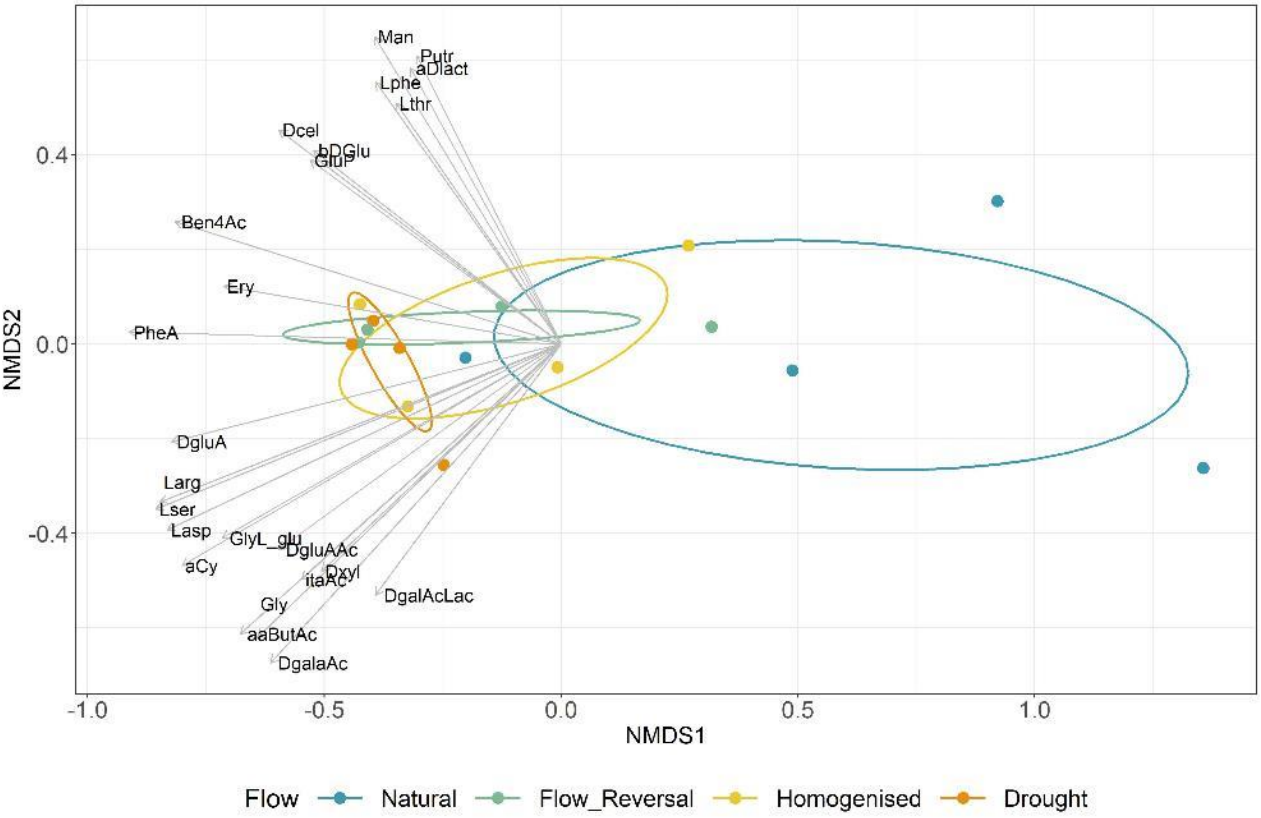

Fig. 2.18. NMDS results from OD of substrates for autumn sampling (Aut) for each of the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue). Ellipses for each season and treatment were drawn using 50% confidence intervals. Kruskal 2D stress was 0.03. Only significant variables are shown: γ-Amino-Butyric Acid (aaButAc), α-Cyclodextrin (aCy), α-D-Lactose (aDlact), β-Methyl-D-Glucoside (bDGlu), 4-Hydroxy-Benzoic Acid (Ben4Ac), D-Cellobiose (Dcel), D-galacturonic Acid (DgalaAc), D-Galactonic Acid γ-Lactone (DgalAcLac), N-Acetyl-D-Glucosamine (DgluA), D-Glucosaminic Acid (DgluAAc), D-Xylose (Dxyl), i-Erythritol (Ery), Glucose-1-Phosphate (GluP), Glycogen (Gly), Glycyl-L-glutamic Acid (GlyL_glu), Itaconic Acid (itaAc), L-Arginine (Larg), L-Asparagine (Lasp), L-Phenylalanine (Lphe), L-Serine (Lser), L-Threonine (Lthr), D-Mannitol (Man), Phenylethylamine (PheA), and Putrescine (Putr).

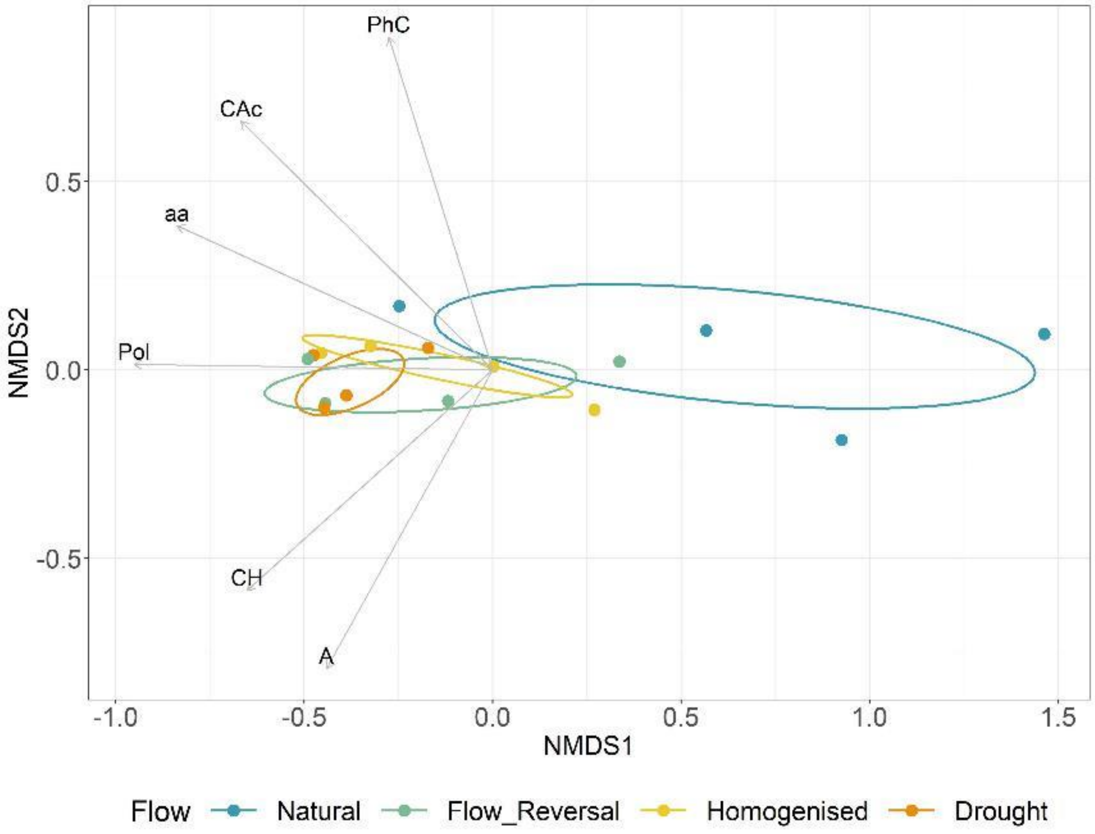

Fig. 2.19. NMDS results from AWCD for functional groups for Aut (Aut) for each of the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue). Ellipses for each season and treatment were drawn using 50% confidence intervals. Kruskal 2D stress was 0.007. Only significant variables are shown: Phenolic Compounds (PhC), Carboxylic Acids (CAc), amino acids (aa), Polymers, (Pol), Carbohydrates (CH), and amines (A).

Flow treatment effects on carbon substrate processing were apparent when considering individual substrates and functional groups (PERMANOVA, Substrates, R^2=0.39, P=0.03; functional groups, R^2=0.44, P=0.0.45). For the individual substrates, these differences were driven by significant changes in use of five substrates: Itaconic Acid (itaAc), α-Cyclodextrin (aCy), i-Erythritol (Ery), D-Xylose (Dxyl) and Glycogen (Gly) (ANOVA: $F_{3,13}$=7.01, P=0.006; $F_{3,13}$=6.55, P=0.007; $F_{3,13}$=6.09, P=0.009; $F_{3,13}$=4.92, P=0.019; $F_{3,13}$=3.89, P=0.037; respectively; Table S2.5). For the carbon functional groups, significant differences were evident for amines (A), carbohydrates (CH) and polymers (Pol) (Fig. 2.13, ANOVA; $F_{3,13}$ =4.79, P=0.02; $F_{3,13}$ =3.54, P=0.04; $F_{3,13}$ =4.74, P=0.21, respectively).

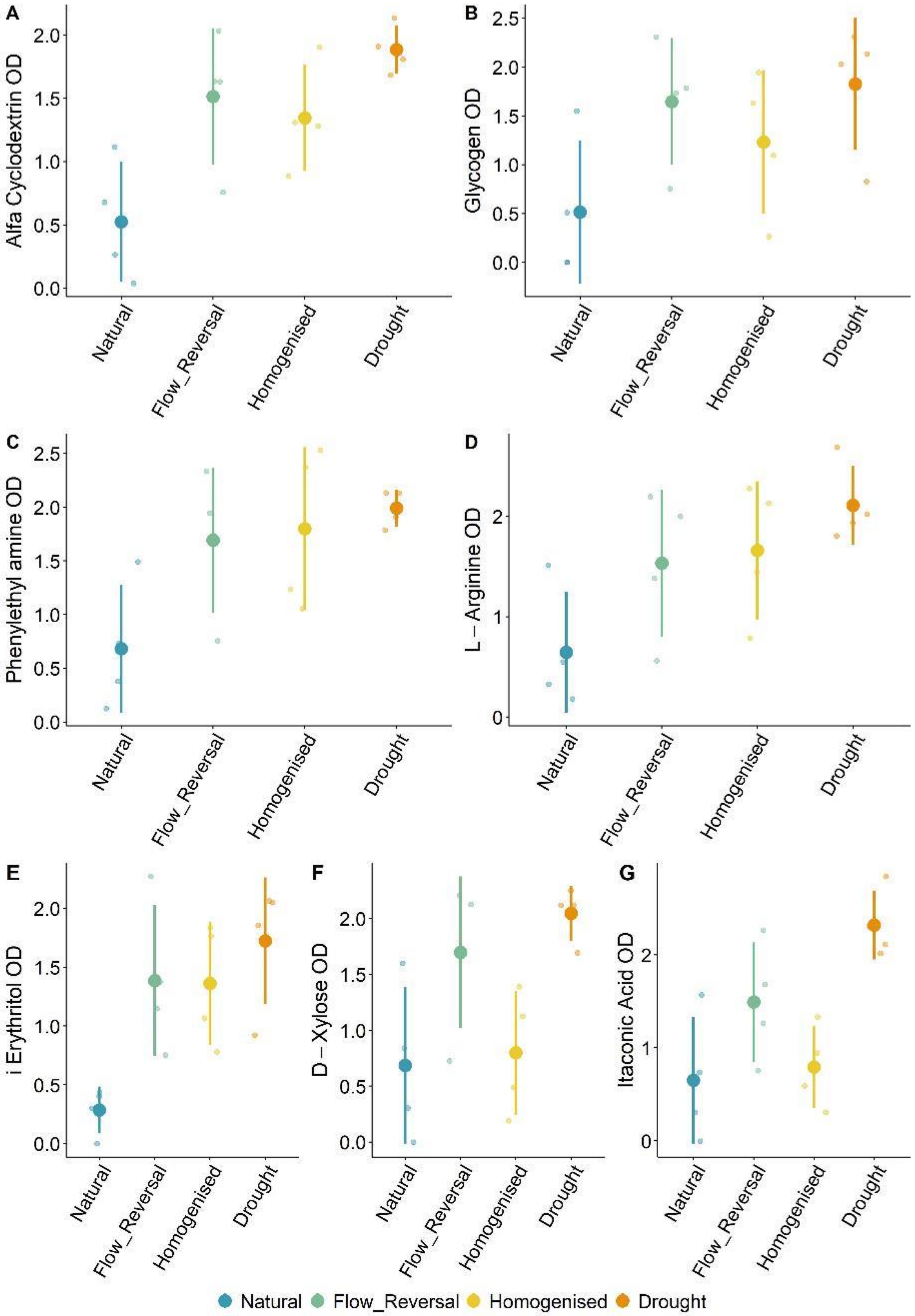

Fig. 2.20. Substrate processing rates (optical densities, OD) for Ecoplates carbon sources. Significant uses of the carbon source substrates are presented for the significantly different polymer-like substrates (Alpha-Cyclodextrin, A and Glycogen, B), nitrogen present substrates (Phenylethyl amine, C; and L-Arginine, D) and carbohydrates and carboxylic acids (i-Erythritol, E; D-Xylose, F; and Itaconic Acid, G).

Flow reversal presented higher S and H than the natural control (S=29.75 ± 0.96; H= 3.31 ± 0.07). In ordination space, the flow reversal treatment presented a slight overlap with the natural control (Fig. 2.10 & Fig. 2.11). For the individual substrates, flow reversal was negatively correlated to the first axis with some dispersion along it (i.e. ellipse area = 0.15). Similarly, for carbon functional groups, flow reversal was negatively correlated to the first axis and presented some dispersion along it (ellipse area = 0.14). Given this dispersion and the small number of samples, only Ery OD was significantly higher in the flow reversal than the control (P=0.03).

The homogenised flow treatment was more similar to the control than other treatments in terms of carbon substrate utilisation. Despite being negatively correlated to the first axis of both NDMS (i.e. carbon substrates and carbon functional groups), the homogenised treatment presented the second largest dispersions (ellipse areas; carbon substrates = 0.17; and carbon functional groups = 0.20). While dispersion in S and H was lower than in the control and means higher (S=28.75 ± 0.96, H=3.23 ± 0.02), overall OD was lower for some substrates and functional groups, with values close to the natural control. Furthermore, itaAc OD was significantly lower in the homogenised than in the drought treatment (P=0.025).

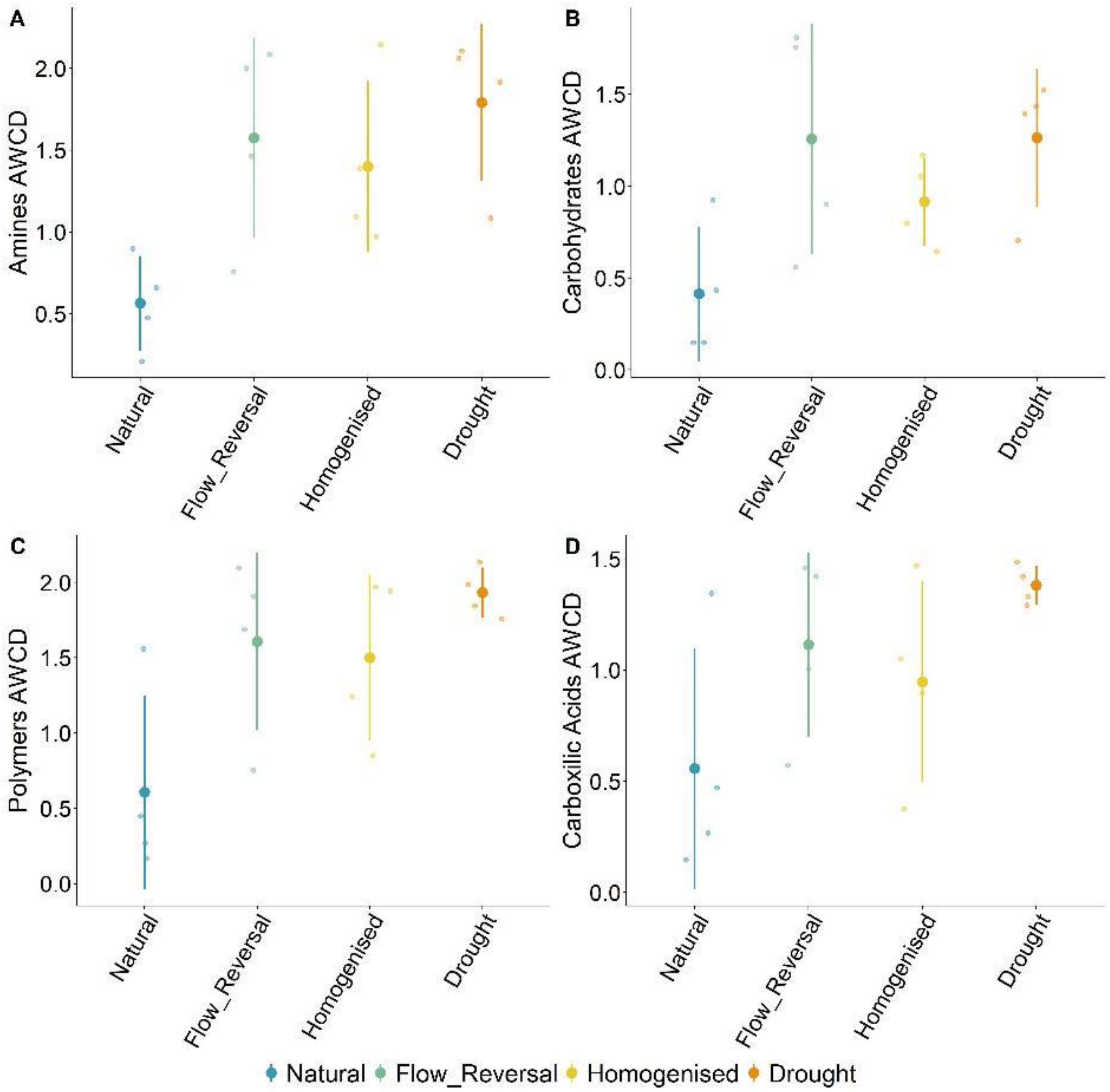

Fig. 2.21. Average substrate processing rates (average well colour development, AWCD) for the carbon functional groups for the natural control (blue) and the three flow treatments (Flow reversal, green; Homogenised, yellow; and drought, orange). Significant uses of the carbon source substrates are presented for the significantly different functional groups: amines (A), carbohydrates (B), polymers (C) and carbolic acids (D).

The drought treatment differed markedly from the control but was similar to the other treatments, all be it with slightly lower S and H and increased within treatment variability (S=28 ± 1.41, H=3.25 ± 0.11). Nevertheless, its dispersion in the NDMS space was the smallest for individual substrates and carbon functional groups (both ellipse area = 0.02) and presented no overlap with the control (Fig. 2.10 & Fig. 2.11). Drought treatment presented significantly higher OD than the N control for aCy, Dxyl, Gly, Ery and itaAc (Fig. 2.12, pairwise comparison, P=0.005, P=0.049, P=0.041, P0.011, and P=0.007, respectively). Also, OD was significantly higher in the drought than in the control for A and Pol (pairwise comparison: P=0.025, P=0.022, respectively).

2.4. Discussion

The main aim of this study was to determine biofilm responses to different degrees of hydrological variability and to alterations in the magnitude and timing of high and low flows. The homogenised flow treatment created a hydrologically stable flow which resulted in similar biofilm growth and decomposition processing rates to the natural control and a more diverse range of carbon substrates processing than the control. This suggests that this stable system might have been controlled by biotic relationships (i.e. competition, predation, parasitism) which limited biofilm growth and processing rates, and that higher competition for resources might have led in higher niche partitioning resulting in a more diversification of carbon processing strategies. Furthermore, carbon substrate processing rates were either similar or higher than the natural, suggesting higher biofilm efficiency. On the contrary, hydrologically unstable flows generated in the drought and the flow reversal treatment resulted in systems being regulated by seasonality of flow dynamics (i.e. abiotic factors). Thus, leading to high temporal variation in ecosystem functioning. As expected, the highly unstable hydrology in the drought treatment resulted in a general reduction of ecosystem processing rates (i.e. growth and decomposition rates) with habitat contraction resulting in a more severe reduction during dry periods. These recurrent drought conditions resulted in biofilm with resistant strategies to desiccation evidenced by higher $\mathrm{Comp}_{\mathrm{chl\text{-}c}}$ (i.e. diatoms) and rapid recovery of microbial functionality on dried biofilm. This resistance of the biofilm preserved a range of carbon processing strategies which was more diverse than the natural control. Furthermore, drought treatment promoted fast growing and opportunistic strategies on the biofilm community when conditions were favourable as evidenced by higher process rates of carbon substrates and higher processing of labile detritus during the wet-period. Flow reversal generated a flow regime with a similar stability to that of natural controls, notwithstanding a reversal in the timing of high and low flow. Nevertheless, this harsh environment did not affect biofilm communities. Only the decomposition rate efficiency of labile detritus was reduced during high-flow. Nevertheless, biofilm accrual dispersion during low flow suggests two stable states in the treatment. Furthermore, the range of carbon substrates processing ability was less variable than the control, suggesting that abiotic factors forced convergent functionality strategies in these systems.

2.4.1. Homogenised flow effects

The homogenised flow treatment created a hydrologically stable flow regime characterized by reduced discharge with limited annual variation and a depth increase. This hydrological stability was expected to increase biofilm biomass, but processing and growth rates would be maintained at similar levels than the control due to resource competition. The results of this chapter might support this hypothesis (H_1). Several studies demonstrated that increase of hydrological stability under dams leads to thicker biofilm (i.e. standing biomass), higher biofilm accrual (i.e. algal and fungal growth rates) and consequently more metabolically active biofilm (Ponsatí *et al.*, 2015; Truchy *et al.*, 2022). Nevertheless, this biomass increase is often linked to higher nutrient levels after hypolimnion dam realises, rather than dependent on hydrological stability alone (Sabater *et al.*, 2008; Ponsatí *et al.*, 2015). Furthermore, it is possible that grazing pressure was enhanced in the system by stability.

The low nutrient concentrations in our experiment were potentially a limiting factor for biofilm growth and processing rates. Thus, increasing the importance of resource portioning and nutrient cycling (Peckarsky, 1983; Poff and Ward, 1989). The relatively high contributions of microbial-source fDOM indicated by the fDOM composition might be the result of biofilm exudates during growing season (summer). Given the lack of sheer stress in the system, macrophytes community's growth might have limited that of epilithic biofilm (Shangguan *et al.*, 2017; Goldenberg-Vilar *et al.*, 2022). Thus, corresponding with the increase of macrophytes observed in the treatments (Arias-Font, unpublished).

The higher diversity of carbon substrates processed in the homogenised flow than in the control suggests an increased functional diversity in this treatment. Thus, partially supporting H_2. Nevertheless, this could have been driven by a thicker biofilm matrix (more cells) which increased the chances of finding more functional strategies (i.e. sample size saturation) and/or by higher competition leading to resource partitioning. Furthermore, given the method used which was independent of cell density (i.e. cell's inoculum was biofilm from cobbles), it is not possible to discern whether the higher decomposition rates of carbon substrates observed in the homogenised flow treatment were the result of more efficient biofilm or a thicker biofilm matrix (i.e. more cells). Biofilm efficiency is often reduced by biofilm matrix thickness which often limits diffusion of nutrients, light and oxygen and might generate redox gradients (Claret, Marmonier and Bravard, 1998; Ponsatí *et al.*, 2015). Thus concurring with previous studies results which found more metabolically active but less efficient biofilms under increase system stability (Sabater *et al.*, 2002; Ponsatí *et al.*, 2015). The methods used in this chapter only gives information on the potential carbon uses of the microbial community, but does not assess efficiency (i.e. cell density/OD) or matrix thickness limitations. However, the higher detritus decomposition of labile substrates observed in autumn may have been the result of this higher efficiency.

2.4.2. Drought effects

The drought flow treatment created a hydrologically unstable flow regime characterised by a reduced discharged with a strong intra-annual variation, with flow intermittency increasing temperature during the hottest months. This highly unstable hydrology resulted in general reduction of biofilm functionality (i.e. growth and decomposition rates) which was exacerbated by dry periods thus supporting H_3 hypothesis. Flow reduction caused habitat contraction sustained across both seasons creating a concentration effect for DOC (i.e. increase concentrations) and reduction in microbial growth and processing rates (Arroita *et al.*, 2017; Arias Font *et al.*, 2021). Given autotrophic biofilm water and light dependency, the limited resilience of their pigments and the limited spatial distribution (i.e. not in the hyporheic zone) resulted in a limited resilience and recolonization capacity (Fischer and Pusch, 2001; Ponsatí *et al.*, 2015; Colls *et al.*, 2021). Thus, leading to a more heterotrophic biofilm (e.g. higher AI ratio). Furthermore, the low dispersion observed in the processing of carbon substrates (i.e. ellipses area) suggests that the unstable hydrology of the system worked as an ecological filter leading to a functional convergence strategy. Under flow intermittency, the hyporheic zone often persists as a highly active processing zone as oxygen limitation is lifted (Burrows *et al.*, 2017; Arias-Real *et al.*, 2020). Thus, supporting processing of DOM and POC within it as suggested by the high degree of fDOM humification in this study. During the dry phase, k_{wood} had a less pronounced reduction than k_{leaf} suggesting that gravel incision of the wood sticks in the hyporheic zone might have promoted their decomposition. Hyporheic zone persistence might also enhance the colonization of new substrates as suggested by the decomposition during non-dry period rates of both labile and recalcitrant material in this study.

During the wet phase, drought treatment endorsed opportunistic strategies on the biofilm community as evidenced by high process rates of carbon substrate (supporting partially hypothesis H_5), which likely resulted in higher processing of labile detritus. Changes in C:N ratios during the dry phase might explain why only labile processing rates increased. During summer, C:N ratios were 22.5 which is above the requirements for epilithic biofilm (i.e. 10.3) but it is below the requirements of litter decomposing biofilm (i.e. 22) (Artigas, Romaní and Sabater, 2008, 2015). During the dry phase, resistant strategies in the biofilm preserved a wide range of carbon processing strategies instead of being lost as habitat contracted (rejecting partially H_5). Some studies have showed that epilithic biofilm can preserve up 20% of live cells during dry events and that high humidity and intermittent precipitation can increase that to 40% (Timoner *et al.*, 2012). Thus, highlighting biofilms resistance to desiccation. Furthermore, after rewetting, biofilm is able to present enzymatic activities equal or higher than pre-drying event (Timoner *et al.*, 2012). Similarly, autotrophic community structure adapted to drying as evidenced by higher $Comp_{chl\text{-}c}$ (i.e. the chlorophyll form associated with diatoms) which confirms H_4 (Ledger *et al.*, 2008).

2.4.3. Flow reversal effects

The flow reversal treatment generated a flow regime with a similar hydrological stability to the natural control, but with the opposite timing of high and low flow. During high flow, we expected that high scouring will reduce biofilm growth and process rates, but only decomposition efficiency of labile organic matter ($k_{dd\text{-}leaf}$) was reduced (i.e. autumn sampling, rejecting H_6). Water quality alterations might have caused this reduction. Temperature increases and increases in humic DOC have been seen to reduce decomposition efficiency of labile materials (Arias Font *et al.*, 2021). Furthermore, flow reversal had the highest values in the range of carbon substances use (S) and higher processing rates than the control (OD) (partially rejecting H_7). Some studies have proved that biofilm are more active under flow reversal (Ponsatí *et al.*, 2015). Furthermore, the carbon substrate usage's dispersion (i.e. ellipses area) was lower than in the control suggesting that convergent functional strategies were generated due to changes of the timing of high and low flows. These flow alteration might have promoted survival strategies based on opportunism homogenization instead of natural rivers spatial specialization (Bernhardt *et al.*, 2017; Palmer and Ruhi, 2019).

Flow reversal resulted in epilithic biofilm growth reaching two clearly differentiated states, specially marked during the low flow. A pair of flumes presented biofilm growth rates 100 times higher than the natural, and the other pair, values similar to the natural. In the high productive flumes, biofilms were matt forming species, instead of crust-like. These specific vegetation cover states are often associated with river flow velocity alterations that trigger a shift from scouring resistant species, to those preferring lower water velocities (Goldenberg-Vilar *et al.*, 2022). This suggest that the matt-forming species probably stabilize in the flumes during low flow. Once the system was established, the algae structure and density reduced flow velocities and scouring. During high flow, bed mobilization would happen in small patches instead of homogeneously (Piqué *et al.*, 2016) and the small openings inhibit recolonization of the crust-like biofilm. This extensive biofilm growth (i.e. river bed greening) is a natural phenomenon increasing worldwide (Piqué *et al.*, 2016) which highlights the fragility of this systems to additional stressors.

2.4.4. Natural control and mesocosm realism

This study was able to mimic the timing and magnitude of medium-high flows of the natural flow regime in UK streams. Undisturbed rivers in the UK are characterised by relatively low flows in summer and a progressive increase during autumn, reaching maximum peaks in March / May (Acreman *et al.*, 2008; House *et al.*, 2017). The set-up used in the study was not able to generate high flow peaks (i.e. maximum Q recorded 14.61 L s^{-1} with a mean velocity of 0.097 m s^{-1} in the pools and 0.192 m s^{-1} in the riffles) of headwater streams (i.e. 600 L s^{-1}; Crips and Robson, 1976). Velocities were too low to move gravel, but sand and fine sediments were mobilised (Piqué *et al.*, 2016). Macronutrients and fDOM reflected the characteristics of the aquifer and were similar to chalk streams (Bowes *et al.*, 2011; Halliday *et al.*, 2014; Stuart and Lapworth, 2016). While the system did not receive any direct run-off, the lower C:N ratios recorded in autumn in the borehole seemed to reflect a remobilization of N on the catchment level (BOREHOLE data, TN: August 2020, 2.66 mg L^{-1}, September 2020, 6.78 mg L^{-1}; and November 2020 4.823 mg L^{-1}). Nevertheless, macronutrient concentrations were quite low in our systems, especially for P. Thus, suggesting that the flumes were P limited and P was likely sequestered in the biomass of the flumes (Bowes, Leach and House, 2005). Nevertheless, these low concentrations of P are realistic and representative of concentrations downstream of dams (Maavara *et al.*, 2015).

Our experimental setup in an outdoor facility enabled us to capture seasonal changes in water quality (i.e. N remobilization) as well as seasonal and stochastic variability in local meteorological conditions (i.e. declining day length, temperature decrease, rainfall events). This includes factors which are often overlooked in indoor experiments (Bedolfe, 2015; Romero *et al.*, 2019; Gionchetta *et al.*, 2020). Furthermore, the relatively large size of our flumes enabled us to capture certain degree of spatial variability (i.e. pools and riffles) which has been achieved in few studies (Elsaesser *et al.*, 2013; Thompson *et al.*, 2013; Saffarinia, Anderson and Herbst, 2022). Here, we found that microbial breakdown rates for labile (mean ± SD: 0.018 ± 0.004; range: 0.031–0.010 d^{-1}) and recalcitrant (mean ± SD: 0.001 ± 0.0004; range: 0.0023-0.0001 d^{-1}) in our flumes were comparable to ranges reported for temperate streams across northern Europe (Chauvet *et al.*, 2016) and comparable to natural leaves (medium and fast decomposing) (Follstad Shah *et al.*, 2017). Microbial breakdown rates for recalcitrant organic matter (mean ± SD: 0.001 ± 0.0004; range: 0.0023-0.0001 d^{-1}) were as well comparable to European streams (Arias-Real *et al.*, 2020). Furthermore, the spread in the carbon processing strategies observed in the control, suggest that the system presented a range of different ecological niches which promote specialization. Thus, being typical of natural streams in which processing of OM and nutrients is based on hot-spots.

2.5. Summary and synthesis

This chapter highlight the importance of hydrological stability and predictability of low and high flows on determining biofilm functioning, reflecting the role of flow regime patterns into shaping ecosystem succession and the importance of biotic and abiotic relationships controlling ecosystems functioning. The results of this chapter revealed that flow stability promotes the ecosystem functioning to be controlled by biotic factors (competition, predation, parasitism) resulting in higher niche partitioning and specialization. On the contrary, lower system stability led to a high temporal variability of the functioning of the ecosystems which might suggest system vulnerability. The results of this chapter suggest that harsh abiotic conditions led to convergent functional strategies. Despite the overall functional reduction in the drought treatment, drought unpredictability did not lead to a functional collapse but generated resilience and resistant strategies in the system promoting ecosystem functions during wet phase and in refugia. The changes on the timing of low and high flows generated a weak effect on ecosystem functioning, but two stable states for the biofilm component were revealed by the results.

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3. Effects of flow alteration on stream macroinvertebrate communities

3.1. Introduction

River impoundment is one of the main drivers of river degradation (Petts, 1984; Zarfl *et al.*, 2015). Worldwide, there are approximately 58,500 large dams (>15m in height) and an estimated 2.8 million small dams, such that only 23 % of rivers globally flowing uninterrupted to the ocean (International Commission on Large Dams, 2011; Lehner *et al.*, 2011; Grill *et al.*, 2019). Given rising human population size and associated energy demands globally, especially in developing countries, dam construction is expected to increase strongly in coming decades. For instance, over 3700 large hydropower dams are known to be either planned or actively under construction worldwide (Zarfl *et al.*, 2015). Whilst dams provide a reliable source of electricity and insure water demands, their environmental impacts on the water courses they modify can be serious and long-lasting. Dams typically fragment rivers (Grill *et al.*, 2019), modify physicochemical conditions (e.g. nutrients, sediment and water temperature) (Maavara *et al.*, 2015, 2017) and change the flow regime of the rivers (Poff *et al.*, 2007). Alterations to the natural flow regime in dammed rivers are often unsynchronized with species life-history adaptations affecting their physiological requirements and/or reproduction (Tennant, 1976; Poff *et al.*, 1997; Lytle and Poff, 2004).

The analysis of assemblage taxonomic composition and functional traits are used widely to quantify responses of communities to hydraulic alterations (Statzner and Bêche, 2010; Tupinambás *et al.*, 2014; Dolédec *et al.*, 2015; White *et al.*, 2017). In comparison with traditional taxonomy, functional traits confer some advantages including (a) spatial consistency (no regional effects), (b) statistical discrimination between tested environmental variables, and (c) mechanistic understanding of community responses to stressors (individual traits responses to stressors) (White *et al.*, 2017). However, taxonomical structure can provide additional information which sometimes is obscured by functional traits analysis such as invasive species presence or extinction of less abundant taxa (i.e. density-dependent extinction being independent of their functional traits) (Bunn and Arthington, 2002; Pimm, Jones and Diamond, 2011; Sarremejane *et al.*, 2021). In rivers, the main focus for assessing biodiversity alterations has been on macroinvertebrates communities due to their sensitivity to change (i.e. bioindicators), wide distribution and relevant role in ecosystem functioning (Menezes, Baird and Soares, 2010).

The effects of dams on macroinvertebrates communities structure is highly variable and dependent on regional patterns (climate, altitude) and dam characteristics (height, residence type, hypolimnetic realise, dam purpose) (White *et al.*, 2017; Wang *et al.*, 2020). Most studies reveal that dams reduces species richness downstream while also impacting the abundance of species that persist (Ellis and Jones, 2013; Wang *et al.*, 2020). These changes are mainly driven by increases in abundance of tolerant taxa below dams (e.g. Hirundinea, Crustacea, Mollusca and Diptera taxa), while susceptible taxa as Plecoptera and Trichoptera are reduced in either abundance and/or richness (Ellis and Jones, 2013; Krajenbrink *et al.*, 2019; Wang *et al.*, 2020). Furthermore, dams often lead to weakening of seasonal flow dynamics (flow homogenisation) which dampens the seasonal patterns of macroinvertebrate communities in impounded rivers (Krajenbrink *et al.*, 2019).

This reduction of macroinvertebrates richness is expected to be the result of selective extinction of species with mal-adapted traits to a given hydraulic alteration (Chase, 2007). Where dams alter flow, physicochemistry and substrate within the streams constitutes physical disturbance (sensu Pickett and White, 1985), and *r*-strategist macroinvertebrate taxa with small body size, generalist diet and multivoltine life histories with will be promoted

(Townsend and Hildrew, 1994). Hydraulic alterations also modify the structure and functioning of food webs (Ledger *et al.*, 2013; White *et al.*, 2017). Below dams and impoundments, macroinvertebrate communities typically present a reduction of macroinvertebrate shredders, filter-feeders and predators, while scrapers are increased (Menéndez *et al.*, 2012; Mbaka and Mwaniki, 2017; White *et al.*, 2017; Sarremejane *et al.*, 2021). These changes are often associated with enhanced primary productivity, substrate change and increased fine sediment deposition in water courses downstream of dams (Benítez-Mora and Camargo, 2014). Where dams reduce water velocity below dams, community composition typically switches from lotic to lentic species (Ellis and Jones, 2013).

Much of our current knowledge of dam impacts is biased toward the stressors generated by hydropower, water supply and multi-purpose dams (Wang *et al.*, 2020). Here, flow regimes are either characterised by significant daily variations and abrupted flow magnitude changes (hydropower), or in contrast, by reductions of the magnitude of low and high flows (water supply). Consequently, our understanding of other flow regime alterations, such as complete flow homogenization (flood control dams or basal flow release) and flow reversals (i.e. alteration of timing/seasonality of low and high flows in irrigation dams) is still relatively limited despite their widespread occurrence (Poff *et al.*, 2007). Furthermore, reduction of flow magnitude below dams, combined with the current climate scenarios, is leading to an increasing number of normally perennial rivers shifting to intermittence during the driest months (Ledger and Milner, 2015). Greater understanding of these flow regime effects is urgently needed to underpin advice to water management practitioners. Furthermore, disentangling how flow interacts with water temperature, nutrients and sediment downstream of dams and impoundments will help inform environmental flow applications (Poff, 2018).

The research reported in this chapter addresses some of these gaps in knowledge by conducting a manipulative experiment in stream mesocosms to compare the effects of contrasting regulated flow regimes on macroinvertebrate community structure and functional trait composition. Mesocosm experiments have enabled testing changes in flow regimes independent of other environmental covariables such as sediment and nutrients which confound field studies. Four flow regimes were evaluated in this stream mesocosm experiment: (1) natural flow (control, summer low flow/ winter high flow), (2) flow 'reversal' (i.e. summer high flow/ winter low flow typical of irrigation dams), (3) homogenised flow (limited seasonal variability) and (4) drought (80% flow reduction).

The flow regimes investigated capture contrasting stability and availability of substrate (homogenised vs drought), and an alteration of the timing of high and low flows (natural vs flow reversal). The substratum stability conferred by homogenised flows could limit physical disturbance to the macroinvertebrate community, potentially promoting biomass accumulation (no scouring or drift) and biotic interactions (e.g. competition and predation) (Poff and Ward, 1989; Townsend and Hildrew, 1994). Under drought flow regimes, by contrast, the stress generated by wetted area reduction and low oxygen levels could reduce overall macroinvertebrate abundance and promote *r*-strategists. Flow 'reversal', that is the inversion of the timing of high and low flows, could enhance macroinvertebrate drift during high-flows and disrupt adult emergence and reproduction (Lytle and Poff, 2004). Consistent with the above, the following hypotheses were tested:

- Relative to the natural control, flow ‘reversal’ will result in:
    - H_1. Lower density and richness of macroinvertebrates, due to drifting events during typically (i.e. long-term) low flow periods which will be unsynchronized with species life-story;
    - H_2 A predominance of larger body size and interstitial taxa, to avoid drifting;
- Relative to the natural control, flow homogenisation will result in:
    - H_3: Higher richness and density of macroinvertebrates, due to stable hydrology providing more permanent niches and species interaction promoting more biodiversity;
    - H_4: A predominance of aquatic-life cycles, grazer-scrapers and lentic species, due to stable hydrology;
- Relative to the natural control, drought will generate:
    - H_5: Lower abundance and richness of macroinvertebrates due to habitat contraction and dewatering events promoting the presence of tolerant taxa;
    - H_6: A predominance of *r*-strategists with small body size, generalist and multivoltine taxa, to prevail in this unstable hydrological ecosystem;

3.2. Methods

3.2.1. Experimental design and sampling

This research was conducted using 16 outdoor stream mesocosm (each 12 m length × 0.5 m width × 0.5 m height, Fig. 2.2) at the University of Birmingham Environmental Change Outdoor Laboratory (EcoLaboratory, Fig. 2.1). Mesocosms were groundwater fed semi-recirculating flumes with three riffle-pool sequences created using commercial gravel (see section 2.2.1 for detailed description of the facility and flume set up). The flumes were seeded with macroinvertebrates from two local streams and were left to colonize for a year before the flow treatments were applied (see 2.2.2). In June 2019, the control and the three flow treatments were applied (i.e. natural control, flow reversal, flow homogenisation and drought; see section 2.2.2 for treatments and section 2.3.1 for hydrographs). After a year of simulated flow treatments, macroinvertebrates sampling was carried out on two occasions, in summer (July 2020) and autumn (November 2020).

3.2.2. Data collection and processing

For each sampling event in a flume, one integrated benthic macroinvertebrate sample was collected for each habitat type (i.e. riffle and pool) using a small modified Surber sampler ($0.0225m^2$, mesh = 300µm). The integrative samples were assessed by pooling three samples (one in each section) per each of habitat type (n=3, $0.0665m^2$). During riffle dewatering in the drought treatment, abundance was assumed to be zero. Samples were preserved in 70% Industrial Methylated Spirit (IMS) and stored for analysis. Samples were sorted from attached detritus and algae, and identified to the lowest practicable taxonomic level (species or genus, except for Oligochaeta and Chironomidae, which were identified to sub-class and family) and counted. Abundance from the two habitat types (riffle and pool) were combined to provide a single abundance measure for each flume (individuals m^{-2}).

Environmental variables linked with food resources and refugia were estimated to determine their association with macroinvertebrate community structure and functional trait composition (biological properties). The percentage cover of macrophytes was estimated for each sampling time per each flume (Fig. 3.1). For each macroscopic plant taxon (algae, Cladophora; and macrophytes, *Myriophyllum spicatum,* Milfoil; and *Veronica beccabunga,* Brooklime), the percentage cover was estimated visually in each riffle or pool (in an area 1.5m × 0.5m). To estimate volume, water depth was measured in the middle of each section to the nearest 0.5 cm. Section water volume was calculated as area of the section multiplied by water depth. If water depth was 0, we assumed an aerial height of 0.01 m to avoid unrealistic estimates (i.e. accounting for macrophytes presence even if depth is 0). Macrophytes volume (m^3) was then estimated as the area of the substratum covered by a given macrophyte and multiplied by the macrophyte stand height (Wood *et al.*, 2012). Stand height was estimated as depth for submerged macrophytes and depth plus 0.01 cm for exposed ones (i.e. brooklime). For null depth, a 0.01cm stand height was also assumed for the macrophytes present. We calculated the percentage of volume inhabited (PVI) by each macrophyte species as macrophyte volume divided by section volume. The percentage of empty space (i.e. without macrophytes, PVI_{em}) was calculated by subtracting the sum of the three macrophytes from 100. The PVI_{em} was calculated to assess possible correlations to gravel affinity species.

Biofilm accrual (AFDM and chl_{tot}) and decomposition (k_{leaf} and k_{wood}) were measured in each of three riffles and three pools per flume in both summer and autumn 2020 (see chapter 1, section 2.5.1). Mean values for each flume were then calculated for: PVI for each taxon (PVI Milifoil, PVI_M; PVI brooklime, PVI_B; and PVI algae, PVI_A), for empty PVI (PVI_{em}), decomposition (k_{leaf}, k_{wood}) and biofilm accrual (AFDM and chl_{tot}). Mean values for all the macronutrients and DOM variables analysis described in chapter 1 were calculated for each flume (see section 2.2.4). For each flume, mean discharge (Q) and depth were calculated for each sampling period using 30 days data (i.e. instant Q and depth) prior to the sampling (see section 2.2.1). A co-linear analysis was used to remove the highly correlated variables from the analysis

(R^2>0.8). The environmental variables selected were: biofilm accrual (AFDM and chl_{tot}), decomposition (k_{leaf} and k_{wood}), DOC quantity and quality (FI, BIX, DOC), nitrogen concentration (TN), water quality (pH, conductivity, DO saturation and temperature) and flow (depth, and Q).

Fig. 3.22 Riffle section used to estimate of macrophytes cover where Brooklime and algae were present.

3.2.3. Processing of macroinvertebrate trait data

Trait values were assigned at the genus level based on fuzzy-coded information. For taxa identified to a higher level (i.e. Chironomidae and Oligochaeta), an average trait was calculated using the genera available belonging to that taxonomic group. Trait genus was assigned using European fuzzy-coded develop by Tachet et al. (2010) for all the taxa, except Diamesinae. Serra et al. (2016) was used for Diamesinae (i.e. Diamesinae and Orthocladiinae share traits in Tachet et al. (2010)). The responses of 16 traits subdivided in 78 functional traits categories were considered (Table S3.1). Maximal potential size traits were initially determined using the categories in Tachet *et al.* (2010). To avoid rare occurrence of given maximal potential size categories (<3 individuals), broader groups were determined (i.e. less frequent size occurrence were grouped with the closest most common size). This was done for the smallest and the largest size defined in Tachet *et al.* (2010). To simplify functional traits, affinity to a generalist diet was calculated as the number of food categories (microorganism, FPOM, CPOM, microphytes, macrophytes, dead animal, microinvertebrates and macroinvertebrates) used by each taxon divided to the total food categories considered (following Chessman, 2015). Similarly, drought resistance was coded as the ratio of the present drought strategies (egg statoblasts, cocoons, housing and diapause) in each taxon to the total strategies considered (following Aspin *et al.*, 2019). To avoid interaction between traits or trait redundancy, only the revised trait was used for diet and drought resistance, and life cycle duration was excluded from the analysis (Verberk, Van Noordwijk and Hildrew, 2013). Given the different responses of biological (i.e. attribute) and ecological traits (i.e. result), these traits were analysed separately (Verberk, Van Noordwijk and Hildrew, 2013), resulting in 14 traits in total (11 biological and 3 ecological) (Table 3.1, Table 3.2, respectively). Prior to analysis, traits categories were standardised from 0 to 1 within each trait to insure equal weight of all the traits. To obtain trait responses in each flume, the abundance data were multiplied by the trait-by-taxon matrix and then once again standardised by trait.

Table 3.8. Functional trait categories based on Tachet *et al.* (2010) analysed in this study.

Functional trait	Functional trait category	Definition
Maximal potential size	small	≤ 0.5 cm
	medium	> .5-1 cm
	large	> 1-2 cm
	vlarge	> 2-4 cm
	vvlarge	> 4 cm
Voltinism	semivoltine	< 1
	univoltine	1
	multivoltine	> 1
Aquatic stages	egg	egg
	larva	larva
	nymph	nymph
	adult	adult
Reproduction	ovoviviparity	ovoviviparity
	isolated eggs	isolated eggs: free and cemented
	clutches	Clutches: cemented or fixed, free, in vegetation and terrestrial
	asexual reproduction	asexual reproduction
Dispersal	aquatic passive	aquatic passive
	aquatic active	aquatic active
	aerial passive	aerial passive
	aerial active	aerial active
Drought resistance	resistant	Presenting resistance forms
	susceptible	No resistance form
Respiration	tegument	tegument
	gill	gill
	plastron	plastron
	spiracle	spiracle
Locomotion	flier	flier
	surface swimmer	surface swimmer
	swimmer	full water swimmer
	crawler	crawler
	burrower	burrower
	interstitial	interstitial
	temporarily attached	temporarily attached
	permanently attached	permanently attached
Generalist diet	generalist	Generalist diet
	specialist	Specialist diet
Feeding habits	absorber	absorber
	deposit feeder	deposit feeder
	shredder	shredder
	scraper	scraper
	filter feeder	filter-feeder
	piercer	piercer
	predator	predator
	parasite	parasite
Thermal preference	cold	cold (< 15°C)
	warm	warm (> 15°C)
	eurythermic	eurythermic

Table 3.9. Habitat preference functional traits

Functional trait	Functional trait category	Definition
Transversal distribution	river channel	river channel
	banks	banks, connected side-arms
	pools	ponds, pools, disconnected side-arms
	marshes	marshes, peat bogs
	temporary waters	temporary waters
	lakes	lakes
	groundwaters	groundwaters
Substrate preference	boulders	flags/boulders/cobbles/pebbles
	gravel	gravel
	sand	sand
	silt	silt
	macrophytes	macrophytes
	microphytes	microphytes
	twigs roots	twigs/roots
	detritus	organic detritus/litter
	mud	mud
Flow velocity preference	null	null
	slow	slow (< 25 cm/s)
	medium	medium (25-50 cm/s)
	fast	fast (> 50 cm/s)

3.2.4. Data processing and statistical analysis

3.2.4.1. Macroinvertebrate community structure

A range of metrics was calculated to assess macroinvertebrate community structure: taxon richness (S), taxonomic diversity, evenness and total density. Taxon richness was calculated as the sum of present taxa in each flume (S). Diversity was calculated as Shannon index (H), and evenness (Ev), as the division of H to the log(S). Total density was calculated as total abundance divided by the area sampled. Repeated-measures linear mixed-effect models were used to test treatment effects on macroinvertebrates community structure metrics. Following Zuur et al. (2010), all variables were assessed using graphical tools to ensure assumptions of regression analysis were fulfilled (e.g. normality and homoscedasticity) and transformed when necessary. After testing for interaction effects, we fitted the most parsimonious model. For each response variable (i.e. metric), I fitted a global model of the following form:

Response ~ Flow × Season + (season | flume [random intercept])

Repeated-measurement two-way ANOVA type II tests were performed for all final models and p-values adjusted to control for Type 1 error following Benjamini and Hochberg (1995). Posteriorly, a non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity was used to assess flow treatment and seasonal effects on macroinvertebrate community relative abundance. A Shepard plot was used to assess how original dissimilarities are preserved in the distance matrix. Overall group similarities were test using an analysis of similarities (ANOSIM). To obtain a stable p-value, 49999 permutation were used in all the test. The effect of flow treatment and season were tested using a PERMANOVA on relative abundances constrained by flume (i.e. permutation). A pairwise-comparison PERMANOVA was used to identify differences between groups.

An indicator species analysis was used to identify the taxa associated to each treatment (De Cáceres and Legendre, 2009). This method estimates association of taxa into previously defined groups based on a point-biserial correlation coefficient (r_{pb}). This provides a correlation coefficient ranging from 1 to 0, with 1 indicating a strong association of the taxa to the grouping. This method was applied on relative abundances using flow treatment as grouping; to identify the taxa associated with each treatment. Posteriorly, each flow treatment and season were grouped independently, to assess seasonal differences in each flow treatments. The significance of this correlation was tested by 49999 permutations.

3.2.4.2. Macroinvertebrate functional traits: biological properties

To assess the strength of the flow treatments effects on macroinvertebrate functionality, the correlation between macroinvertebrates biological trait categories and environmental factors (i.e. quantitative and qualitative) was assessed using a combination of RQL analysis (Dolédec *et al.*, 1996) and fourth-corner test (Dray and Legendre, 2008). The RQL analysis provides an ordination method to maximizes the covariation of trait data (Q) and the environmental data (R), by constraining it by species abundances-data (L). To perform the RQL, three tables were created: table L containing abundance data in each flume and season; table Q containing biological trait-by-taxon data; and R containing environmental data for each flume and season. To assess how taxa attributes were affected by the flow treatments, only biological properties were used in this analysis (following Verberk, Van Noordwijk and Hildrew, 2013). A combination of quantitative and qualitative data was included in the environmental data (R table). Based on the prior co-linear analysis (see 3.2.2), the quantitative environmental variables considered in the analysis were: k_{leaf}, k_{wood}, chl_{tot}, FI, BIX, DOC, TN, pH, conductivity, DO saturation, temperature, PVI_A, PVI_M, PVI_W, PVI_{em}, depth, Q. Flow treatment and season were considered as qualitative variables (i.e. factors). Prior to the RQL analysis, table R (environmental-data) was subjected to a Multivariate Correspondence Analysis (MCA) using the Hill and Smith method for a combination of factors and numerical variables (Hill and Smith, 1976). The table L (species abundance-data) was transformed to a Correspondence Analysis (CA), and table Q (trait data) to a Fuzzy Correspondence Analysis (FCA). The RLQ analysis on two-axis was performed providing a spatial distribution of traits and environmental variables. The fourth-corner analysis was then used to determine significance of correlations between trait and environmental variables on the RLQ axis (Dray *et al.*, 2014). Thereafter, the fourth-corner model was used to detect correlations between environmental variables and traits variables. Model 6 was used which combines the two hypotheses of: (model 2) species abundance with fixed traits is related to environmental-data; and (model 4) species abundance with fixed environmental descriptors is related to species traits. The statistical significance was tested by 49999 permutations and corrected for Type I error. Posteriorly, correlations of traits and environmental variables were explored to obtain a more detailed information of flow effects. Depending on the nature of the environmental variable, its correlation to the trait category was explored using an RM-ANOVA for categorical variables (i.e. flow treatment and season); and correlation coefficient (R^2) for continuous variables. For continuous variables, a threshold of $R^2<0.4$ was set-up to avoid unrealistic interpretations.

3.2.4.3. Macroinvertebrate functional traits: ecological traits

For all ecological traits, a repeated measured mixed-effect models were used to test treatment effects on each of the ecological trait categories. All trait categories were assessed using graphical tools to ensure assumptions of regression analysis were fulfilled and transformed when necessary (Zuur, Ieno and Elphick, 2010). For each trait category, a global model of the following form was fitted:

Response ~ Flow × Season + (1 | flume [random intercept])

Repeated-measurement two-way ANOVA type II tests were performed to test significance and p-values adjusted to control for Type 1 error. All analysis were performed using R version 4.2.1. (R Core Team) and the packages *lme4* (Bates *et al.*, 2015) for the mixed effect models, *bestNormalize* (Peterson and Cavanaugh, 2020) for data transformation, *sjstats* (Lüdecke, 2020) for regression and ANOVA, *vegan* (J Oksanen, FG Blanchet, R Kindt, 2022) for diversity indexes and NDMS, *ade4* (Dray and Dufour, 2007) for RQL analysis and fourth-corner; and *indicspecies* (De Cáceres and Legendre, 2009) for indicator species analysis.

3.3. Results

3.3.1. Macroinvertebrate community composition

Twenty-seven macroinvertebrate taxa were identified among the different flow treatments and seasons (Table 3.3). Taxon richness per flume (integrated sample) ranged from 6 to 18, and density from 2170 to 31,022 individuals per m^{-2}. A core group of 7 taxa dominated the samples, by order of relative abundance, namely: *Asellus aquaticus* (Isopoda, Asellidae), *Gammarus pulex* (Amphipoda, Gammaridae), *Potamopyrgus antipodarum* (Gastropoda, Tateidae), *Crangonyx pseudogracilis* (Amphipoda, Crangonyctidae), Oligochaeta, *Ancylus fluviatilis* (Gastropoda, Planorbidae) and *Radix peregra* (Gastropoda, Lymnaeinae). These taxa represented between 82.3% to a 99.7% of the total abundance for 93% of the samples (Table 3.3, Table S3.2).

Table 3.10 Relative abundance of the recorded taxa in each of the flow treatments (flow reversal, homogenised and drought) and the control (natural). Data are presented as mean and SD for relative abundance (0-1) for each season. Taxa is order based on the mean relative abundance for all the treatments. See Table SX for total densities.

	Natural		Flow Reversal		Homogenised		Drought	
	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn
Asellus aquaticus	0.229±0.164	0.091±0.1	0.184±0.078	0.134±0.131	0.41±0.276	0.251±0.209	0.656±0.08	0.407±0.23
Gammarus pulex	0.197±0.102	0.085±0.076	0.163±0.186	0.517±0.208	0.11±0.068	0.249±0.188	0.037±0.009	0.024±0.024
Crangonyx pseudogracilis	0.06±0.05	0.027±0.035	0.113±0.085	0.108±0.11	0.209±0.138	0.17±0.11	0.192±0.07	0.399±0.213
Potamopyrgus antipodarum	0.326±0.337	0.637±0.354	0.013±0.021	0.074±0.088	0.038±0.059	0.034±0.036	0.002±0.002	0.017±0.021
Oligochaeta	0.029±0.028	0.028±0.039	0.096±0.1	0.057±0.061	0.045±0.017	0.134±0.131	0.053±0.024	0.13±0.115
Ancylus fluviatilis	0.078±0.079	0.082±0.12	0.112±0.2	0.005±0.004	0.01±0.015	0.029±0.035	0.005±0.006	0.014±0.026
Radix peregra	0.004±0.004	0.004±0.005	0.162±0.172	0.062±0.03	0.013±0.026	0.059±0.081	0.005±0.007	0.001±0.001
Orthocladiinae	0.004±0.007	0±0	0.016±0.021	0±0	0.083±0.157	0±0	0.004±0.003	0±0
Polycelis nigra tenuis	0.004±0.005	0.015±0.02	0.002±0.004	0.002±0.004	0.008±0.008	0.021±0.041	0.027±0.025	0.003±0.004
Baetis rhodani	0.02±0.018	0.014±0.02	0.037±0.055	0.009±0.011	0±0.001	0.002±0.003	0±0	0±0
Plectrocnemia conspersa	0.007±0.007	0.002±0.003	0.038±0.048	0.016±0.016	0.004±0.003	0.009±0.011	0.001±0.001	0±0
Physa fontinalis	0.02±0.027	0.013±0.026	0±0	0±0	0.006±0.011	0.021±0.041	0±0	0±0
Tanytarsini	0.002±0.002	0±0	0.003±0.003	0±0	0.04±0.071	0±0	0.012±0.011	0±0
Polycentropus flavomaculatus	0.004±0.003	0.001±0.001	0.027±0.02	0.003±0.004	0.004±0.004	0.007±0.009	0±0	0±0
Dugesia polychroa	0±0	0±0	0.013±0.024	0.01±0.02	0.001±0.002	0.007±0.009	0.001±0.001	0±0
Diamesinae	0.004±0.003	0±0	0.004±0.002	0±0	0.008±0.013	0±0	0.002±0.002	0±0
Hydroptila	0.003±0.003	0±0	0.013±0.022	0±0	0±0.001	0±0	0.001±0.002	0±0
Planorbarius corneus	0.004±0.005	0.001±0.001	0±0	0±0.001	0.001±0.001	0.005±0.008	0.001±0.002	0.001±0.002
Chironomini	0±0.001	0±0	0±0	0±0.001	0.008±0.01	0±0	0±0	0±0
Glossiphonia	0.004±0.006	0±0	0.002±0.002	0±0.001	0.001±0.001	0.001±0.001	0.001±0.001	0.001±0.001
Limoniidae	0±0.0005	0±0	0±0	0±0	0±0.0004	0±0	0±0	0.002±0.0022
Dendrocoelum lacteum	0±0	0±0.0006	0±0	0±0	0±0.0004	0±0.0009	0±0.0004	0±0
Empididae	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0.001±0.0024
Sympetrum	0±0	0±0	0±0	0±0	0±0.001	0±0	0±0	0±0
Batracobdella paludosa	0±0	0±0	0±0	0±0.0006	0±0	0±0	0±0	0±0
Psychodidae	0±0	0±0	0±0	0±0	0±0	0±0	0±0.0004	0±0
Stratiomyidae	0±0	0±0	0±0	0±0	0±0.0003	0±0	0±0	0±0

Total macroinvertebrate density was significantly influenced by flow treatment (RM-ANOVA, Table 3.4). The control channels supported higher densities than other flow treatments, with stronger effects in autumn; Fig. 3.2), while the flow reversal treatment channels supported the lowest densities (marginal effect; P<0.1, Table S3.3). Macroinvertebrate taxon richness was significantly affected by season with lower values recorded in autumn (Table 3.4, Table S3.3, Fig. 3.2). Taxon richness was weakly affected by flow treatment (P<0.1). Nevertheless, the RM pairwise comparison indicated significantly lower richness in the drought treatment in autumn (MMD= -0.25, P=0.04, Table S3.3). Macroinvertebrate diversity (H) and evenness were unaffected by the flow treatment or the season (Table 3.4, Table S3.3).

Table 3.11. Results of the RM-ANOVA with REML estimation of the macroinvertebrates diversity index for the mix effect models. Note, total density and Shannon index were log(x+1) transformed. Parameters showed: degrees of freedom (Df), chis-square (Chi-sq), adjust p-value (p-adj) and level of significance (sig). Levels of significance presented are: non-significant (ns), <0.05 (*), <0.01 (**), and <0.001 (***). Significant results are showed in bold.

Index	Factor	Df	Chi-sq	p-adj	sig
Total density	**Flow**	**3**	**11.970**	**0.022**	*****
	Season	1	1.937	0.246	ns
	Flow:Season	3	3.096	0.377	ns
Richness (S)	Flow	3	7.334	0.093	ns
	Season	**1**	**71.753**	**<0.001**	*******
	Flow:Season	3	5.563	0.135	ns
Shannon Index (H)	Flow	3	8.567	0.107	ns
	Season	1	3.237	0.108	ns
	Flow:Season	3	5.311	0.15	ns
Evenness (ev)	Flow	3	5.743	0.187	ns
	Season	1	0.035	0.853	ns
	Flow:Season	3	6.964	0.187	ns

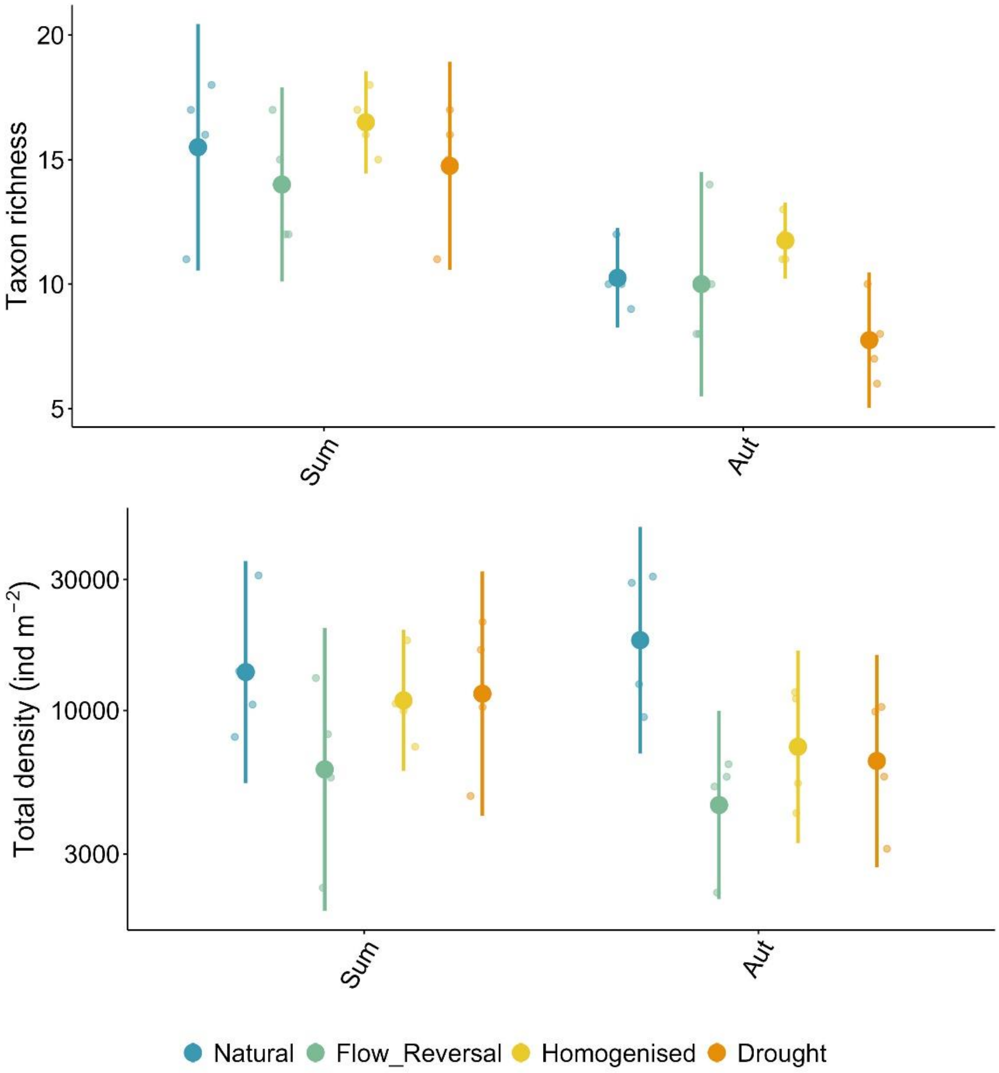

Fig. 3.23. Total richness (S, top) and total density (bottom). Means and CI_{95} are presented for the three treatments (flow reversal, green; homogenised, yellow; and drought, orange) and the control (natural, blue) during the summer (Sum) and autumn sampling (Aut) in 2020. Dots represent the values of each sample.

There was a clear differentiation of community structure among the flow treatments and seasons as revealed by NMDS (Fig. 3.3). The control and the flow treatments were separated along the Axis 1 of the NMDS ordination for both seasons (KD-stress=0.16). The NMDS captured the majority of the original data variation (Shepard plot, non-metric R^2=0.97, Fig. S3.1). There was a significant dissimilarity/differentiation of macroinvertebrate communities among flow treatment and seasons (ANOSIM; R=0.41, P<0.001). Both flow treatment, season and their interaction had a significant effect on macroinvertebrate community structure (Table 3.5). All the treatments were significantly different from the control (Pairwise PERMANOVA, Table S3.4). Community structure in the homogenised flow treatment overlapped strongly with the other flow treatments, and was not significantly different from the flow reversal and only significantly different to the drought (R^2=0.14, P=0.049, Table S3.4).

Table 3.12. Permutational Multivariate Analysis of Variance (PERMANOVA) results for the Bray-Curtis distance matrix on structural composition of macroinvertebrate communities. Parameters showed: degrees of freedom (Df), sum of squares (Sum Of Sqs), R^2, F-statistics (F) and adjust p-value (P).

Variable	Df	Sum Of Sqs	R^2	F	P
Flow	3	2.387	0.388	6.432	0.001
Season	1	0.290	0.047	2.346	<0.001
Flow x Season	3	0.506	0.082	1.363	0.018
Residual	24	2.969	0.483		
Total	31	6.153	1.000		

The control was characterised by greater densities of *Gammarus pulex*, *Physa fontinalis*, *Potamopyrgus antipodarum*, *Plectrocnemia conspersa and Baetis rhodani* (Indicator Species Analysis (ISA), Table S3.5). In autumn, differences in community structure between controls and the other flow treatments was mainly attributable to high densities of *P. antipodarum*

(ISA, Fig. 3.3, Table S3.6). Flow reversal community composition overlapped with controls along axis 1, sharing high densities of *G. pulex*, *P. conspersa* and *B. rhodani*, but differed along axis 2 with higher densities of *R. peregra* than controls (ISA, Fig. 3.3, Table S3.5). The homogenised flow treatment was similar in composition to controls and the flow reversal treatment, reflecting high densities in *G. pulex* (ISA, Table S3.5), but had higher densities of *C. pseudogracilis* and Chironomini, especially in summer when *A. aquaticus* was also abundant (Table S3.6). Drought-impacted communities were also characterised by high densities of *C. pseudogracilis* and *A. aquaticus* in summer (Table S3.5 and S3.6). A clearer differentiation in autumn was associated with the presence of Limoniidae in the drought treatment (Table S3.6). Consistent with taxon richness data (Fig. 3.2), summer was characterised by more taxa, independently of treatment, notably *A. aquaticus*, Hydroptila, Orthocladiinae, Tanytarsini, Chironomini, Diamesinae and *Glossiphonia* (Table S3.7).

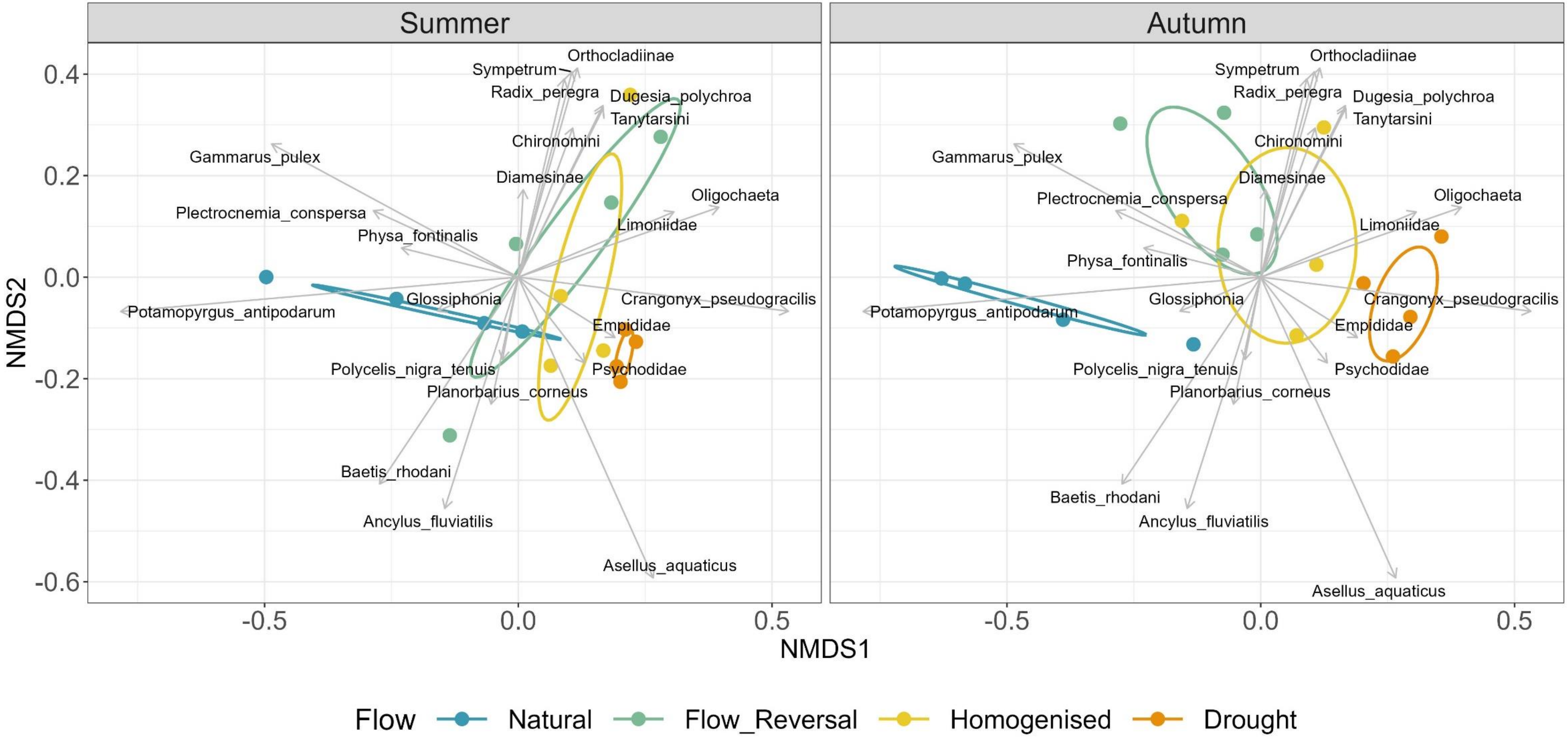

Fig. 3.24. NMDS plot of macroinvertebrate community composition based on Bray-Curtis distance on relative abundance. For each of the three treatments (Flow reversal, green; Homogenised, yellow; and Drought, orange) and the control (blue), summer and autumn are presented. Kruskal 2D stress was 0.16. To avoid excessive overlap, only the more contributing species are shown (P<0.7).

3.3.2. Macroinvertebrates biological functional traits

The RQL analysis explained 56% and 27.4% of the total variance along the ordination axis one and two, respectively, and retained a considerable amount of inertia of the table ordinations (Table 3.6). There were significant relationships between RLQ axis and some of the environmental and trait variables. From the environmental variables (table Q), axis RLQ1 was negatively correlated with discharge (Q), percentage of empty volume (PVI_{em}) and the control (natural); and positively correlated to drought (Fig. 3.4). Axis RLQ2 was positively correlated to total chlorophyll (chl_{tot}) and flow reversal. Regarding functional trait variables, axis RLQ1was negatively correlated to egg (aquatic life stage), scraper (feeding strategy), aerial passive and aquatic active (dispersal); and positively to larva, aquatic passive, and interstitial (Fig. 3.5). Axis RLQ2 was positively correlated to cold, clutches and very vlarge (>2cm) and negatively to ovoviviparity. Nevertheless, while the fourth-corner analysis revealed a significant relationship between species abundance and environmental variables (P=0.002, L × R, model 2), there was no significant relation between environmental variables and functional traits (P=0.98, R × Q, model 4).

Table 3.13. Summary of the ordinations for the R, L and Q tables and the RQL analysis. Variance is presented as a percentage and eigen values within brackets.

Analyses	Variable	Axis 1	Axis 2
R/MCA	Variance (λ)	21.8 (4.8)	18.4 (4.06)
L/CA	Variance (λ)	33.2 (0.6)	18.4 (0.3)
Q/FCA	Variance (λ)	24.3 (0.26)	13.5 (0.15)
RLQ	Variance (λ)	56.0 (0.17)	27.3 (0.08)
	Covariation	0.41	0.28
	Correlation	0.55	0.37
	R -Variance (λ)	78.8 (3.8)	77.8 (6.9)
	Q (Variance)	54.7(0.14)	80.5 (0.33)

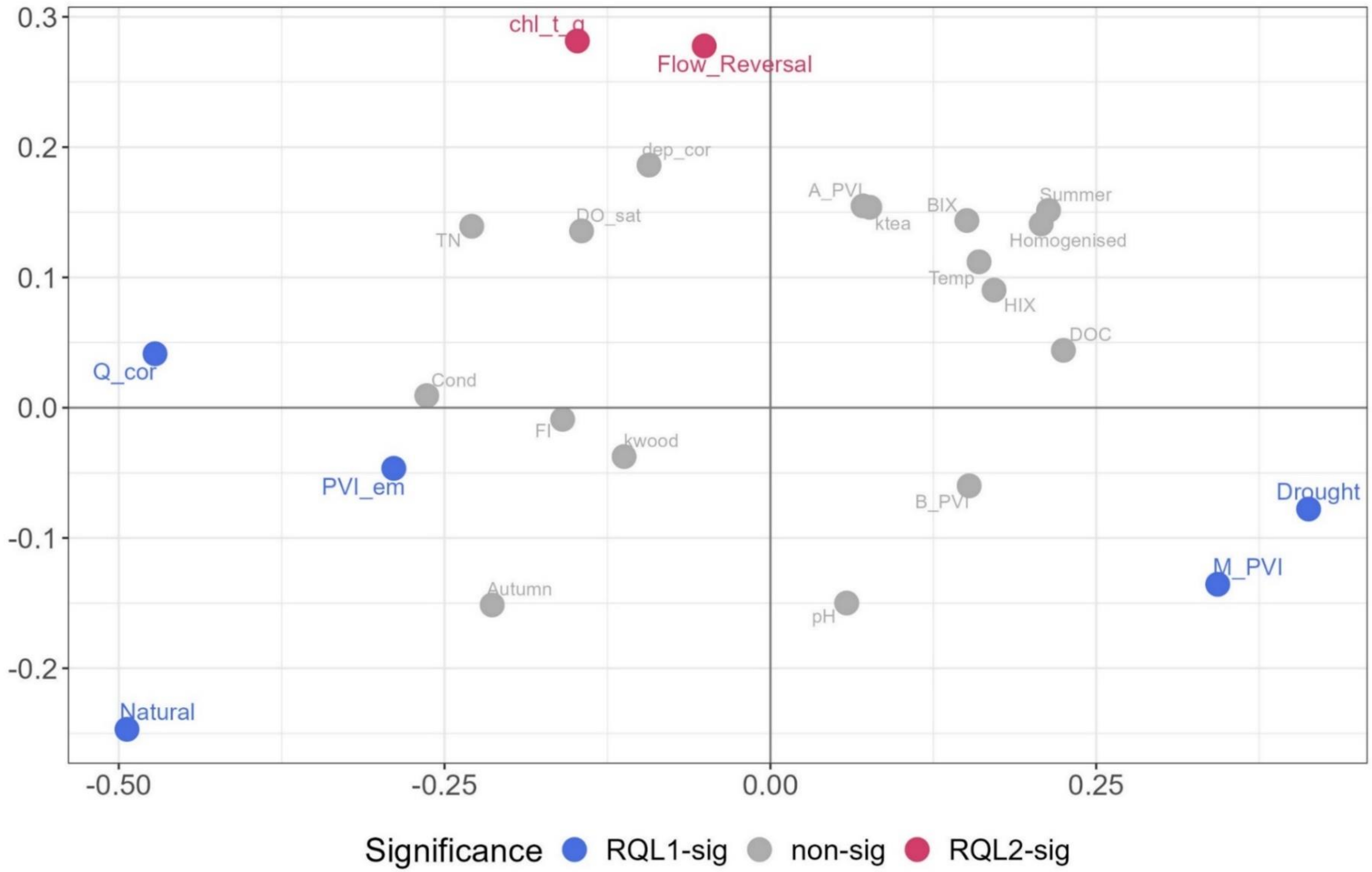

Fig. 3.25. Ordination of environmental variables (R loadings) along the RQL axis one and two showing the fourth-corner analysis results between RLQ axis and environmental variables. The different colours of the environmental variable denote whether the trait is not significant (non-sig, grey), significantly correlated to axis 1 (RQL1-sig, blue) and significantly correlated to axis 2 (RQL2-sig, pink). The environmental variables considered in the analysis were: k_{leaf} (ktea), k_{wood} (kwood), chl_{tot} (chl_t_g), FI, BIX, DOC, TN, pH, conductivity (Cond), DO saturation (DO_sat), temperature (Temp), PVI_A (A_PVI), PVI_M (M_PVI), PVI_W (W_PVI), PVI_{em} (PVI_em), depth (dep_cor), Q (Q_cor), flow treatment (natural, drought, flow_reversal and homogenised) and season (summer and autumn).

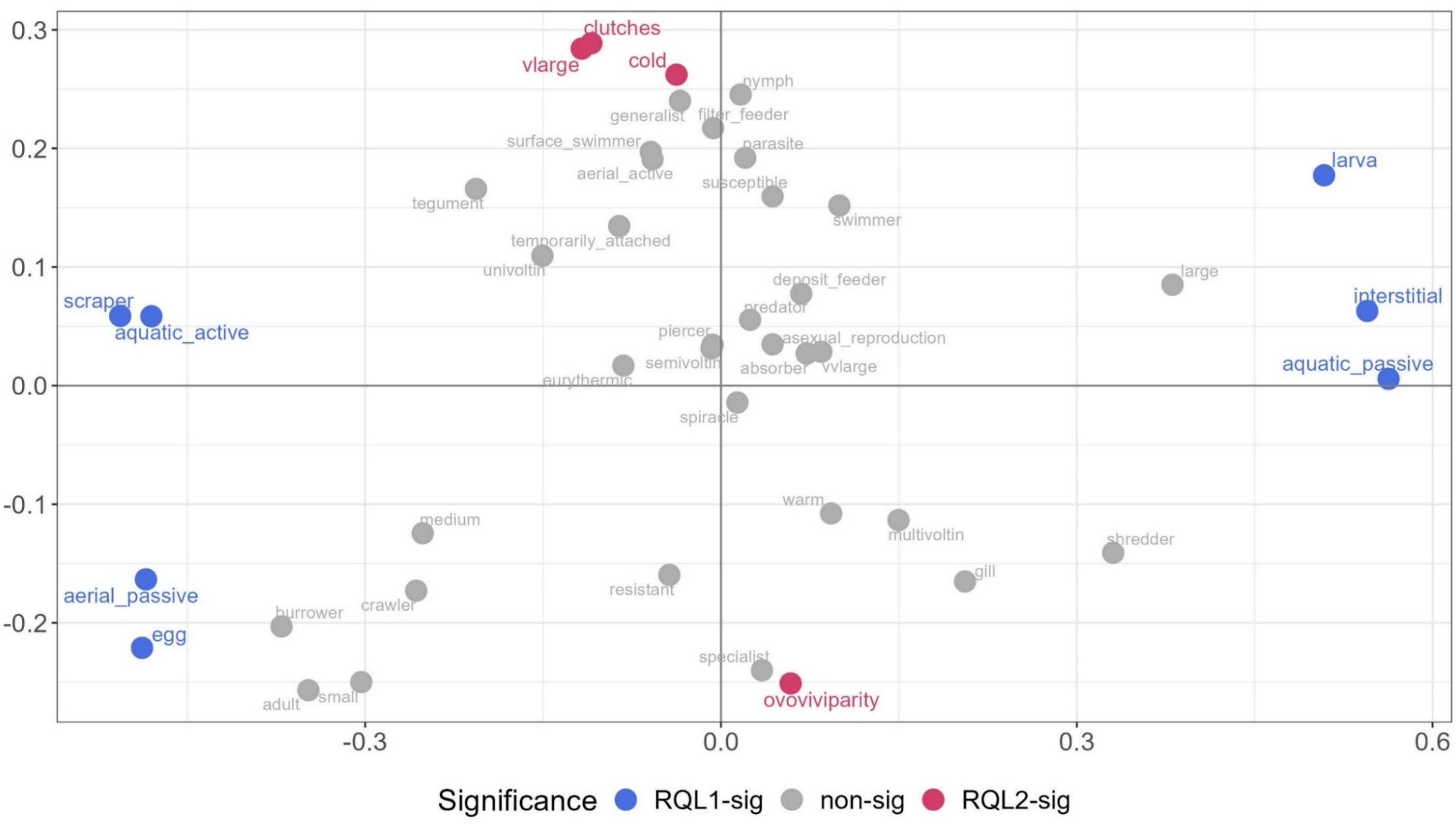

Fig. 3.26. Ordination of biological traits (Q loadings) along the RQL axis one and two showing the fourth-corner analysis results between RLQ axis and biological trait categories. The different colours of the biological trait category denote whether the trait is not significant (non-sig, grey), significantly correlated to axis 1 (RQL1-sig, blue) and significantly correlated to axis 2 (RQL2-sig, pink).

The RQL analysis discriminated the flow treatments and the control along the RQL axis (Fig. 3.6B). The controls were negatively correlated with axes 1 and 2 and were associated with *P. antipodarum* (Fig. 3.6A) and to aerial passive dispersion and eggs as life history attributes. Drought was correlated positively with axis 1 and negatively with axis 2; and was associated with *A. aquaticus* and *C. pseudogracilis* and with interstitial, aquatic passive and ovoviviparity functional traits. The homogenised flow treatment was positively correlated to axis 1 and 2 and were associated with Empididae, Chironomini, *Sympetrum*, *Batracobdella paludosa*, Stratiomyidae, Oligochaeta, *G. pulex*, Psychodidae and Limoniidae, and to larvae as an aquatic life stage. The flow reversal was negatively correlated to axis 1 and positively to axis 2, which showed a strong overlap with *P. conspersa*, and was associated with the remaining macroinvertebrate taxa. These were the three chironomid families (Tanytarsini, Diamesinae, Orthocladiinae), *P. flavomaculatus*, *B. rhodani*, the remaining mollusc taxon (*R. peregra*, P. *fontinalis*, *A. fluviatilis* and *P. corneus*), *Glossiphonia*, all the flat worms (*Dendrocoelum lacteum*, *Dugesia polychroa* and *Polycelis nigra/tenuis*) and Hydroptila. Flow reversal was associated to very large, aquatic active, and scraper functional traits. For all the flow regimes, autumn sampling had more negative scores for both axes in line with the lower richness observed.

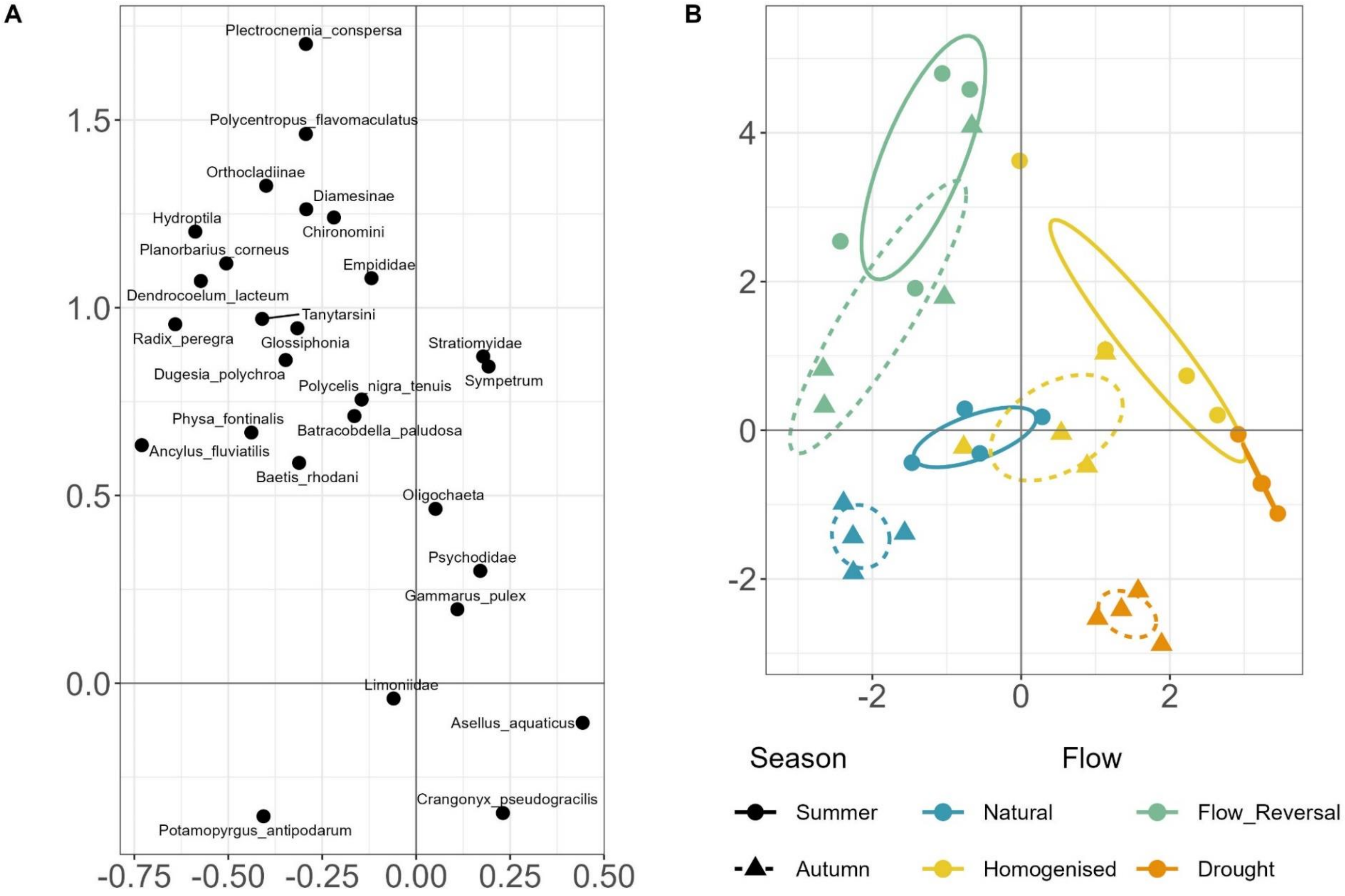

Fig. 3.27. RQL axis plot showing species scores (A, Q row scores) and flumes (B, R scores). Values are presented for the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue) during the summer (Sum, round) and autumn sampling (Aut, triangle) in 2020. Coloured dots represent the values of each flume and black one's values for species.

The fourth-corner analysis (model 2) was used to identify correlations between functional traits and qualitative or quantitative environmental factors. From the 44 trait categories analysed, 16 biological traits were affected by the flow treatments and 10 traits were affected by four other environmental variables (Q, k_{leaf}, chl_{tot}, PVI_M) (Fig. S3.2). Consistent with this, two-way RM-ANOVAs were used to test the effect of flow treatment on the significant 16 biological traits detected by the four-corner analysis (Table S3.8). All the 16 traits were significantly affected by flow treatment (RM-ANOVA, Table S3.8). These significant effects were on the following traits: maximum potential size (small and vlarge), aquatic stages (egg, larva and adult), dispersal (aquatic passive, aquatic active and aerial passive), resistance forms (diapause), locomotion (swimmer, crawler, burrower and interstitial), generalist diet (generalist and specialist) and feeding habits (shredder and scraper). Significant seasonal effects occurred in 8 of the trait categories (RM-ANOVA, Table S3.8). The significant seasonal effects were on potential size (small), aquatic stages (egg, larva and adult), dispersal (aquatic active) and locomotion (swimmer, burrower and interstitial).

In the controls, small and large were the predominant body size (Fig. 3.7A and B) and egg and adult were the more common aquatic stages (Fig. 3.7F-H). Dispersion in controls was dominated by aquatic passive strategies (Fig. 3.7I), but aquatic active and aerial passive dispersal was also evident (i.e. relative abundance >0.1, Fig. 3.7G-I). Specialists represented a large proportion of the functional diet strategies in controls, and shredders and scrapers represented around the 80% of the feeding groups (Fig. 3.8). Crawlers was the most common locomotion strategy representing around 60%, followed by burrowers, swimmers and interstitial fauna (Fig. 3.9D-F).

In the drought treatment, large was the most common body size (Fig. 3.7 A-C) and very large taxa were less abundant than the controls, especially in summer (Table S3.9). Aquatic life stages were evenly distributed across egg, larva and adult; creating a significant difference to the control as eggs and adult were lower in the drought, and larva higher (Fig. 3.7 D-E, Table S3.9). Dispersion under drought was mainly aquatic passive, with significantly lower relative abundance of aquatic active and aerial passive than controls (Fig. 3.7 G-I, Table S3.9). While generalist and specialist traits were found in similar proportions to those of the control, scrapers were scarcer (Fig. 3.8, Table S3.9). Crawlers dominated locomotion strategies under drought, but were significantly less common than in controls. Interstitial locomotion was common (~30% of taxa) and significantly higher than in controls (Fig. 3.9F, Table S3.9). Swimmers occurred commonly in the drought treatment whereas burrowers were less abundant than in controls (Fig. 3.9C-F, Table S3.9).

In the homogenised flow treatment, large maximal size was the most common, with small being significantly lower and very large higher than the control in autumn (Fig. 3.7A-C, Table S3.9). Aquatic stages were evenly distributed between egg, larva and adult (Fig. 3.7D-F). Similar to the drought treatment, larva was significantly higher than the control while adult and egg were lower (Table S3.9). Aquatic passive was the dominant locomotion strategy with a considerable contribution of aquatic active. Aquatic passive was significantly higher in the homogenised flow than the control and aerial passive lower (Fig. 3.7G-I, Table S3.9). Generalist and specialist taxa were evenly distributed in summer, but dominance of generalists was apparent in autumn with significantly higher relative abundance than the control; and a reduction of scrapper taxa (Fig. 3.8, Table S3.9). Crawler was the most common locomotion strategy but with significantly lower abundances than in the control, in favour of swimmer and interstitial which were significantly higher than the control (Fig. 3.9C-F, Table S3.9).

In the flow reversal treatment, large was the most common maximal size followed by vlarge, with small body size being significantly lower and very large significantly higher than the control in autumn (Fig. 3.7A-C, Table S3.9). Similar to the other flow treatments, aquatic stages were evenly distributed (Fig. 3.7D-F). Larva was significantly higher than the control and adult and egg were significantly lower (Table S3.9). Aquatic passive was the dominant dispersion strategy, followed by aquatic active. Nevertheless, none of them were significantly different than the control and only aerial passive was lower in autumn (Fig. 3.7G-H, Table S3.9). Generalist dominated during both season with a stronger effect in autumn, which resulted significant differences to the control (Fig. 3.8A, Table S3.9). Similar to the homogenised treatment, scrapper relative abundance was lower in autumn (Fig. 3.8A-G, Table S3.9). Locomotion presented similar responses as the flow homogenised treatment. While crawler was the most common locomotion strategy, this was significantly lower than in the control and swimmer and interstitial were higher (Fig. 3.9C-F, Table S3.9).

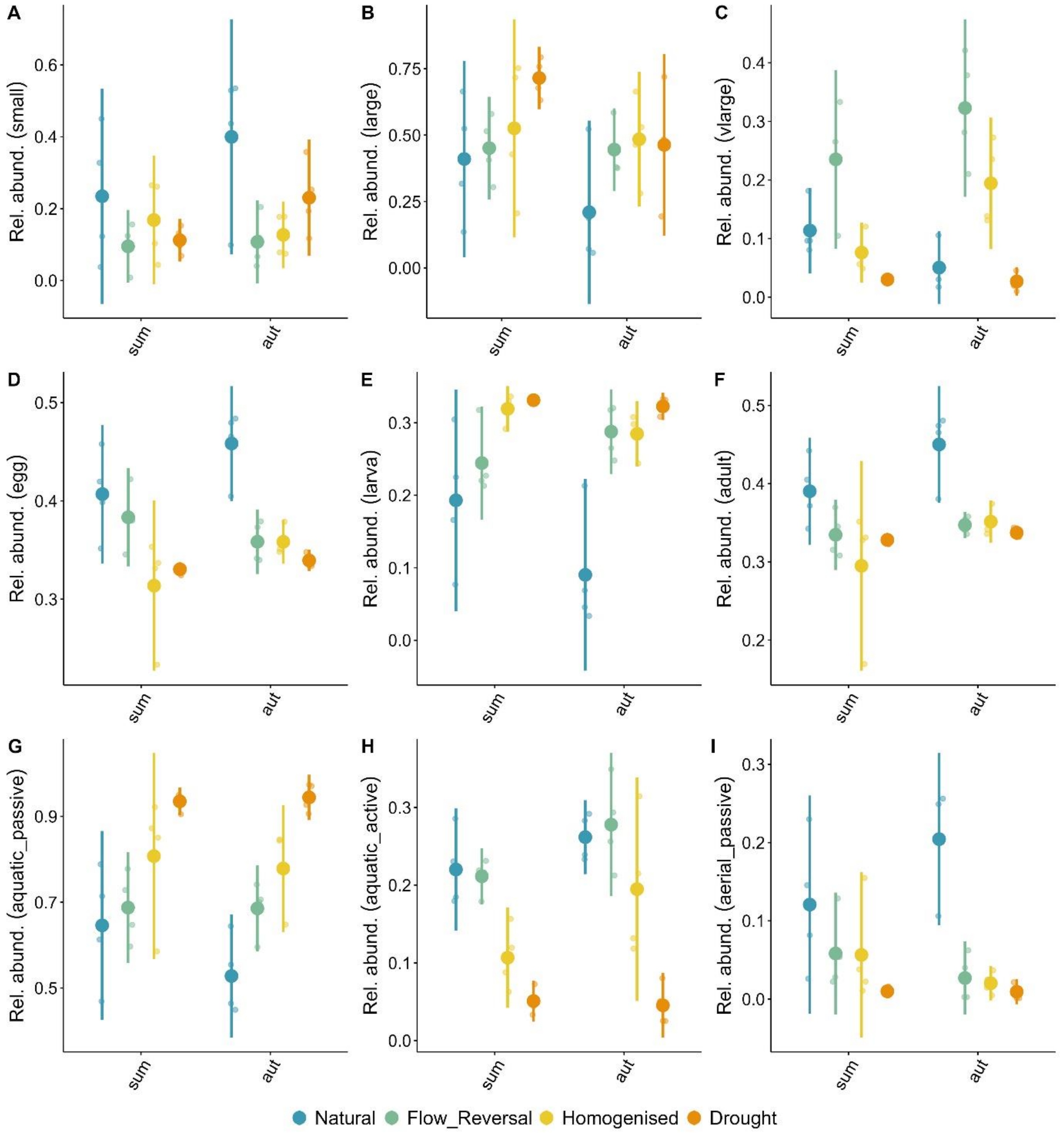

Fig. 3.28. Life cycle functional traits. Only the functional trait categories with a significant effect of flow treatment (ANOVA) are presented. Functional trait categories are organised by trait: potential size (A, B, C), life cycle duration (D, E), aquatic life stages (F, G, H) and dispersal (I, J, K). Notice that large is not significant but it is kept to visualise trait distribution. Means and CI_{95} are presented for the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue) during the summer (Sum) and autumn sampling (Aut) in 2020. Dots represent the values of each sample.

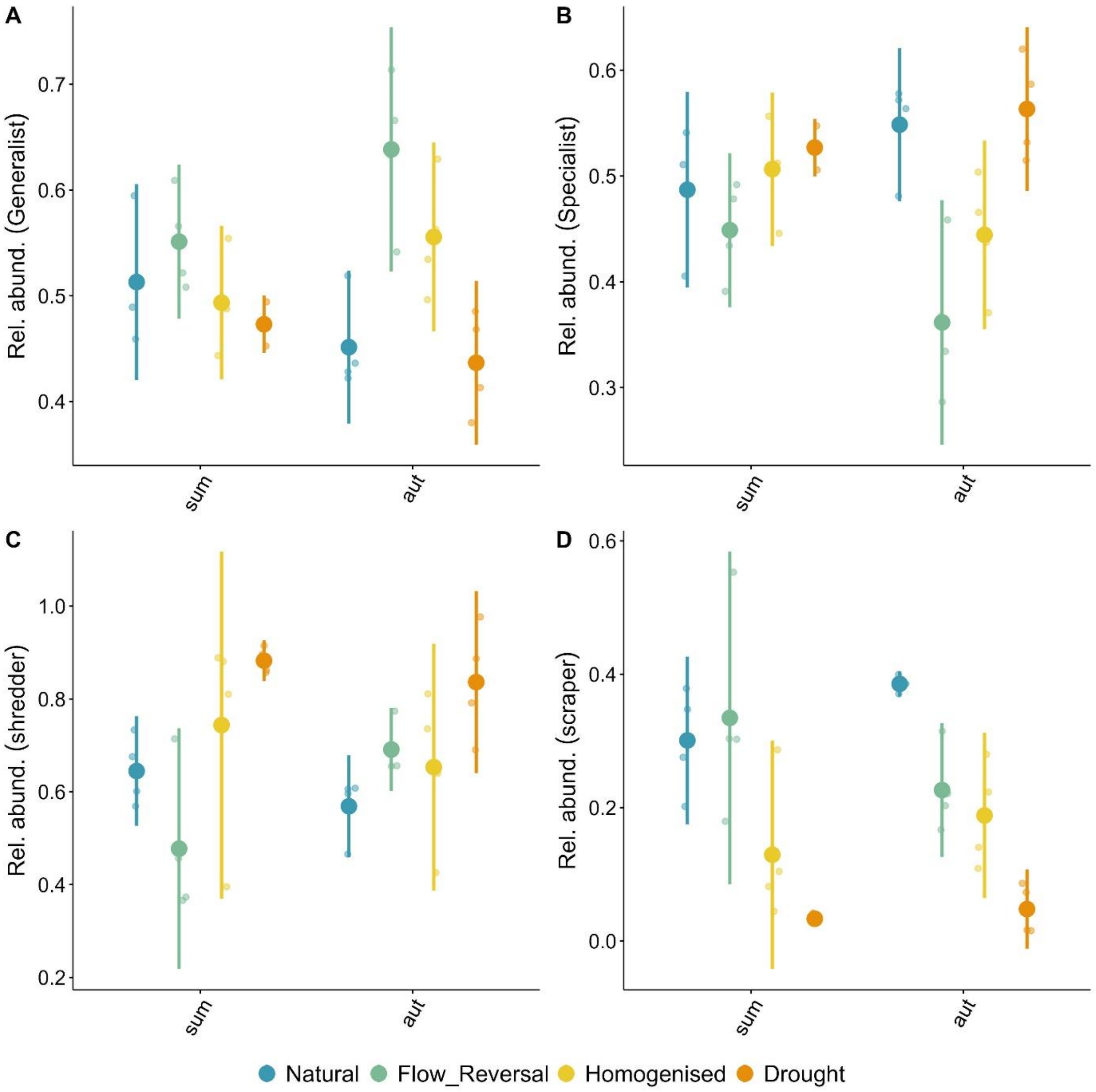

Fig. 3.29. Feeding functional traits. Only the functional trait categories with a significant effect of flow treatment (ANOVA) are presented. Functional trait categories are organised by trait: food preference (A, B, C), feeding strategy (D, E) and food specialisation (F, G). Means and CI_{95} are presented for the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue) during the summer (Sum) and autumn sampling (Aut) in 2020. Dots represent the values of each sample.

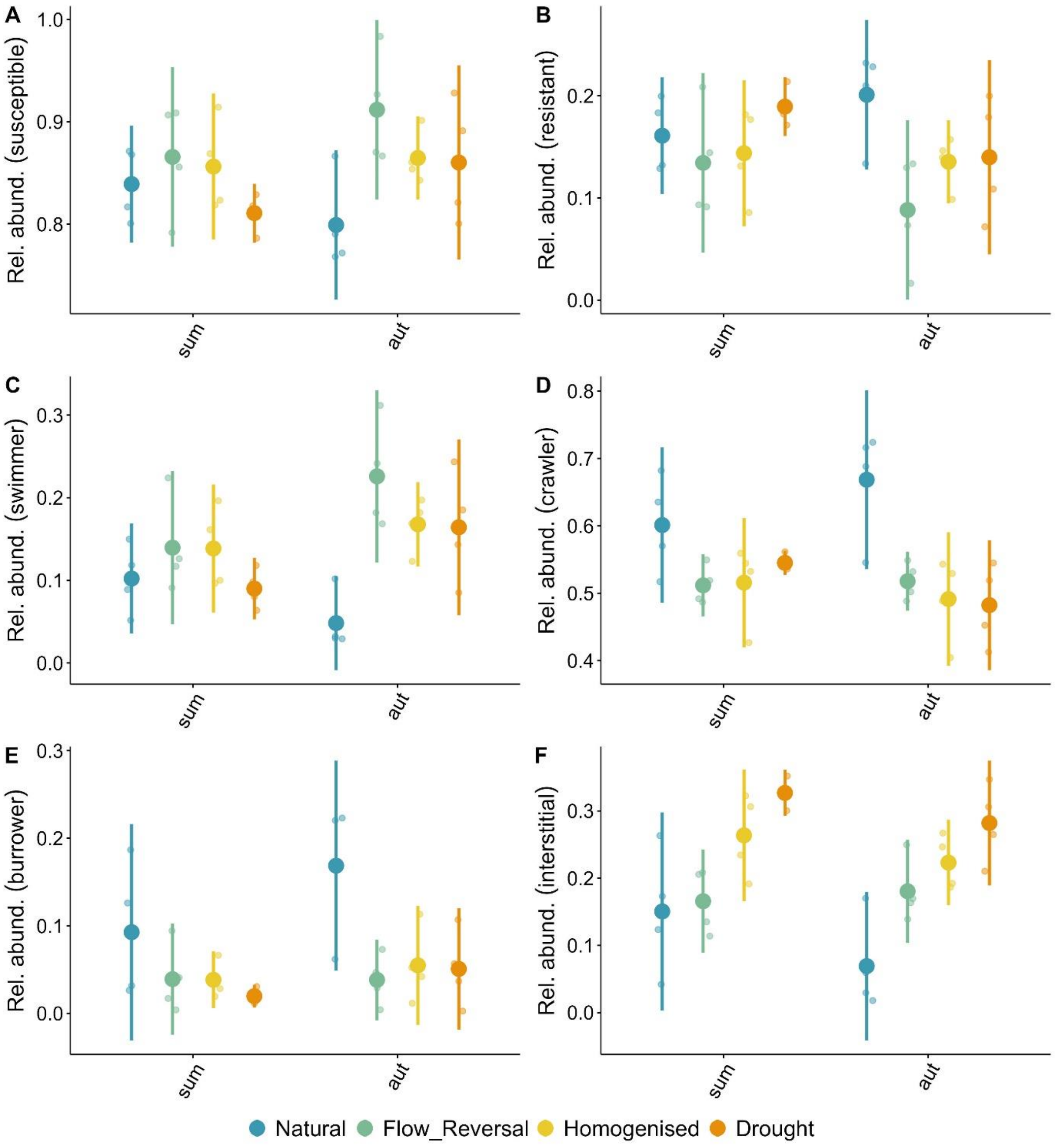

Fig. 3.30. Drought-resistant strategies and locomotion functional traits. Only the functional trait categories with a significant effect of flow treatment (ANOVA) are presented. Functional trait categories are organised by trait: drought resistant (A, B) and locomotion (C, D, E, F). Means and CI_{95} are presented for the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue) during the summer (Sum) and autumn sampling (Aut) in 2020. Dots represent the values of each sample.

From the 10 traits identified by the fourth-corner analysis to be related to continuous environmental variables, 4 of them presented R^2 higher than 0.4 and were all related to discharge (Fig. 3.10). The effects of discharge affected dispersal (aquatic active and passive), locomotion (interstitial) and feeding habits (scraper) Discharge (Q) was negatively correlated to aquatic passive, interstitial; and it was positively correlated to aquatic active and scraper.

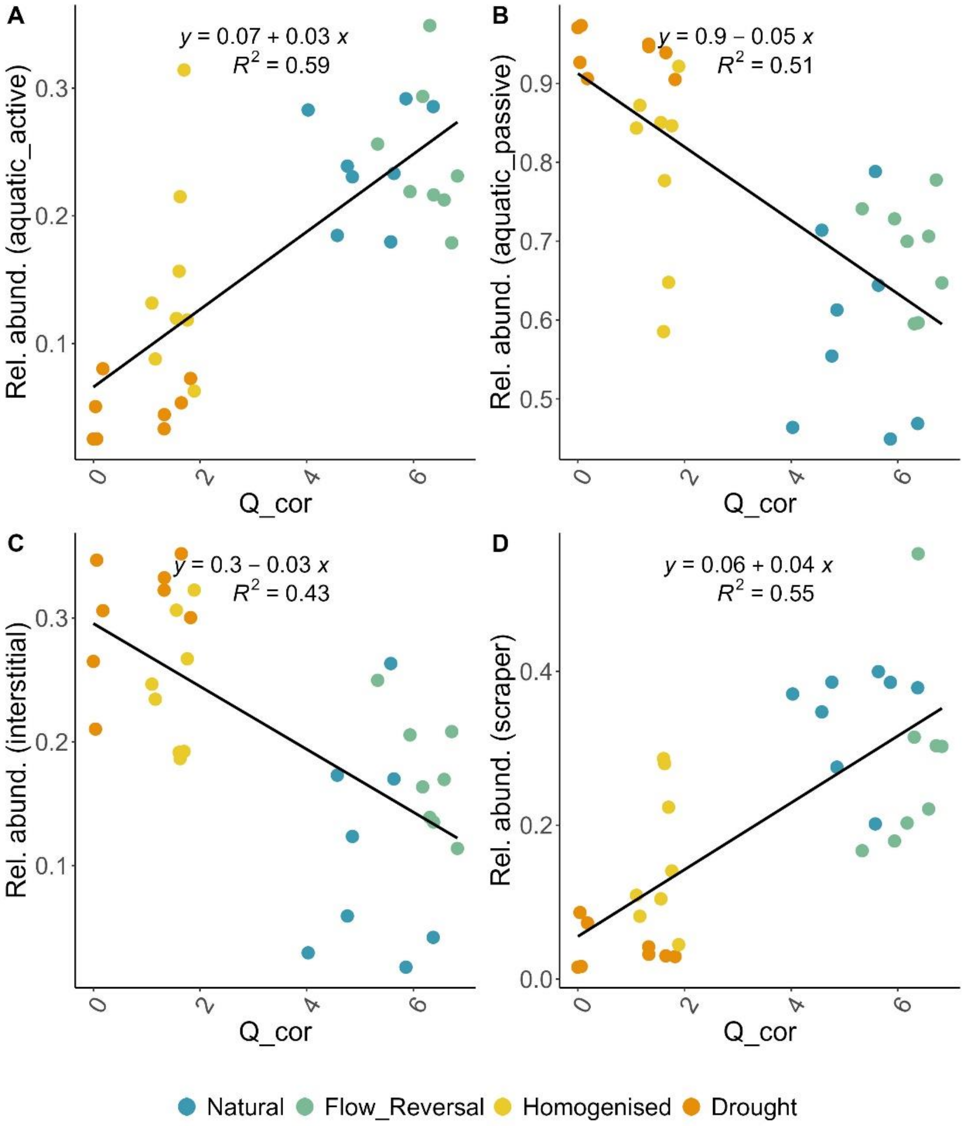

Fig. 3.31. Correlations of trait categories to discharge (Q_cor): aquatic active (A), aquatic passive (B), interstitial (C) and scrapper (D). Notice that only R^2 higher than 0.4 are presented. Values for each flume are presented for the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue).

3.3.3. Macroinvertebrates ecological traits

All three ecological traits (transversal distribution, substrate preference and flow velocity) were significantly affected by the flow treatment (Table 3.7, Table S3.10). Transversal distribution trait was affected by flow treatment on five trait categories (river channel, banks, pools, temporary waters and lakes). Substrate preference trait was affected by flow treatment on eight trait categories (boulders, gravel, silt, macrophytes, microphytes, twigs-roots, detritus and mud). Flow velocity preference was affected by flow treatment on all four categories (null, slow, medium and fast). Out of these categories, five of them were also affected by season (temporary waters, silt, macrophytes, null and medium). In the natural treatment, macroinvertebrate community was mainly composed taxon preferring river channel and banks distribution, with pools and lakes representing lower proportions (Fig. 3.11A-E). Substrate preference was quite evenly distributed between boulders, gravel, macrophytes, microphytes, detritus and mud (Fig. 3.11F-M). Similarly, flow velocity preference was evenly distributed between null, slow and medium (Fig. 3.11K-M).

Table 3.14. Results of the RM-ANOVA with REML estimation of the macroinvertebrates ecological functional trait categories. Parameters showed: degrees of freedom (Df), chis-square (Chi-sq), adjust p-value (p-adj) and level of significance (sig). Levels of significance presented are: non-significant (ns), <0.05 (*), <0.01 (**), and <0.001 (***). Significant results are showed in bold. (next page)

Trait	Trait category	treat	Df	Chi-sq	p-adj	sig
Transversal distribution	river channel	**Flow**	**3**	**15.132**	**0.005**	**
		Season	1	0.466	0.742	ns
		Flow_Season	3	0.315	0.957	ns
	banks	**Flow**	**3**	**18.947**	**0.001**	***
		Season	1	0.000	0.991	ns
		Flow_Season	**3**	**14.944**	**0.003**	**
	pools	**Flow**	**3**	**17.566**	**0.002**	**
		Season	1	1.490	0.333	ns
		Flow_Season	3	1.106	0.776	ns
	temporary waters	**Flow**	**3**	**24.227**	**<0.001**	***
		Season	**1**	**5.077**	**0.036**	*
		Flow_Season	3	2.943	0.4	ns
	lakes	**Flow**	**3**	**23.588**	**<0.001**	***
		Season	1	1.432	0.232	ns
		Flow_Season	**3**	**19.791**	**<0.001**	***
Substrate preference	boulders	**Flow**	**3**	**50.042**	**<0.001**	***
		Season	1	0.219	0.64	ns
		Flow_Season	3	5.381	0.219	ns
	gravel	**Flow**	**3**	**28.372**	**<0.001**	***
		Season	1	0.480	0.488	ns
		Flow_Season	3	2.485	0.488	ns
	silt	Flow	3	0.631	0.889	ns
		Season	**1**	**12.201**	**0.001**	**
		Flow_Season	**3**	**10.962**	**0.018**	*
	macrophytes liv	**Flow**	**3**	**30.324**	**<0.001**	***
		Season	**1**	**9.395**	**0.003**	**
		Flow_Season	**3**	**13.885**	**0.003**	**
	microphytes liv	**Flow**	**3**	**11.520**	**0.014**	*
		Season	1	2.257	0.133	ns
		Flow_Season	**3**	**20.662**	**<0.001**	***
	twigs roots	Flow	3	2.767	0.429	ns
		Season	1	1.436	0.346	ns
		Flow_Season	**3**	**20.574**	**<0.001**	***
	detritus	**Flow**	**3**	**38.709**	**<0.001**	***
		Season	1	0.007	0.935	ns
		Flow_Season	**3**	**24.212**	**<0.001**	***
	mud	**Flow**	**3**	**11.687**	**0.026**	*
		Season	1	1.374	0.241	ns
		Flow_Season	**3**	**8.939**	**0.045**	*
Flow velocity preference	null	**Flow**	**3**	**35.375**	**<0.001**	***
		Season	**1**	**4.873**	**0.041**	*
		Flow_Season	3	1.624	0.654	ns
	slow	**Flow**	**3**	**24.335**	**<0.001**	***
		Season	1	2.574	0.163	ns
		Flow_Season	3	0.713	0.87	ns
	medium	**Flow**	**3**	**41.530**	**<0.001**	***
		Season	**1**	**9.401**	**0.003**	**
		Flow_Season	3	2.746	0.433	ns
	fast	**Flow**	**3**	**13.075**	**0.013**	*
		Season	1	0.199	0.655	ns
		Flow_Season	3	2.942	0.601	ns

In the drought treatment, taxon distribution along the river bed was evenly distributed between river channel, banks, pools and lakes. The banks proportions were significantly lower than the control, while the lakes and pools were higher (Fig. 3.11 A-E, Table S3.10). Substrate preference was mostly dominated by macrophytes and detritus, with both being significantly higher than the control; and boulders, gravel, microphytes and mud being significantly lower (Fig. 3.11 F-J, Table S3.10). Flow velocity preference was dominated by null and slow flow, with both being significantly higher than the control and medium being significantly lower (Fig. 3.11K-L, Table S3.10).

In the homogenised treatment, river distribution was evenly distributed between river channel, banks, pools and lakes (Fig. 3.11A-E). The relative abundance of bank was significantly lower than the control and lakes was higher (Table S3.10). Substrate preference was dominated by macrophytes and detritus, both being significantly higher than the control (Fig. 3.11F-J, Table S3.10). Flow velocity preferences were dominated by null and slow, with medium relative abundance being significantly lower than the control but higher than the drought (Fig. 3.11K-L, Table S3.10).

In the flow reversal treatment, river distribution functionality was relatively similar to the control with no apparent differences (Fig. 3.11A-E). Nevertheless, temporary water was higher than in the remaining flow treatments, presenting a significantly higher relative abundance than the drought flow treatment (i.e. drought has the lowest abundances, Table S3.10). Substrate preference was similar to the control, with differences only occurring in autumn for microphytes and mud which were significantly lower; and detritus higher (Fig. 3.11F-J, Table S3.10). Flow velocity preferences were evenly distributed between null, slow and medium with no differences to the control (Fig. 3.11K-L, Table S3.10).

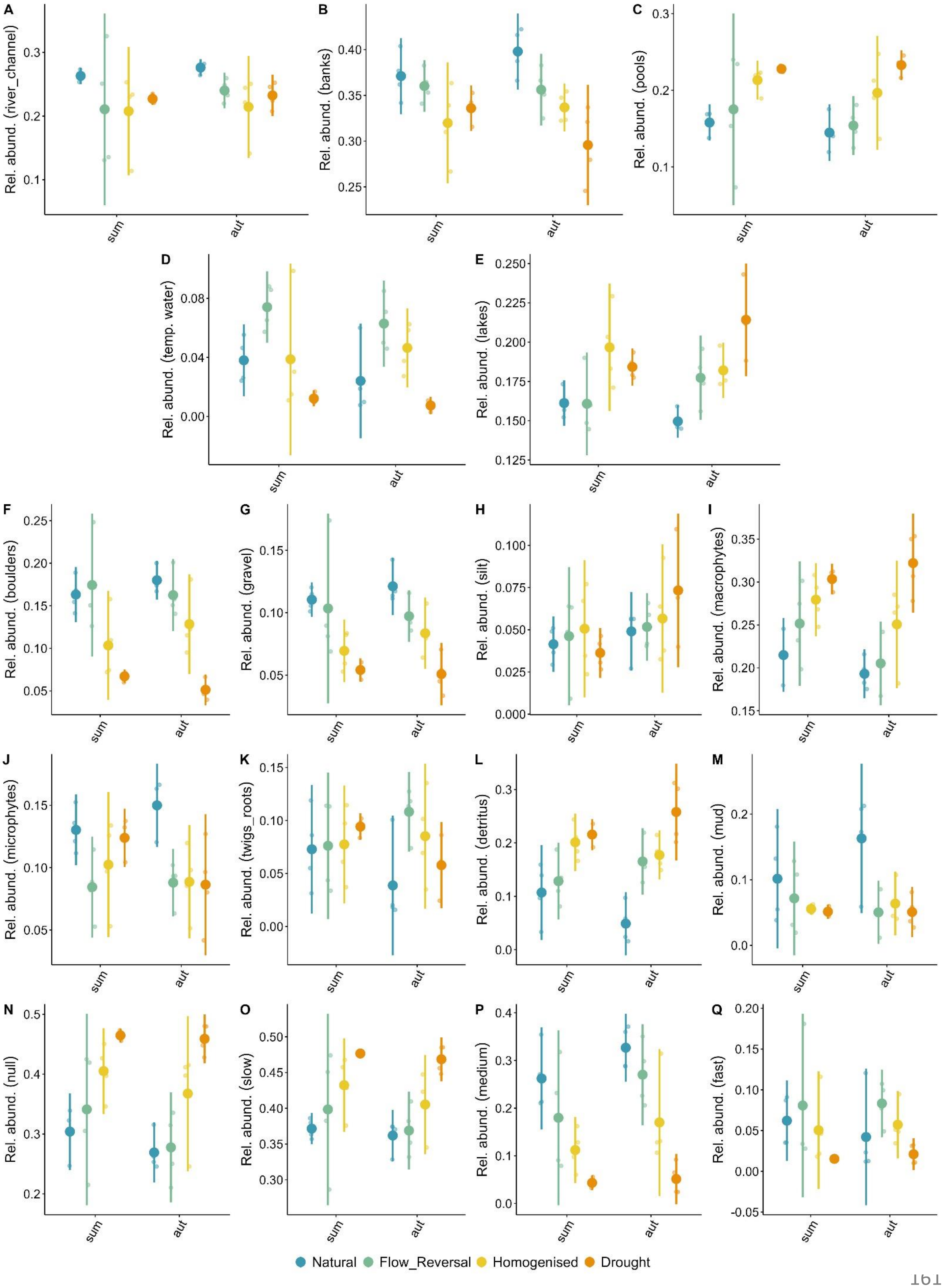
A
Rel. abund. (river_channel)
0.1
0.2
0.3
B
Rel. abund. (banks)
0.25
0.30
0.35
0.40
C
Rel. abund. (pools)
0.1
0.2
0.3
D
Rel. abund. (temp. water)
0.00
0.04
0.08
E
Rel. abund. (lakes)
0.125
0.150
0.175
0.200
0.225
0.250
F
Rel. abund. (boulders)
0.05
0.10
0.15
0.20
0.25
G
Rel. abund. (gravel)
0.05
0.10
0.15
H
Rel. abund. (silt)
0.000
0.025
0.050
0.075
0.100
I
Rel. abund. (macrophytes)
0.15
0.20
0.25
0.30
0.35
J
Rel. abund. (microphytes)
0.05
0.10
0.15
K
Rel. abund. (twigs_roots)
0.00
0.05
0.10
0.15
L
Rel. abund. (detritus)
0.0
0.1
0.2
0.3
M
Rel. abund. (mud)
0.0
0.1
0.2
N
Rel. abund. (null)
0.2
0.3
0.4
0.5
O
Rel. abund. (slow)
0.30
0.35
0.40
0.45
0.50
P
Rel. abund. (medium)
0.0
0.1
0.2
0.3
0.4
Q
Rel. abund. (fast)
-0.05
0.00
0.05
0.10
0.15
0.20
sum
aut
● Natural ● Flow_Reversal ● Homogenised ● Drought

Fig. 3.32. (previous page). Only the functional trait categories with a significant effect of flow treatment (ANOVA) are presented. Ecological trait are presented organised by trait categories: distribution along the river (A, B, C, D, E), substrate preference (F, G, H, I, J) and flow velocity (K, L, M). Notice that river channel is not significant but it is kept to visualise the trait distribution. Means and CI_{95} are presented for the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue) during the summer (Sum) and autumn sampling (Aut) in 2020. Dots represent the values of each sample.

3.4. Discussion

This study is the first to definitively show that flow regimes modify the functional traits of the macroinvertebrate community. While the majority of the flow regimes examined maintained similar total densities and richness relative to the control, the flow reversal regime (i.e. reversal of seasonality of high and low flows) and the drought treatment (i.e. dry-period, riffles dewatering) reduced density and richness, respectively. Nevertheless, all the flow regime treatments supported different macroinvertebrate communities characterised by different densities of core taxa and presence of rare species.

The effect in species composition) rather than changes in biological traits seemed to prevail (fourth-corner analysis). The limited number of species present in the four treatments may have generated functional redundancy of biological traits on the metacommunity, thereby limiting the trait pool. Nevertheless, some trait categories presented a gradual progression between treatments (i.e. natural to flow reversal to homogenised to drought, see Fig. 3.9 and Fig. 3.10) dependant on discharge. This indicates a gradual procession of alteration of the biological traits of the community — which diminished the power of the analysis used (see Pompeu *et al.*, 2022). While biological traits presented a less apparent response, ecological traits had a more consistent pattern and also showed the same progression between treatments than biological traits. There was a clear turnover between lotic species (gravel, medium velocity and river channel) in the natural control to species more associated with lentic habitats (sand, null and pools) in the drought.

3.4.1. Flow reversal

Only total density of macroinvertebrates, and not richness, was affected by flow reversal partially confirming H_1 that this treatment would support lower richness and density than the control. Contrasting results have been found in impounded streams for which richness, as well as abundance, were reduced by flow reversal (Salmaso *et al.*, 2018; Pompeu *et al.*, 2022). Actually, inversion of flow seasonality is seen to cause the greatest reduction in diversity when compared to hydropower, water supply or flood protection dams (Mellado-Díaz *et al.*, 2019; Pompeu *et al.*, 2022). Nevertheless, other studies have shown that the effects can be highly variable (Wang *et al.*, 2020) and that general degradation of the area might diminish the change in macroinvertebrates richness produced by damming (i.e. West Midlands UK, Krajenbrink *et al.*, 2019).

In our study, the stronger reduction in total density observed in autumn might indicate enhanced drift during the high-flow season. The increase of maximal body size (i.e. large and vlarge) suggests that small species (e.g. Chironomidae) and passive dispersers drifted readily out of the treatment (Consoli *et al.*, 2022). This finding, partially confirms the 2nd part of hypothesis H_2 that larger body sizes would be evident in this treatment. The inversion of flow seasonality has the potential to disrupt the development of the macroinvertebrates in a critical moment of their life cycle (Lytle and Poff, 2004). Under drifting events, traits associated with high mobility or better holding capacity concede species drift-resistance capacity (Poff, 2018; Consoli *et al.*, 2022). This might explain the higher abundance of *P. conspersa* and *R. peregra* in this treatment. Furthermore, the higher relative abundance of swimmer and interstitial than the control suggested that strategies to avoid drift were promoted (Pompeu *et al.*, 2022), thereby confirming an element of H_2 that interstitial taxa would be higher.

Furthermore, the reduction of aerial dispersal taxa might indicate a disruption of reproduction of these species, as high flows might limit the success of emergence of adults as well as the ability of flying adults to lay their eggs (Poff, 2018). In contrast with other studies, deposit feeders or filterers did not increase (Pompeu *et al.*, 2022) suggesting that this functional trait is more related to the changes of DOM and FPOM generated by dams, than by flow alterations (Benítez-Mora and Camargo, 2014). Nevertheless, the flow reversal treatment created a dominance of generalist species, indicating functional homogenization of the systems (Mondy and Usseglio-Polatera, 2014) and an increase of opportunistic species. This opportunistic strategy was further supported by the increased number of temporal water species found in the treatments (Bonada, Rieradevall and Prat, 2007).

3.4.2. Homogenised

The findings supported the rejection of H_3 as richness and density was not enhanced by the hydrological stability of the homogenised flow treatment compared to the control as predicted. Abundance typically increases in impounded streams with stable flow as a result of increase resources, refugia and lower drifting events (Lake, 2000; Lobera *et al.*, 2017; Robinson, Siebers and Ortlepp, 2018; Mellado-Díaz *et al.*, 2019). As discussed in Chapter 2, an increase in biofilm biomass did not occur in the homogenised flow treatment, partially explaining the lack of higher abundance (Poff et al., 1990; Ponsatí *et al.*, 2015). In temperate regions, the effects of dams on abundance downstream have found to be small, and often dominated by changes in abundances of tolerant species (Hirundinea, Crustacea, and Mollusca) (Wang *et al.*, 2020) or by the colonization of invasive species (Bruno *et al.*, 2019). Furthermore, in the homogenised flow treatment, habitat homogenization (i.e. similar riffles and pools) together with the low physical habitat complexity, thereby reduced niche availability. This effect resembles the structure of channelized rivers which can markedly reduce the macroinvertebrate community richness and diversity (Horsák *et al.*, 2009).

Given the hydrological stability of the treatment, the system was expected to be highly regulated by biotic interactions (resource partitioning and predation) dominated by competitive species (Poff and Ward, 1989; Townsend and Hildrew, 1994). The harsh-benign theory predicts that under a stable environment, taxa with long-life span, large body size and diet specialisation (k-selection) dominate. While body size tended to be larger than the control, evidence for longer life spans or diet specialization were not found. Some other studied have showed similar responses with no functional differences between homogenised flow and control sites (Lobera *et al.*, 2017) or even increases in sensitive taxa (less adapted to altered flow conditions) (Martínez *et al.*, 2020). This might be a consequence of habitat homogenisation and opportunistic species combined with increased resources availability and stability.

Functional redundancy and limited pool of recolonization sources might have limited the possibility of traits' selection for specialist species. Nevertheless, macroinvertebrate ecological traits analysis confirmed the second part of H_4 that there would be a preference for lentic habitats (pool and lake distribution and null and slow water velocities). This might indicate that species ecological traits (i.e. habitat preference) might be the main driving factor for fitness in systems not exposed to critical disturbances. Therefore, morphological trait selection will be more important when physiological limits are exceed (Aspin *et al.*, 2019). In the homogenised flow, no increase of biofilm occurred with an associated increase in scrappers. This finding suggests that the widely accepted increase in scrappers observed below dams is a consequence of a combination of both flow stability and nutrients alteration, rather than related to stable flow alone (Benítez-Mora and Camargo, 2014).

3.4.3. Drought

Contrary to our expectations, lower macroinvertebrate densities were not observed compared to the control and only richness was lower during the dry period (autumn) thus partially rejecting H_5. In this study, the drought flow regime generated an overall differentiation of the community, as indicated by the NDMS and RLQ analysis, suggesting that the macroinvertebrate community adapted to the flow intermittency. This adaptation maintained similar biodiversity than the control treatment with a different species composition. Similar results have been found in intermittent streams, in which predictable flow interruption promoted similar biodiversity levels than perennial streams but community structure was modified (Bonada, Rieradevall and Prat, 2007). The reduction of richness during the dewatering of the riffles (autumn) highlights the importance of drought threshold into modifying community structure (Aspin *et al.*, 2019; Sarremejane *et al.*, 2021; Chanut *et al.*, 2022). During dewatering events, reduction in richness seemed to be driven by a combination of density-dependent extinction (i.e. predominance of core species while rare species go extinct) together with a trait-dependent extinction. Some dry-sensitive taxa were eliminated (e.g. *P. conspersa* and *P. flavomaculatus*) and some drought-resistant taxa prevailed (i.e. Limoniidae and *A. aquaticus*) (Arias-Real *et al.*, 2022).

The overall reduction of wetted area and the lack stability of this flow treatment likely increased the importance of refugia and resource partitioning during dewatering events and of subsequent recruitment (Huttunen *et al.*, 2017; White *et al.*, 2017; Van Looy *et al.*, 2019). In this study, macrophytes in the pools acted as a refugia (i.e. RQL correlation between the drought treatment and the percentage of volume inhabited by Milfoil) together with the hyporheic zone of the riffles. This attribute shaped the ecological-traits of the community which showed higher relative abundances of interstitial locomotion and macrophytes and detritus as substrate preference. Furthermore, maintained abundances during both sampling times and the high relative abundance of specialist clearly suggest that resource portioning was relevant in the systems. Nevertheless, no evidence of recruitment was found as no increase in aerial dispersed taxa occurred which is expected in unstable environments (Bonada, Rieradevall and Prat, 2007; Verberk, Van Noordwijk and Hildrew, 2013).

Contrary to expectations in this treatment, there was no domination of r-strategists (small body size, multivoltine and generalists), so thus H_6 was rejected. Furthermore, no effect on drought resistant strategies was evident suggesting that the extent of the drought duration and/or magnitude was not sufficient to trigger physiological trait selection, but only behavioural traits (i.e. locomotion and feeding) and ecological ones (Aspin *et al.*, 2019; Chanut *et al.*, 2022). As a result of biofilm dewatering (see chapter 1), there was a lower relative abundance of scrapers and the food chain was mainly maintained by shredders, increasing the system heterotrophy and carbon process ability. Furthermore, there was a clear tendency for lentic water velocities (i.e. null and slow).

3.4.4. Natural control and mesocosm realism

The experimental design enabled us to capture macroinvertebrate community structural and functional differences under different flow regimes scenarios. Macroinvertebrate abundances were comparable to those observed in the UK (White *et al.*, 2017) and mesocosm experiments in river-connected set-ups (Aspin, 2018). Nevertheless, richness was lower than that recorded in local rivers or other mesocosm set-ups (White *et al.*, 2017; Aspin, 2018), suggesting a limitation in the colonization of the streams (Krajenbrink *et al.*, 2019). There was a clear dominance of Crustaceans and Molluscs which are often associated with impounded rivers (Salmaso *et al.*, 2018; Consoli *et al.*, 2022). Given the high abundance of *P. antipodarum* (invasive species of perennial rivers) in the controls, our results highlight the control of invasive species is likely dependant on bed mobilizing flows (Bunn and Arthington, 2002; Arscott *et al.*, 2010). Nevertheless, the species present in the system responded to the flow regime by means of biological and ecological traits; with the natural control presenting traits characteristics of lotic areas.

3.5. Summary and synthesis

The research in this chapter highlights the importance of flow magnitude and timing of low and high flows in triggering changes in the macroinvertebrate community, highlighting their sensitivity to extreme events (i.e. dewatering and high-flow). The experiment additionally revealed that responses to the disturbance are highly dependent on the disturbance predictability (i.e. recurrence) and their impacts. In the flow reversal, unpredictable high-flow promoted a general strategy of survival (i.e. avoiding drift, with opportunistic and generalist species remaining), indicating system fragility. Contrastingly, in the drought treatment, the overall strategy was focused on the refugia and resource partitioning, indicating overall stability of the system. The results highlight flow regimes as a structural component on determining species distribution, with ecological traits being more relevant. Overall, these findings underline the importance of disentangling stable flow effects from regulated flow effects (e.g. water temperature and nutrients), and studying potential mitigation strategies to put in place.

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4. Low flow and heatwaves alter ecosystem functioning in a stream mesocosm experiment

4.1. Introduction

Multiple stressors resulting from climate change and rapid human population growth threaten the biodiversity and functioning of freshwater ecosystems across the globe (Albert et al., 2020; Stillman, 2019; Ummenhofer and Meehl, 2017). In rivers and streams, climate change is likely to increase the occurrence and severity of human-induced low flows associated with water abstraction and hydromorphological alterations (Oki and Kanae, 2006; EEA, 2012). The ecological consequences of low flow stress (e.g. hypoxia, habitat emersion) may ramify across multiple levels of organisation (Lake, 2000; Boulton, 2003), especially where substratum dewatering leads to significant mortality (e.g. Ledger et al., 2011; Dewson et al., 2007). Most of the focus has been on the structural responses of macroinvertebrate communities to low flow stress (e.g. Aspin et al., 2019; Lancaster and Ledger, 2015; Ledger et al., 2013), but impacts on ecosystem functioning remain poorly resolved, especially for multiple functions at the whole-system scale (Sabater et al., 2018; Death et al., 2009).

Although climate change research has focused principally on the consequences of shifting mean conditions, especially gradual warming, extreme events, not trends, may have the most significant impact on ecosystems globally (Ummenhofer and Meehl, 2017; Woodward et al., 2016). Heatwaves are historically rare and occur unpredictably, but record-breaking events, such as the European heatwave of 2003 (Mouthon and Daufresne, 2015), are increasing in frequency and intensity across large parts of Europe, Asia, and Australia (IPCC, 2012; Fischer and Schär, 2010). The ecological impacts of heatwaves on stream systems are not well understood but could be significant and long-term, as evidenced for macroinvertebrates through the 2003 European heatwave (Mouthon and Daufresne, 2015).

Heatwave timing may be critical (Woodward et al., 2010), acting potentially as a subsidy by providing energy inputs which enhance temperature-dependent ecosystem processes (e.g. decomposition and respiration), or as a stressor, in which the exceedance of thermal maxima drives mortality of functionally significant biota (Odum, Finn and Franz, 1979). The timing of heatwaves will potentially affect their interaction with other stressors, such as low flow events (e.g. Ledger and Milner, 2015). Resistance, the ability of a community or population to withstand a disturbance unchanged (Holling, 1973), may be affected by one or more of the stressors and progressively decline through a series of recurrent events. Consequently, heatwaves that occur late in a sequence may result in the most ecological impact (Hughes *et al.*, 2019). However, in some cases, sequential events can lead to increased community tolerance, with impacts less pronounced in systems with ecological memory of previous events (e.g. a legacy effect; Guest et al., 2012). Overall, community responses across a range of stressor combinations present a complex picture with few generalisations (Piggott et al., 2015b; Matthaei et al., 2010).

Understanding of individual and combined effects of stressors that occur unpredictably can be derived from mesocosm experiments that combine the control and replicability of laboratory experiments with the realism of field surveys (e.g. Gossiaux et al., 2020; Romero et al., 2019; Piggott et al., 2015a; Stewart et al., 2013; Woodward et al., 2010). Microbial communities (attached algae, bacteria and fungi or biofilms) are ideally suited to manipulation at the mesoscale (Petersen and Englund, 2005); being small relative to the size of the mesocosms, they capture both intra- and inter-generational responses to stressors over a timescale of months (Stewart *et al.*, 2013). Microbial communities also play a pivotal role in many ecosystem processes, from nutrient cycling and carbon sequestration to bottom-up regulation of food web processes (Allan and Castillo, 2007). In systems with limited resource input, metabolism is largely driven by the microbial community, with the balance between ecosystem respiration (ER) and gross production (GPP) reflecting the ratio of autotrophs to heterotrophs. Hence, these communities represent an ideal level of biological organisation for experimental studies seeking to disentangle impacts of perturbations or stressors on ecosystem function and services (Sabater *et al.*, 2007; Cheng *et al.*, 2017; Freixa *et al.*, 2017).

Despite the potential for extreme events to alter stream ecosystem functioning, knowledge of the impact of heatwaves, and their interaction with other stressors, is still scarce. This study reports the findings of a stream mesocosm experiment to determine the individual and combined effects of low flows and heatwaves on multiple ecosystem processes (i.e. multifunctionality) in attached microbial communities (biofilms). Low flows (simulating flow reduction from dams without gravel dewatering), and a sequence of three heatwave events, were applied in mesocosms during autumn (of 2018) when elevated water temperature might accelerate ecosystem process rates in what is typically a quiescent period. I expected the heterotrophic component of food webs to show the greatest responses as process rates are temperature-dependent (Bastos et al., 2020; Orsenigo et al., 2015; O'Gorman et al., 2012) and short day length (low light) limit algal production. I expected low flows to act as a press disturbance that constrains ecosystem functioning through habitat contraction (Arroita *et al.*, 2017). To test whether sequence effects and event history governed responses to stressors (i.e. 'ecological memory'; Hughes et al., 2019), three heatwave events were applied sequentially, each consisting of a heating episode and an intervening unheated recovery period. In streams, the response of whole-system metabolism to a specific heatwave event could be contingent on previous events, with the high plasticity of microbial communities (e.g. their potential for acclimation) enhancing process rates (Guest *et al.*, 2012). The acclimation to a given stressor may be driven by physiological responses (i.e. changes in the functioning of the microbial community) or rather genetical (i.e. filtering of non-adapted individuals) (Lake, 2000). However, heatwave interactions with low flow are likely to be synergistic due to the legacy effects of previous heatwave events. Specifically, I hypothesise that:

H_1: Heatwaves will stimulate heterotrophic processes more than autotrophic processes, thereby increasing the rates of detrital decomposition and ecosystem respiration.

H_2: Low flows will reduce gross primary production and ecosystem respiration due to habitat contraction;

H_3: The stimulatory effect of heatwaves on ecosystem respiration and gross primary production will be annulled due to the decelerating effect of low flow.

H_4: Ecosystem acclimation to individual heatwaves will enhance ecological responses to heatwave events later in the sequence (ecosystem memory). Where the heatwave sequence is combined with low flow, acclimation will be offset by reduced resistance to low flow, resulting in an antagonistic interaction.

4.2. Materials and methods

4.2.1. Experimental facility and design

Research was conducted over two months (October-December 2018) in 24 outdoor stream mesocosms fed by borehole water at the University of Birmingham Environmental Change Outdoor Laboratory (EcoLaboratory), U.K. (52.45° N, 1.93° W). The experiment aimed to create analogues of local groundwater fed streams (i.e. urban headwater systems). Each mesocosm was a fiberglass oval raceway channel (total width 0.4 m, inner channel width 0.15m, length 2 m, depth 0.15 m; see Lee et al., 2016) raised 0.5 m aboveground on galvanized steel tables (Fig. 4.1). Mesocosms received groundwater pumped from the borehole (tapping a Permo-Triassic Sandstone aquifer) via a 2000L, sealed header tank. Continuous monitoring using a multi-parameter sonde (Aquatroll 400, In-situ Inc, Fort Collins, USA) demonstrated that the quality of the groundwater was stable during the experiment, being cool (water temperature [mean ± standard deviation] 10.7 ± 0.7 °C) and of moderate nutrient status (conductivity 448.9 ± 41.1 µS cm^{-1}; total nitrogen 3.8 mg L^{-1} and phosphate 0.013 mg L^{-1}). Oxygen levels in the header tank were maintained using a pond aeration kit (DO saturation: 86.9% ± 9.23).

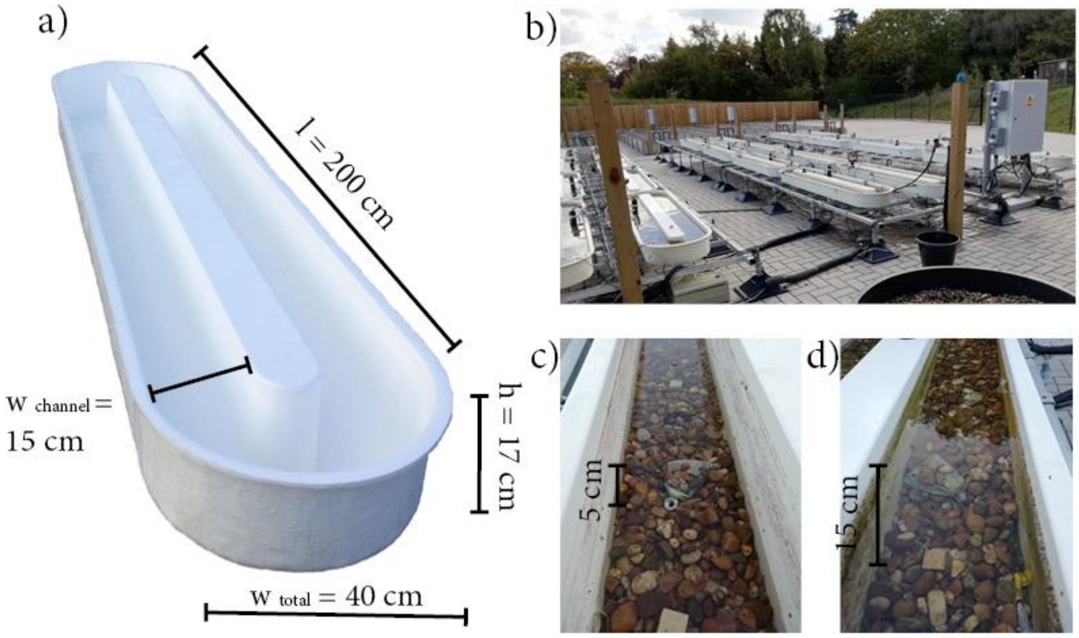

Fig. 4.33. Experimental set-up in the EcoLaboratory. (a) The semi-recirculating flumes used for this experiment. (b). The flumes were distributed across three raised tables and were left to colonize for two months before the experiment started. In early October, experimental treatments were applied (heatwave, low flow (c) and heat have & low flow) and a control (d) was maintained to enable comparison.

From the header tank, water was delivered to each mesocosm via underground piping with inputs controlled using a programmed solenoid valve and irrigation dripper. The irrigation drippers were 2 cm above the water surface, with a flow rate of 5 mL/min when activated. Water in each mesocosm was recirculated in the channel using a small aquarium pump (NWA 1.6Adj 2.6W, Newa Wave Industria, Loreggia, Italy) with a flow rate ~0.2 m s^{-1} (Electromagnetic current meter, Valeport Ltd., Devon, UK), and exited under gravity through a small outlet port, yielding a turnover of 3.5 days. The continuous recirculation by the pump system ensured that re-aeration potential was high and the flumes were well oxygenated Mesocosm channels were filled to 3 cm depth with washed commercial gravel (particle size 10-20 mm). In July 2018, each mesocosm was seeded with a homogenised biofilm slurry scraped from stones of a local stream (Bourn Brook), together with 10 g of coarse particulate organic matter (coarsely ground litter of Oak, *Quercus robur,* and Sycamore, *Acer pseudoplatanus)*, and left to establish for 3 months. Two weeks prior to application of the treatments, water temperature and oxygen levels in all flumes were assessed using in-situ optical dissolved oxygen sensors (RDO PRO-X, In-Situ, Inc. Fort Collins, USA – 15 min resolution). Mean water temperature across all flumes was 11.4 ± 2.8 °C (mean ± SD) and there was no significant difference between the assigned treatments (ANOVA; [Cohen's d ± 95% confidence intervals], C_d=0.168 ±1.43, P = 1). Flumes were well oxygenated (mean ± SD, 99.7 % ± 5.08) with no significant difference between the assigned treatments (ANOVA; heatwaves, C_d = -0.547 ± 2.80 , P = 0.24; low flow, C_d = -0.687 ± 1.24 ,P = 0.21; heatwave & low flow, C_d= 0.0171 ± 1.20, P = 1).

Following acclimation, a full-factorial experiment was initiated, with a replicated (n = 6) randomised blocks design to test the main effect of low flow and heatwaves, and their combination, in a 63-day mesocosm experiment (spanning mid-late autumn). The experiment was organised in three spatial blocks of eight mesocosms (24 mesocosms total), with two mesocosms within a block assigned randomly to each of three manipulative treatments (i.e.: low flow only, heatwaves only, heatwaves & low flow combined) or an unmanipulated control. There were thus two fixed factors (low flow, heatwaves) and two levels of each factor (presence or absence of the stressor). At the onset of the experiment (12 October 2018), low flows were applied by a) slowly reducing water level to 33% of that in controls (50% reduction of the wetted perimeter, but no gravel dewatering), and b) halting water inputs via irrigation drippers (Fig. 4.1). With this treatment, the aim was to mimic low flow associated with reduced flow magnitude below dams. Heatwave events were applied as three 8-d episodes of +5 °C above controls (i.e. consistent with the definition of a heatwave in IPCC (2007), with 10-15 days recovery between each episode. Warming was achieved using an aquarium heater (Newa Therm VTX 200W TNT, Newa Wave Industria) controlled via a programmed CR1000 data logger (Campbell Scientific, Inc., Logan, USA). Due to the relatively low volume of water, that was well mixed, the thermal gradients in the flumes were minimal. Water temperature was measured at 10 cm intervals along the flume and the maximum temperature difference between these points was <0.2°C.

4.2.2. Monitoring, sampling and processing

Continuous monitoring of DO and water temperature occurred in each mesocosm (RDO PRO-X) and photosynthetically-active radiation (PAR; SKP215 Quantum Sensor, Campbell Scientific) was measured at 2 m. Measurements were 15 min means based on scans every 5 minutes. Water quality parameters (pH, electrical conductivity, water temperature and dissolved oxygen) were also measured weekly in each flume using a handheld meter (Thermo Scientific Orion Star A329, Thermo Fisher Scientific, Waltham, USA). At the end of the experiment, 20 ml water samples were collected from each mesocosm, filtered at 0.45 µm (Whatman GD/X disposable filter device), and frozen for subsequent analysis of dissolved organic matter (DOM).

4.2.3. Functional indicators of stressors

A total of nine key ecosystem processes (response variables) were determined to test the effect of the stressor treatments on the functioning of the autotrophic and heterotrophic compartments of the whole system and the associated food web (Death, Dewson and James, 2009). Epilithic biofilm was characterised by the accrual of chlorophyll concentration (function 1) and ash-free dry mass (AFDM; function 2), and the autotrophic index (function 3). The functioning of the heterotrophic compartment was determined as decomposition activity measured by microbial decomposition of leaf litter (function 4) and wood (function 5), and their ratio (function 6). The whole system response was measured as gross primary production (GPP, function 7), ecosystem respiration (ER, function 8), and net ecosystem production (NEP, function 9). Four further variables associated with changes in dissolved organic matter quantity and quality were measured; dissolved organic carbon (DOC), specific ultraviolet absorbance $SUVA_{254}$, and two fluorescence indices, the ratio of Peak C: T and Humification Index (HIX), both indicators of microbial degradation (see below for more details).

Microbial breakdown of leaf litter and wood. Alder (*Alnus glutinosa*) leaf packs and wooden craft sticks (i.e. birch) were incubated in triplicate in each mesocosm for the full duration of the experiment (63 d). Leaf packs were empty paper teabags (dimensions 7x9 cm) filled with ~1 g of air-dried litter. At the end of the experiment, both substrates were oven-dried at 65 °C for 48 h and weighed to the nearest 0.001 g. Detrital and wood standard decomposition rates (k_{wood} and k_{leaf}) were calculated based on a single exponential decay model (e.g. Wieder and Lang, 1982).

Given the variability of leaf structural constituents (cellulose, hemicellulose and lignin) and its lability, single decay models for fresh leave materials tend to underestimate decomposition rates of labile material and neglected slow decomposing and non-decomposable fractions when used over short periods (Wieder and Lang, 1982). To account for this limitation, the proportion of slow decomposition rates (i.e. recalcitrant organic matter with a high lignin content) relative to the detrital decomposition rates was calculated, by using the ratio of k_{wood}: k_{leaf}. To determine the decomposition effect excluding the direct effect of temperature (i.e. to enable comparison of processing efficiency), decomposition per degree-day was also calculated ($k_{dd\text{-}wood}$ and $k_{dd\text{-}leaf}$) following Chauvet et al. (2016).

Biofilm accrual. The effect of the stressors on the biofilm community was assessed as the rate of accrual of chlorophyll *a* and ash-free dry mass (AFDM) on artificial substrates (small [4 cm^2] unglazed tiles) incubated in the mesocosms for the duration of the experiment. After a 63-d incubation, tiles (n = 4x2 per mesocosm) were collected and biofilm was removed using a toothbrush and washed into 60 mL sample bottles. The slurry was then filtered through a 0.7 µm glass fibre filter (Whatman GF/F 47mm) and the filtrate frozen. Chlorophyll *a* was quantified spectrophotometrically as outlined in ASTM D3731 (ASTM International, 2020). Total biofilm biomass was estimated as AFDM (Hauer and Lamberti, 2017). Defrosted filters were oven-dried for 1 h at 105 °C then weighed to the nearest 0.1 mg, then combusted in a muffle furnace at 500 °C for 1 h hour and reweighed at room temperature (e.g. Ledger and Hildrew, 1998). Accrual rates (mg cm^{-2} d^{-1}) were calculated simply as the mass of material sampled at the end of the experiment divided by the number of experimental days (63 days). The autotrophic index (AI) was calculated as the ratio of biofilm accrual biomass to algal accrual, with lower values indicating higher levels of autotrophy (Hauer and Lamberti, 2017).

Dissolved organic matter (DOM). DOC was determined using a Shimadzu TOC-V CSH total organic carbon analyser (Kyoto, Japan). For each sample, replicate DOC readings (n = 3–5) were undertaken and ≤2% coefficient of variation was observed. Absorbance spectra (200–850 nm) were measured using a Jenway 6800 dual-beam spectrophotometer (Stone, UK; cuvette path length 10 mm) and quartz cuvettes that were triple rinsed with sample water. A Cary Eclipse Spectrofluorometer (Varian Inc., Palto Alto, USA) was used to measure fluorescence with instrument settings outlined in Khamis et al., (2015). A Raman blank was measured at the start of each instrument run to calibrate fluorescence intensity (Lawaetz and Stedmon, 2009). Excitation Emission Matrices (EEMs) were determined for each sample (plus an ultrapure water blank) overexcitation range 200–400 nm (5 nm slit width) and emission range 280–500 nm (2 nm slit width). Samples were diluted with ultrapure water before analysis where DOM concentrations were observed (absorbance 254 nm > 0.3 AU). $SUVA_{254}$, an index of carbon aromaticity (Weishaar *et al.*, 2003), was calculated as the absorption coefficient at 254 nm divided by DOC concentration. The ratio of fluorescence Peak C (Ex. 340 nm, Em. 440 nm) to Peak T (Ex. 270 nm, Em. 304 nm was used to provide an indication of recalcitrant to labile fluorescence DOM (fDOM, C: T, Baker, 2001). HIX, a fluorescence-based index that is indicative of microbial processing or humification of fDOM was calculated following Hansen et al. (2016). All EEMs were processed, and indices calculated using R version 3.6.3 (R Core Team) and the *staRdom* package (Pucher et al., 2019).

Whole-system metabolism: GPP/ER/NEP. For each mesocosm, metabolic rates were estimated from diel DO fluctuations using the *streamMetabolizer* package (Appling, Hall, *et al.*, 2018). Bayesian state-space models were fitted to estimate GPP, ER and the O_2 specific gas exchange rate coefficient (K_{600}). These variables were modelled at daily time steps using 15 min records of

DO, water temperature, PAR, and water column depth. The equilibrium concentration of DO was calculated using R version 3.6.3 (R Core Team) and the *lakeMetabolizer* package (Baldwin *et al.*, 2019). For modelling purposes, the following deterministic relationship between three process rates was assumed:

$$\frac{dO_t}{dt} = P_t + R_t + D_t \quad \text{Equation 1}$$

where t is time (h); P_t is a function of daily mean GPP (g O_2 m^{-2} d^{-1}), mean water depth (m) and PAR; R_t is a function of daily mean ER (g O_2 m^{-2} d^{-1}) and mean water depth (m); D_t is the gas exchange rate and is the product of the daily reaeration rate, K_{600} (d^{-1}), and the deficit between actual and equilibrium concentrations of DO (g O_2 m^{-3}). More specifically,

$$P_t = GPP \times \frac{1}{\bar{z}_t} \times \frac{(t_1 - t_0) \times PPFD_t}{\int_{u=t_0}^{t_1} PPFD_u \, du} \qquad \text{Equation 2}$$

$$R_t = ER \times \frac{1}{\underline{z}_t} \qquad \text{Equation 3}$$

Z is the mean water depth across the flume (m), PPFD is the photosynthetic photon flux density (µmol photons m^{-2} d^{-1}), t_0 and t_1 are the beginning and end of the day and du is the continuous time within a day when t is already taken. For further information or more specific details of the process rate equations see Appling et al., (2018a). The Bayesian State space models incorporated both observation and process error and the trapezoid rule was used to solve the ordinary differential equation for DO. Bayesian models were run using 1000 burning and 2000 saved steps. GPP and ER were constrained to be always positive and negative, respectively. Net ecosystem metabolism (NEM) was estimated by aggregating GPP and ER with their corresponding sign. Accordingly, negative NEM values indicate heterotrophy (net carbon release) and positive values, autotrophy (net carbon mineralization). Data for one of the mesocosms were omitted from the analysis due to a sensor error. To ensure measures of functions were comparable (i.e. integrative measures, such as detrital decomposition rates, vs quasi-instantaneous measurements such as GPP) cumulative ER, GPP and NEM for each flume to integrate metabolism across the experiment duration were calculated.

4.2.4. Statistical analysis

Data for nine indicators of ecosystem functioning were collated to calculate aquatic ecosystem multifunctionality, including six measured at the patch-scale (occurring at a discrete location within the flume) and three at the whole-system scale. At the patch-scale, the indicators measured were: benthic algal accrual (chlorophyll *a*, mg m^{-2} d^{-1}), total biofilm accrual (AFDM, mg m^{-2} d^{-1}), autotrophic index (AI), detrital decomposition (k_{leaf}, d^{-1}), wood decomposition (k_{wood}, d^{-1}), ratio k_{wood}:k_{leaf}. At the whole-system scale, cumulative GPP (GPP, mg O_2 m^{-2}), cumulative ER (ER, mg O_2 m^{-2}), and cumulative NEP (NEP, mg O_2 m^{-2}) were determined. The cumulative values for the three whole-system metabolism variables were used to ensure they were comparable with the other patch-scale functions integrated over the duration of the experiment. To capture how all these functions respond to the stressors, a standard metric of multifunctionality (see Byrnes et al., 2014) which averages the standardized values of all measured functions into a single index was used. Where loss of functioning was evidenced by higher process rates (e.g. ER), values were 'reflected', before standardizing between 0 and 1. The averaged multifunctionality index (MF) was calculated as:

$$\mathrm{MF}_a = \frac{1}{F}\sum_{i=1}^{F} g(r_i(f_i)) \qquad \text{Equation 4}$$

Where F is the total number of functions, f_i are the values of each function i, r_i is a mathematical function that reflects f_i where appropriate, and g is a transformation to standardize the values of each process to a common scale.

Linear mixed effect models were used to test treatment effects on the functional indicators (k_{wood}, k_{leaf}, k_{wood}:k_{leaf}, AFDM, chl-a, AI, GPP, ER, NEM, MF) and indicators of DOM quality and quantity (DOC, $SUVA_{254}$, HIX and C : T ratio). Following methods outlined by Zuur et al. (2010), all variables were assessed using graphical tools to ensure assumptions of regression analysis were fulfilled (e.g. normality and homoscedasticity) and transformed when necessary. For each response variable (i.e. function) I fitted a global model of the following form:

Response ~ heatwaves + low flow + heatwaves : low flow + block (random intercept)

When more than one replicate was available per channel (e.g. decomposition rates, pH, EC, temperature), mean values were used to run the model. Model fit was assessed using conditional R^2 following Nakagawa and Schielzeth (2013) (Table 4.1).

Table 4.15. Model fit assessment for the patch-scale and whole-system responses. Degrees of freedom (df) and conditional R^2 (R^2) are presented for each of the mixed effect models. For each response variable, block was used as random effect with three levels and heatwave and low flow were fixed effects with two levels each.

Response	df	R^2
Leaf decomposition (k_{leaf})	20	0.398
Wood decomposition (k_{wood})	19	0.471
Ratio k_{wood}:k_{leaf}	19	0.259
Chl-a accrual	19	0.610
AFDM accrual	19	0.104
Autotrophic Index (AI))	19	0.590
DOC	19	0.221
$SUVA_{254}$	19	0.169
HIX	19	0.275
C:T ratio	19	0.320
Cum. GPP	19	0.866
Cum. ER	19	0.576
Cum. NEM	19	0.147
Multifunctionality Index (MF)	19	0.467

To assess the impact of three sequential heatwaves periods on metabolism, linear mixed effect models were used with cumulative GPP, ER and NEP as response variables. These variables were chosen as they integrate finer scale processes driving heterotrophic and autotrophic energy pathways and were monitored at an increased temporal resolution compared to the patch-scale processes. For each of the heatwave events (i.e. HWE 1, HWE 2, HWE 3) cumulative GPP, ER and NEP (n = 72) was calculated. All data were transformed to meet normality and homoscedasticity assumptions (Peterson and Cavanaugh, 2020) and responses were assessed using the following model:

Response ~ heatwaves * low flow * event+ event (random intercept) + flume (random intercept).

ANOVA type II tests were performed for all final models and p-values adjusted to control for Type 1 error following Benjamini and Hochberg (1995). Statistical significance was determined at 0.05. For all the models including a temporal component (i.e. period event), repeated-measurements three-way ANOVA analysis was performed. To quantify the magnitude of the treatment effects, standardized effects sizes were used. Cohen's d and confidence intervals (CI) were calculated to estimate the differences between two treatment groups (see Equation S4.1), and the effect size was classified following Nakagawa and Cuthill (2007): trivial <= 0.20, weak > 0.50, moderate > 0.80 and large > 0.80.). Since Cohen's d only calculate the differences between two groups, Cohen's f was used to determine` effect sizes for the three-way ANOVA (i.e. temporal analysis). While Cohen's d reports the standardized differences between two given groups, Cohen's f describes the proportion of variability accounted for each treatment. For Cohen's f, the effect size was classified following Cohen (1988): trivial <= 0.10, weak > 0.10, moderate > 0.25 and large > 0.40. All analyses were performed using R version 3.6.3 (R Core Team) and the packages *lme4* (Bates *et al.*, 2015) for mixed effect models, *bestNormalize* (Peterson and Cavanaugh, 2020) for data normalization*, piecewiseSEM* (Lefcheck, 2016) for the calculation of conditional R^2, and *sjstats* (Lüdecke, 2020) for regression and ANOVA.

4.3. Results

4.3.1. Physicochemical responses

Thermal regimes differed markedly between the stressor treatments (Fig. 4.2). ANOVA revealed a statistically significant main effect of heatwaves (C_d = 15.28 ± 70.74, $P < 0.001$), but not low flow (C_d = -0.975 ± 1.28, $P = 0.20$), on mean water temperature and there was a statistically significant interaction between heatwaves and low flow (C_d = 1.47 ± 6.915, $P <0.001$). Over the course of the experiment, mean water temperature in heatwaves and heatwaves & low flow treatments were 1.8 ℃ and 2.1 ℃ higher than the control respectively. Thermal regimes in both heatwaves treatments were right-skewed, with more hot days (maxima = 25.5 ℃ vs 20.3 ℃ in controls) and thus the 90th percentile of water temperature (T_{90}) was 5.2 ℃ greater than controls as a consequence (Fig. 4.2). Electrical conductivity (cross-treatment mean ± CV = 324.3 µs cm^{-1} ± 12.7 %) was higher in heatwaves (mean 338.3 ± SD 23.8 µs cm^{-1}) and heatwaves & low flow treatments (340.6 ± 29.5 µs $cm^{-1)}$ than in low flow (306.8 ± 29.0 µs cm^{-1}) and control treatments (311.4 ± 31.8 µs cm^{-1}; C_d = 0.64 ± 3.18, *P* = 0.02). By contrast, pH (cross-treatment mean pH ± CV = 8.6 ± 3.4%) and DO saturation (95.47 % ± 4.67) did not vary significantly among the treatments (ANOVA, *P* > 0.05; see Supplementary Material, Table S4.1). Nevertheless, heatwaves significantly reduced the DO minima (C_d = -1.40 ± 6.58, *P* = 0.023) with the 5th percentile of DO concentration being 1.14 mg O_2 lower than the control for heatwaves and 0.65 mg O_2, for heatwaves & low flow.

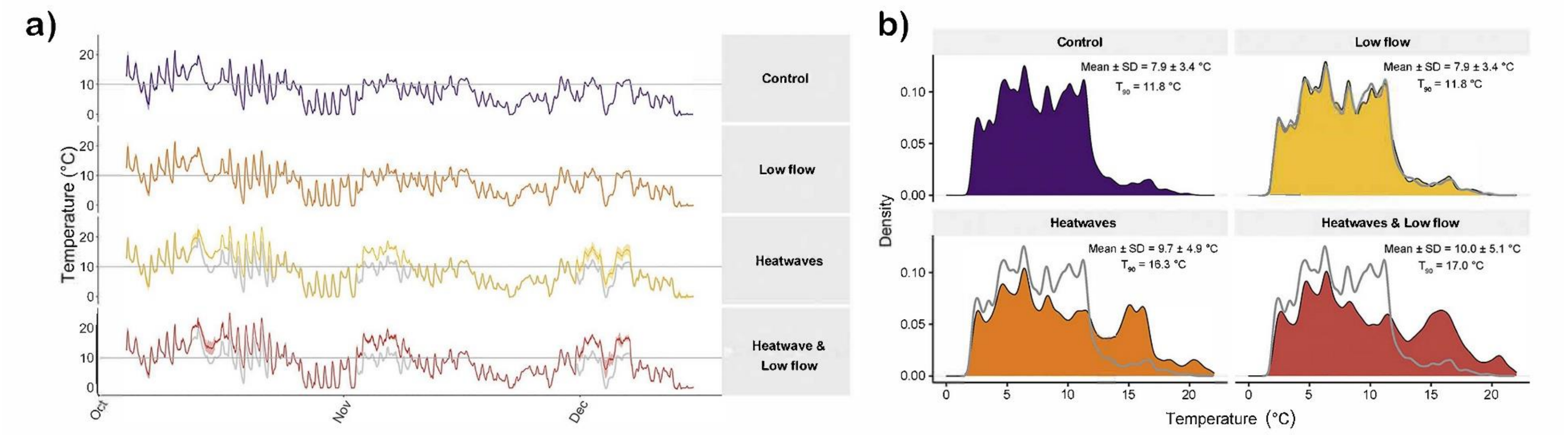

Fig. 4.34. Water temperature during the experimental period. a) Daily temperature oscillations between October and December for the controls and three stressor treatments: heatwaves, low flow and heatwaves & low flow. Means and standard deviations are presented for each treatment (mean, coloured line; and SD, coloured shaded area). b) Water temperature density distributions for the controls and the experimental treatments. Mean, SD and the 90th water temperature percentile (T_{90}) are presented for each distribution. For both plots, the grey line represents the control temperature.

4.3.2. Stressor effects on ecosystem processes

Our statistical analysis revealed some but not all response variables were significantly affected by heatwaves (n = 5; 38 %) and low flows (n = 3; 23 %), whereas interactions between heatwaves and low flows were non-significant for all nine ecosystem processes and the four DOM variables measured in the study (Table 4.2 & 4.3, Fig. 4.3 & 4.4).

Microbial detritus processing. Heatwaves significantly increased the rate of microbial decomposition of leaf litter and wood, both presenting large effect sizes (Table 4.2, Fig. 4.3). The ratio between leaf and wood decomposition was not significantly affected, despite heatwaves presenting a biologically-relevant weak effect size. When the temperature was included in the calculation of process rates (i.e. degree days vs days), heatwaves decreased the efficiency of leaf decomposition rates per degree day ($k_{\text{dd-leaf}}\, C_d = -0.531 \pm 4.38$, $P = 0.009$, Fig. S4.1), but it did not affect the other decomposition measurements. By contrast, low flow did not affect leaf or wood processing rates significantly (Fig. 4.3; Table 4.2).

Biofilm accrual. Heatwaves increased the rate of accrual of chlorophyll *a*, whereas responses for AFDM were non-significant (Fig. 4.3, Table 4.2). Heatwaves generated a biologically-relevant moderate effect on the autotrophic index, despite being non-significant (P=0.08). Low flow did not significantly affect these patch-scale descriptors of biofilm accrual, nevertheless, a strong whole-system response was evident, as outlined below.

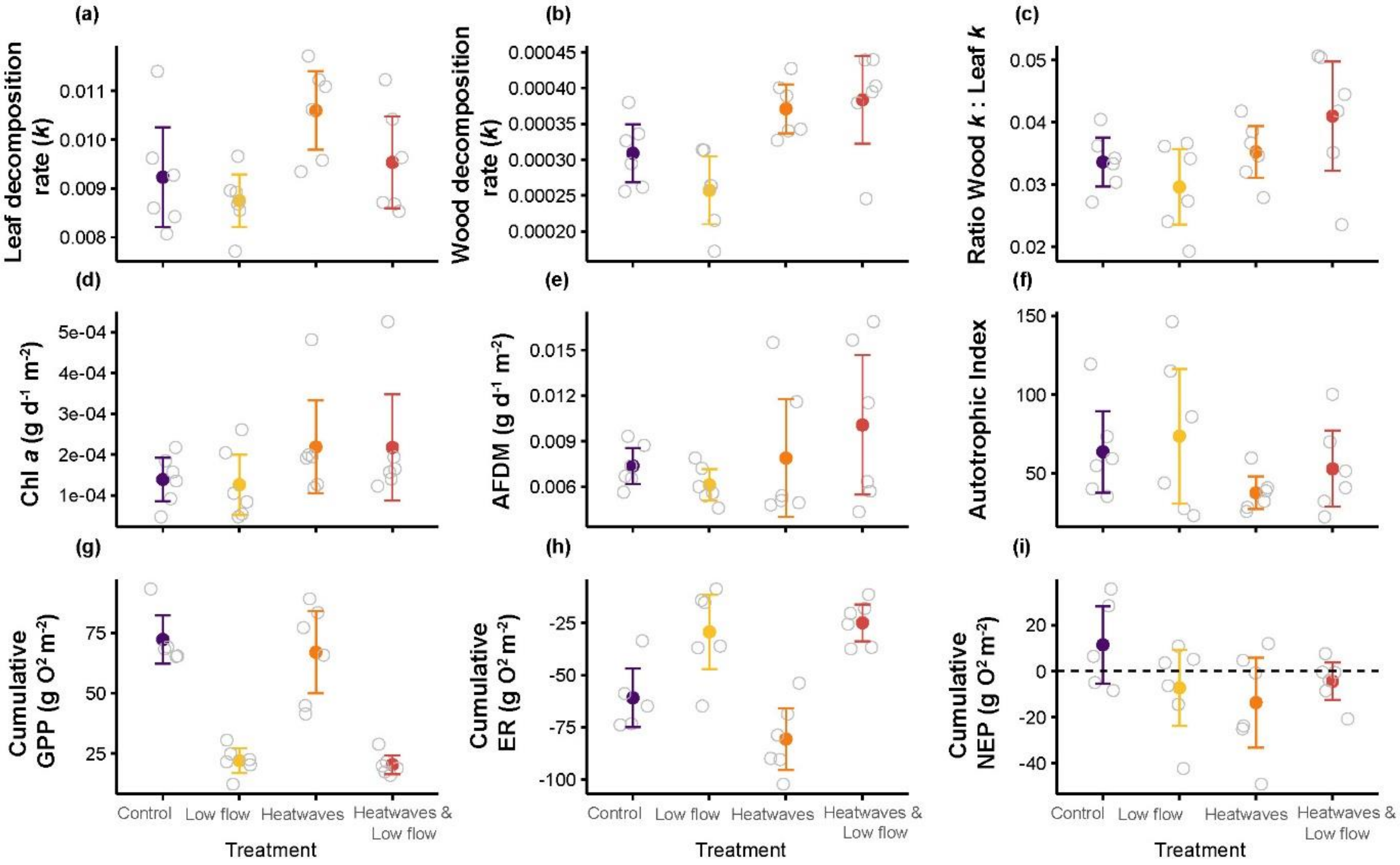

Fig. 4.35. Indicators of ecosystem processes across stressor treatments and controls. Data are plotted as mean ± CI_{95} for six patch-scale (panels a-f) and whole-system scale (g-i) functional indicators. GPP = Gross Primary Production, ER = Ecosystem Respiration, NEP = Net Ecosystem Production, AFDM = Ash Free Dry Mass and Chl a = Chlorophyll a. Grey circles on plots are data for mesocosm channel.

Table 4.16. Results of the mixed-effect ANOVA type II to test the effect of the treatments on the patch-scale indicators: leaf decomposition, wood decomposition, ratio of leaf:wood decomposition, autotrophic biomass accrual (i.e. *Chl a* concentration on tiles), total biofilm accrual (AFDM on tiles), autotrophic index (ratio of autotrophic and heterotrophic growth rates), dissolved organic carbon concentration (DOC), specific absorbance at 254 nm (SUVA_{254}), ratio of Peak C (humic-like fluorescence) to Peak T (tryptophan-like fluorescence) (C:T ratio) and Humification Index (HIX). Standardized effect sizes (Cohen's d) are presented for each factor and categorized following Cohen (1988). All p-values were calculated using Chi-squared likelihood ratio tests and were adjusted to control for type 1 errors (see methods). Bold text indicates statistically significant results and underlined text, biologically relevant results (i.e. d category >trivial and p<0.20).

Response	Treatment	Cohen's d	±CI	d category	p
Leaf decomp. (k_{leaf})	**Heatwave**	**1.480**	**6.952**	**Large**	**0.001**
	Lowflow	0.031	1.195	Trivial	0.720
	HW&LF	-0.165	1.418	Trivial	0.720
Wood decomp. (k_{wood})	**Heatwave**	**1.117**	**5.059**	**Large**	**<0.001**
	Lowflow	-0.826	1.316	Large	0.483
	HW&LF	0.520	2.602	Moderate	0.293
Ratio k wood: k leaf	Heatwave	0.288	1.781	Weak	0.131
	Lowflow	-0.623	1.289	Moderate	0.574
	HW&LF	0.516	2.588	Moderate	0.312
Chl-a accrual	**Heatwave**	**0.668**	**3.314**	**Moderate**	**0.006**
	Lowflow	-0.108	1.196	Trivial	0.641
	HW&LF	0.049	1.216	Trivial	0.641
AFDM accrual	Heatwave	0.136	1.349	Trivial	0.453
	Lowflow	-0.328	1.204	Weak	0.891
	HW&LF	0.451	2.403	Weak	0.453
Autotrophic Index	Heatwave	-0.754	3.689	Moderate	0.078
	Lowflow	0.287	1.202	Weak	0.413
	HW&LF	0.077	1.246	Trivial	0.413
DOC	Heatwave	-0.495	2.584	Weak	0.677
	Lowflow	0.446	1.212	Weak	0.063
	HW&LF	0.406	2.177	Weak	0.620
SUVA254	Heatwave	0.805	3.911	Large	0.285
	Lowflow	0.969	1.276	Large	0.285
	HW&LF	-0.365	2.034	Weak	0.285
C:T ratio	Heatwave	1.019	4.863	Large	0.079
	Lowflow	0.931	1.270	Large	0.109
	HW&LF	-0.179	1.478	Trivial	0.455
HIX	Heatwave	0.241	1.635	Weak	0.842
	Lowflow	**1.212**	**1.320**	**Large**	**0.007**
	HW&LF	-0.013	1.254	Trivial	0.916

4.3.3. Whole-system responses

DOM quantity and composition were affected by low flow and heatwaves, but no interaction was apparent (Fig. 4.4; Table 4.2). Low flow significantly increased the HIX, an indication of microbial degradation of DOM. While it was not significant at 0.05, low flow did increase DOC concentration and C: T ratios, with more humic-like compounds relative to protein-like compounds. A similar effect was found on heatwaves treatment where a biologically relevant large effect size on C: T ratios was apparent.

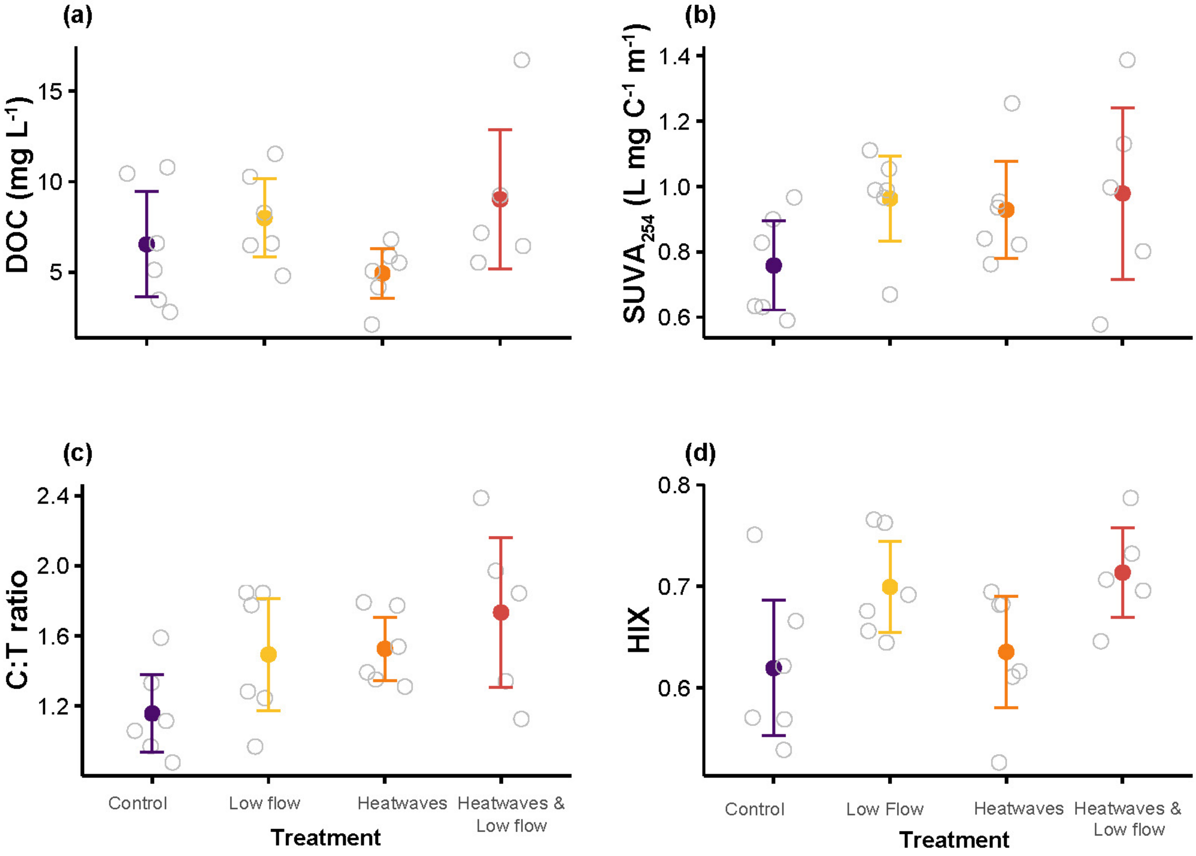

Fig. 4.36. Indicators of dissolved organic matter responses across stressor treatments and controls. Data are plotted as mean ± CI_{95} for dissolved organic matter quantity and quality indices: a) DOC concentration; b) $SUVA_{254}$ index; c) Peak C:T ratio; and d) Humification Index (HIX). Grey circles represent values for individual channels.

Both GPP and ER were strongly reduced in low flow and heatwaves & low flow treatments (Table 4.3, Fig. 4.3). GPP presented a larger effect with effect size doubling the values for ER. While the reduction associated with low flow was significant, neither the effect of heatwaves alone nor their interaction with low flow was statistically significant for either descriptor. Despite these impacts, NEP was not significantly affected by the stressors (Fig. 4.3). Nevertheless, an assessment of NEP temporal dynamics revealed a general shift from autotrophy to heterotrophy as the experiment progressed (mean NEP_{cum}: low flow -9.94 mg O_2 m^{-2}; heatwaves -16.16 O_2 m^{-2} and heatwaves & low flow -6.23 mg O_2 m^{-2}; Fig. 4.5). This was weakest for the control which was net autotrophic (mean NEP = 5.31 mg O_2 m^{-2}). The highest daily NEP values were recorded on day 4 of the experiment for the control and heatwaves treatment (2.27 and 2.16 mg O_2 m^{-2} day^{-1} respectively) while the maximum for low flow and heatwaves & low flow treatments were > 50% lower (0.6 and 1.08 mg O_2 m^{-2} day^{-1} respectively). The minimum NEP for all treatments was recorded after day 30 and were in the order control < heatwaves & low flow < low flow < heatwaves (-1.93, -2.88, -4.47, -5.43 mg O_2 m^{-2} day^{-1}).

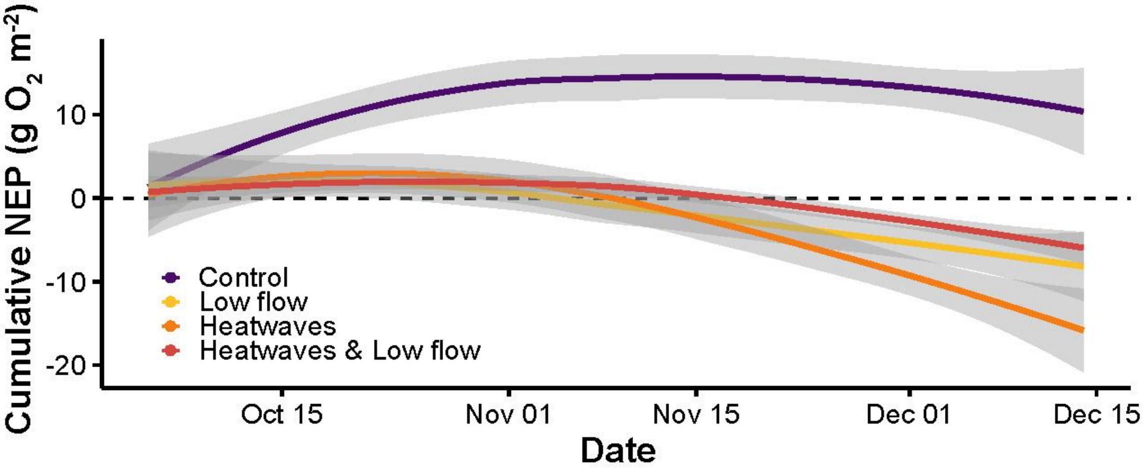

Fig. 4.37. Net ecosystem production for the control (purple) and the three stressors (heatwaves, orange: low flow, yellow; and heatwaves & low flow, red) treatments during the experimental period. The solid lines represent a LOESS smoother fitted with span = 0.9 and the grey shading is the associated 95% confidence interval.

Table 4.17. Results of the mixed-effect ANOVA type II to test the effect of the treatments on whole system functioning; specifically, cumulative metabolism (ER, GPP and NEP) and multifunctionality index. Standardized effect sizes (Cohen's d) are presented for each factor and categorized following Cohen (1988). All p-values were calculated using Chi-squared likelihood ratio tests and were adjusted to control for type 1 errors (see methods). Bold text indicates statistically significant results and underlined text, biologically relevant results (i.e. d category >trivial and p<0.20).

Response	Treatment	Cohen's d	±CI	d category	p
Cum. ER	Heatwave	1.128	5.107	Large	0.519
	Lowflow	**-1.804**	**1.531**	**Large**	**<0.001**
	HW&LF	-0.689	3.273	Moderate	0.519
Cum. GPP	Heatwave	-0.415	2.211	Weak	0.625
	Lowflow	**-3.937**	**2.293**	**Large**	**<0.001**
	HW&LF	0.145	1.405	Trivial	0.805
Cum. NEP	Heatwave	-1.297	5.828	Large	0.350
	Lowflow	-0.965	1.338	Large	0.350
	HW&LF	0.720	3.401	Moderate	0.398
Multifunc. Index	Heatwave	0.258	1.689	Weak	0.080
	Lowflow	**-1.984**	**1.583**	**Large**	**0.001**
	HW&LF	0.494	2.504	Weak	0.219

The impact of the sequential heatwaves on metabolism varied among heatwave events, with the strongest responses observed for GPP and NEP (Fig. 4.6; Table 4.4). There was a significant effect of heatwave event (HWE) on GPP and a significant interaction between HWE and low flow (Table 4.4). A reduction for all treatments was apparent from values during HWE 1 to those in HWE 2 and HWE 3 (Fig. 4.6). The interaction between the heatwaves and low flow treatments was not significant despite presenting a moderate effect size (C_f, Table 4.4). Likely, the limited sample size might have reduced the power of the model to generate significant results. In the treatment with both stressors, the cumulative reduction in GPP represented the sum of the individual effects (i.e. interaction C_d close to 0; Fig. 4.6). The response to the sequential heatwaves was less pronounced for ER, with no significant three-way ANOVA terms (Table 4.4). There was, however, a non-significant large effect size on ER, with ER increasing in each successive heatwave event

($C_{d\ HWE\ 1}$= -0.0940 ± 1.32, $C_{d\ HWE\ 2}$= - 0.442 ± 2.31 and $C_{d\ HWE\ 3}$ = -0.487 ± 2.48). The interaction between heatwaves and low flow was not significant at the 5 % level but was presented a large effect size (Table 4.4, Fig. 4.6). The effect size was larger during HWE 2 ($C_{d\ HWE2}$= 0.801 ± 3.73). NEP was affected by the sequential heatwave events (Table 4.4) with reductions apparent not only for all treatments, but for the interaction (Fig. 4.6). There was a significant interaction between low flow & event (Table 4.4; $C_{d\ HWE\ 1}$= -1.39 ± 1.42, $C_{d\ HWE\ 2}$= -1.20 ± 1.45 and $C_{d\ HWE\ 3}$ = 0.085 ± 1.25) and a significant 3-way interaction (Table 4.4).

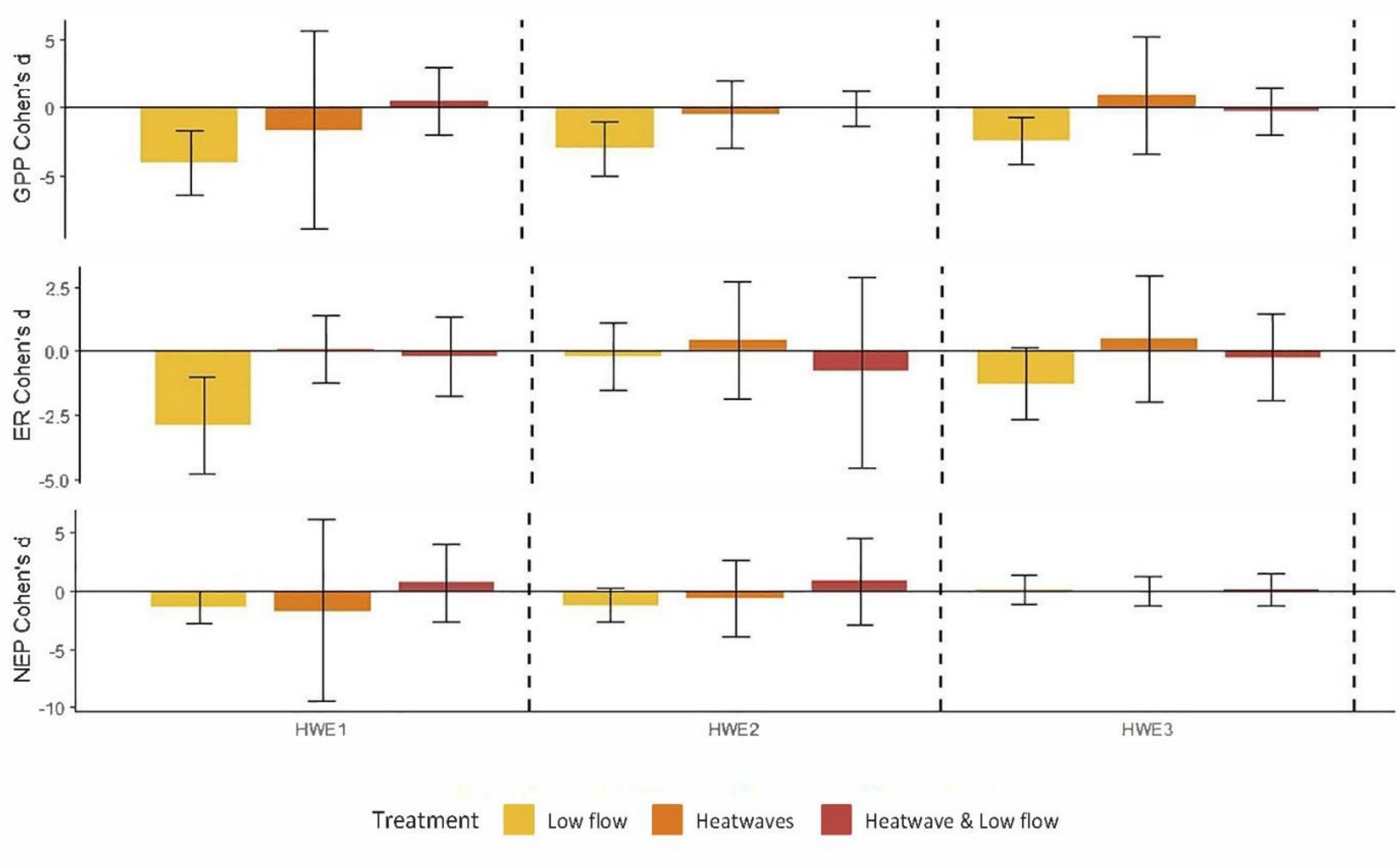

Fig. 4.38. Metabolism responses relative to the control for the three sequential heatwaves (HWE1, HWE2, HWE3). For each of the period events, the standardized effect size, Cohen's d ± CI_{95}, are shown for each metabolism measurements – gross primary productivity (GPP, top), ecosystem respiration (ER, middle) and net ecosystems productivity (NEP, bottom). The effect of the treatments and the interaction are presented: low flow treatments (yellow), heatwaves (orange) and heatwave low flow interaction (red). Notice that ER axis has been reverse and is reported as loss in ER to facilitate interpretation.

Table 4.18. Results of the three-way ANOVA to test the effect of the three sequential heatwaves events on metabolism dynamics. For each of the metabolism measurements (GPP, ER, NEP), 8-days cumulative values for each heatwave-flume were calculated and the effect of the heatwaves treatment (HW), low flow treatment (LF) and the interaction of the two (HW&LF) was analysed. To assess changes of this effects between heatwave events, we assess the effects of heatwave event (HWE) and its interactions with the treatments (HW:HWE, LF:HWE, HW:LF:HWE). Standardized effect size (Cohen's f) is presented for each factor and categorized following Cohen (1988), describing the proportion of the variability accounted for each treatment. All p-values were calculated using Chi-squared likelihood ratio tests and were adjusted to control for type 1 errors (see methods). Bold text indicates statistically significant results and underlined text, biologically relevant results (i.e. d category >trivial and p<0.20).

	Cum GPP			Cum ER			Cum NEP		
Treatment	Cohen's f	f category	P	Cohen's f	f category	P	Cohen's f	f category	P
HW	0.153	Weak	0.263	0.184	Weak	1.000	0.115	Weak	0.218
LF	**1.590**	**Large**	**<0.001**	0.777	Large	0.182	**0.045**	**Trivial**	**0.013**
HWE	**0.245**	**Weak**	**<0.001**	0.146	Weak	1.000	**0.273**	**Moderate**	**0.017**
HW&LF	0.073	Trivial	0.180	0.211	Weak	0.182	0.207	Weak	0.223
HW: HWE	0.823	Large	0.161	0.498	Large	1.000	0.367	Moderate	0.129
LF:HWE	**1.123**	**Large**	**0.003**	0.633	Large	1.000	**0.743**	**Large**	**0.006**
HW:LF:HWE	0.326	Moderate	0.180	0.455	Large	0.182	**0.417**	**Large**	**0.047**

4.3.4. Multifunctionality

Overall, the low flow treatment significantly reduced multifunctionality with a large effect size (Table 4.3, Fig. 4.7). Despite being insignificant, the sequential heatwaves treatment presented a weak effect size which could be considered biologically relevant, (P= 0.080). There was no interaction between the main effects (i.e. not statistically significant neither biologically significant).

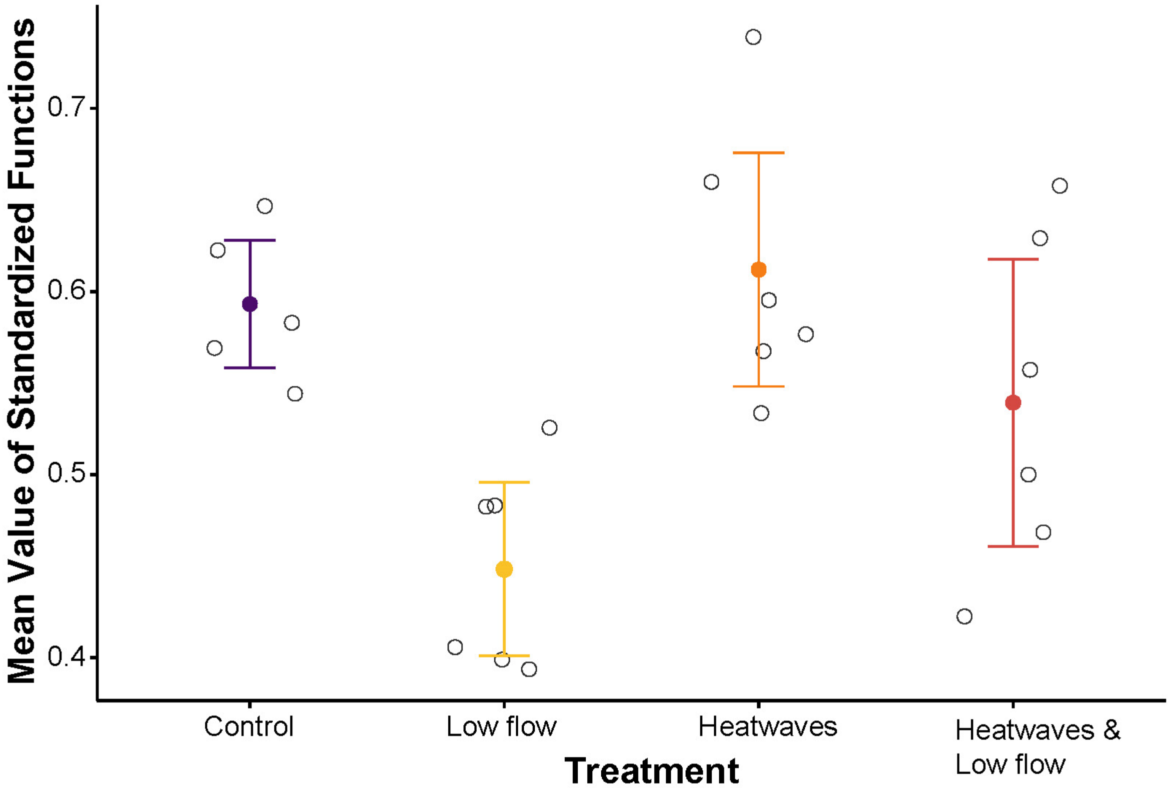

Fig. 4.39. Multifunctionality presented as the standardised value of patch-scale and whole ecosystem processes across the stressor treatments and controls. Data are plotted as mean ± CI_{95} with grey circles representing values for individual channels.

4.4. Discussion

This study used a multifunctional approach to develop an integrative understanding of the impact of heawaves and an additional stressor, as low flow, on aquatic systems (Giling et al., 2019; Alsterberg et al., 2014). Major differences occurred in the responses at different spatial scales (patch-scale vs whole system metabolism) but taken together indicated that multifunctionality was suppressed by low flows but enhanced by heatwaves with respect to functional processes.

4.4.1 Effects of heatwaves and low flow on ecosystem processes

As heatwaves stimulated heterotrophic process rates (detrital decomposition and DOC aromaticity) to a greater degree than autotrophic processes this supported our first hypothesis H_1 that heatwaves will stimulate heterotrophic processes more than autotrophic ones, thereby increasing the rates of detrital decomposition and ecosystem respiration. Two-thirds of the patch-scale process rates observed were elevated by the heatwaves treatment. Enhanced microbial detrital processing with higher water temperature is expected under metabolic theory (Brown, 2004; Arrhenius, 1915; Van't Hoff, 1896), being primarily a physiological response (e.g. increased enzymes activity or respiration). Previous experiments applying constant warming have recorded elevated microbial processing rates (Gossiaux et al., 2020; Jabiol et al., 2020; Ferreira and Chauvet, 2011) that increase exponentially as enzyme activation energy is fulfilled (Gossiaux et al., 2020; Jabiol et al., 2020; Freixa et al., 2017; Romaní et al., 2014; Ferreira and Chauvet, 2011). Hence, the strongest decomposition responses likely occur at warmer water temperature than observed during this study (i.e. 10-25 °C), however, this can be modulated by litter quality and diversity of the heterotroph community (Ferreira and Canhoto, 2014; Martínez et al., 2014; Fernandes et al., 2012). For the heatwave treatments, water temperature was below

10 °C for ~50% of the experiment, resulting in a more modest increase in process rates than that produced by sustained warming. Thus, lower efficiencies of leaf litter breakdown were observed when taking into consideration decomposition rates per degree-day. Interestingly, increases in processing rates for wood were greater, relative to leaf detritus in the heatwaves treatments, suggesting that enzyme activity related to episodic temperature increases may be coupled with more recalcitrant organic matter sources (Jabiol et al., 2020; Fierer et al., 2005). Similar responses may occur due to changes in microbial community structure. As species with higher thermal tolerance limits become more abundant, the biologically-active temperature range might be amplified or shifted (Jabiol et al., 2020; Manning et al., 2018; Dang et al., 2009). Overall, the observed elevated processing rates and potential for more recalcitrant organic carbon to be mineralised has important implications for increased greenhouse gas emissions from streambeds under scenarios of future climate change (Romeijn *et al.*, 2019).

Heatwaves also increased autotrophic production, but with a smaller effect size than those reported for decomposition thereby further supporting H_1. With higher rates of chlorophyll-*a* accrual and lower autotrophic index values (greater algal biomass relative to heterotrophic biomass), our study highlights the potential for autumnal heatwave events to stimulate autotrophic biofilm growth in rivers. Warming experiments in streams typically show that productivity increases are more related to resource use efficiency (Hood *et al.*, 2018) than shifts in community composition (Villanueva *et al.*, 2011), while there is substantial variance modulated by environmental covariates such as solar radiation, seasonality and nutrient concentrations (Gossiaux et al., 2020, 2019; Witteveen et al., 2020; Delgado et al., 2017). Enhanced autotrophic growth might be a response specific to autumn heatwaves, as the stimulatory effect of heatwaves is likely to be highest in colder months (Delgado *et al.*, 2017), and dependence on solar irradiation is often constrained by shading from riparian vegetation during late spring and summer in smaller streams (Myrstener *et al.*, 2020).

In our experiment, low flows were applied as a reduction in water depth and wetted area without gravel dewatering, a scenario that replicates flow responses to flow reduction in permeable river catchments. A reduction in the wetted area during low flow phases can represent a strong press disturbance on biofilm and microbial activity as the river active area contracts, however, there was no response in patch-scale functions measured at or close to the gravel substrate (e.g. detrital decomposition and biofilm accrual). In other low flow experiments, reductions in biofilm process and growth rates have been observed but generally in response to extensive gravel dewatering (Sabater et al., 2018; Arroita et al., 2017). In our experiment, limited evidence for density-dependent effects on patch scale processes was found (i.e. associated with a reduction in wetted area). This is likely due to second-order stressors (e.g. anoxia, temperature tolerance exceedance), which can trigger changes in microbial physiology or community structure, being less pronounced during autumn (Schimel, Balser and Wallenstein, 2007; Romero *et al.*, 2019).

4.4.2. Effects of heatwaves and low flow on whole system metabolism

Low flow strongly reduced whole system metabolic processes (i.e. cumulative GPP, ER and NEP) thereby supporting our second hypothesis H_2 that low flows will reduce gross primary production and ecosystem respiration due to habitat contraction. The habitat contraction associated with the low flow conditions reduced cumulative GPP and ER through direct mortality of dewatered autotrophs and heterotrophs (i.e. biofilm on the flume walls) and a reduction in habitat volume and area for both benthic and suspended organisms. Consistent with previous research, the uneven distribution of autotrophs and heterotrophs over the dewatered area (i.e. the autotroph biofilm community is distributed on walls/banks and surface gravel, while heterotrophs can also colonise the underside of gravel) translated into an asymmetric response of GPP and ER (Sabater *et al.*, 2016). The observed reduction of GPP to one-third of the control treatments and ER to one-half pushed the system into a more heterotrophic state (lower/negative NEP). Low flow also modified the dissolved organic matter quantity and composition (i.e. higher DOC, C: T and HIX) suggesting a concentration effect possibly coupled with elevated extracellular release of DOM from algal cells (Villacorte *et al.*, 2015). The increased HIX may represent the biofilm/algal stress response with the release of higher molecular compounds than previously observed for stressed populations in laboratory cultures and natural environments (Elliott, Lead and Baker, 2006).

Habitat loss was the key driver in the decrease in GGP and ER observed in the heatwaves & low flow treatment, as responses were similar to those in the low flow treatment. This finding supports H_3 that the decelerating effect of low flow offsets the stimulatory effect of heatwaves on ER and GPP. Nevertheless, patch-scale processes and Peak C:T ratios showed similar positive responses in the heatwaves & low flow and heatwave treatments. Thus, the functioning of the biofilm community was strongly determined by the wetted channel area (Arroita *et al.*, 2017). Given the potential for elevated heterotrophic processing rates under warming scenarios (Yvon-Durocher *et al.*, 2010; Song *et al.*, 2018) and the majority of OM located in the bottom of the flumes (wetted gravels), a relative increase in ER for both heatwaves treatments was expected. Indeed, this increase in ER was present in the heatwaves treatment, but not in the combined heatwaves & low flow treatment. The absence of this increase suggests that additional stressors might be inhibiting ER (Keller *et al.*, 2020).

Short-term responses to the discrete heatwave events can help unravel the drivers or timing of these additional stressors. The autotrophic response to each heatwave varied between events, with temporal differences in GPP across the three heatwaves (i.e. a greater increase was apparent for the third heatwave event relative to control than for previous events). While the approaches used in this experiment do not allow us to specifically identify a mechanism for these differences, the finding suggests that either ambient water temperature was a limiting factor during the last heatwave (Woodward *et al.*, 2016) or acclimation of the microbial communities occurred (Freixa et al., 2017; Barthès et al., 2015; Romaní et al., 2014). These findings partially confirm H_4 that sequential heatwaves will generate an enhanced response during the later heatwaves indicating community acclimation, albeit this acclimatisation was altered when combined with low flow. The system became less heterotrophic as a strong reduction of ER in the interaction treatment drove this effect (Fig. 4.6). These findings further support H_4, suggesting that low flow weakened ecosystem resilience that manifested as a stronger reduction in ER during heatwaves. While heatwaves stimulated GPP and ER, in combination with an additional stressor, this had a detrimental effect and those effects accumulated over time. Some studies suggest that self-extracellular DNA release regulates biofilm formation and enhances competition responses within the biofilm matrix (Mazzoleni *et al.*, 2015; Ibáñez de Aldecoa, Zafra and González-Pastor, 2017). Exudates from the dried biofilm may have led to modified behaviour in the submerged biofilm (microbial collective modified behaviours; Yin et al., 2019; Papenfort and Bassler, 2016; Stanley et al., 2004), resulting in a reduction of respiration. Similar reductions of ER and GPP are found in intermittent streams prior to complete dewatering (von Schiller et al., 2011; Acuña et al., 2005). The concept of microbial collective behaviours might partially explain the temporal changes in ER and GPP observed for the low flow treatment. Specifically, the decrease in metabolism during each subsequent event suggests that direct mortality of microbes due to a reduction in the wetted area was not the only factor constraining metabolism.

4.4.3. Mesocosm experiments: experimental design and realism

Our experimental setup in an outdoor facility enabled us to capture seasonal and stochastic variability in local meteorological conditions (i.e. declining day length, temperature decrease, rainfall events) as well as diel patterns (i.e. in air temperature and light) which are often overlooked when experiments are conducted indoors (Gionchetta et al., 2020; Romero et al., 2019; Bedolfe et al., 2015). Here, microbial breakdown rates (mean ± SD: 0.0095 ± 0.001; range: 0.0078 – 0.0117 d^{-1}) observed in our small recirculating flumes were comparable to ranges reported for temperate streams across N. Europe (Chauvet *et al.*, 2016) and from a global synthesis of *Alnus* breakdown rates: 0.0169 ± 0.0128; 0.0011 – 0.0876 d^{-1} (Follstad Shah et al., 2017). Similarly, mesocosm GPP (0.63 ± 0.58; 0.0077-5.17 g O_2 m^{-2} d^{-1}) was comparable to a study of 365 temperate rivers in the USA (Appling et al., 2018b; GPP_{P50} = 0.659 g O_2 m^{-2} d^{-1}). However, mesocosm ER (0.72 ± 0.74; 0.016 - 5.89 g O_2 m^{-2} d^{-1}) was relatively low by comparison (ER_{P83} = 0.75 g O_2 m^{-2} d^{-1}) and likely constrained by the finite input of allochthonous material and lack of primary and secondary consumers. Our experimental setup and groundwater input may constrain the recolonization capacity of the system as drift into the channels from upstream areas was not possible (Wagenhoff, Townsend and Matthaei, 2012; Bruder *et al.*, 2017). Nevertheless, aerial colonization of emerging adults and groundwater bacteria were still happening. Mesocosm experiments inevitably represent a simplification of ecological complexity but this trade-off enables increased replication and investigation of environmental stress gradients that are not possible using survey-based approaches (Stewart *et al.*, 2013). Mesocosms avoid the confounding variability normally associated with field experiments (i.e. geographical effects, differences in anthropogenic pressures), and can adequately capture the biocomplexity of riverine ecosystems (Ledger *et al.*, 2009; Brown *et al.*, 2011; Bruder *et al.*, 2017; Romero *et al.*, 2019; Sarremejane *et al.*, 2020).

4.5. Summary and synthesis

This is one of the first mesocosm experiments to assess the impact of autumn heatwaves, in combination with low flow, on stream ecosystem multifunctionality. These results suggest autumn heatwaves are likely to stimulate heterotrophic processes over autotrophic processes, and increase processing rates of recalcitrant organic matter. This in turn has the potential to increase greenhouse gas emissions from streambeds under likely scenarios of future climate change. Conversely, low flow suppressed whole-system processes, reducing the stimulatory effect of heatwaves on ecosystem respiration and gross primary production. Finally, the identification of legacy effects of heatwaves (i.e. their position in a sequence of events) and interactions with low flow have important implications as we move into a period of increased climate extremes and highlights the need for research on heatwaves across annual to inter-annual time scales.

4.5. References

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5. Synthesis and future perspectives

5.1. Closing remarks

River impoundments result in a wide range of flow modifications which can interact with additional stressors and result in a variety of ecological responses (Stubbington *et al.*, 2022). This study used mesocosms to explore flow regulations on headwater streams without cofounding variables derived by dams, regional variation or human activities. The impacts of flow modification on functional and structural properties of biofilm and macroinvertebrate communities were explored for different components of the flow regime (timing of high and low flow, reduced flow magnitude and intermittence, and annual homogenisation of flow dynamics). Additionally, the interaction between low flow and heatwaves was explored on biofilm functionality. Given the experimental design used in this research, the sample size (n=4) was determined based on ecologic standards and experimental feasibility. Its limitations need to be considered when interpreting the outputs of the statistical models. The research identified five key impacts of flow regulation commonly associated with impoundments:

1) all flow regulations increased spatial homogenisation of heterotrophic biofilm functionality and increased OM processing potential;

2) intermittency and heatwaves both increased the processing rate of CPOM/ detritus, with effects being systematically higher for recalcitrant material;

3) the altered timing of high and low flows increased the stochasticity of benthic biofilm growth rates and increased the probability of extensive algal growth;

4) species turnover due to flow regulations was the result of ecological preferences (habitat and flow velocity) rather than filtering of biological traits;

5) contrary to drought-ecology theories, our results suggest perennial streams might be more resistant to moderate drought intensity (intermittency, channel fragmentation) than previously thought as resistance and resilience strategies were evident for biofilm and macroinvertebrate communities, despite an overall functional reduction.

5.2. Future scenarios: increased intermittency and heatwaves

River intermittency is widely increasing globally, with six of the largest rivers on Earth becoming intermittent in the main stream and dewatering events happening earlier and for longer in Europe (Larned *et al.*, 2010; Tramblay *et al.*, 2021). Artificial and natural causes might cause dewatering, but drying patterns (timing and magnitude) are normally indistinguishable (Snelder *et al.*, 2013). Despite the symmetries, drying in perennial streams, such as streams in the UK, might have stronger effects than in those streams occurring naturally (Zipper *et al.*, 2022). In perennial streams, drying might lead to tipping points as the communities are not adapted to drying disturbances (drought resistance strategies).

Despite dewatering generating reduced functionality due to habitat contraction and instability (Arroita *et al.*, 2018), the results of this study highlight the ability of the ecosystem to adapt to moderate drought intensity (chapter 2 and 3), as evidenced by the combination of strategies present in the drought treatments: resistant strategies, refugia utilization, resource partitioning and temporal adaptation to the wet-dry phases (Van Looy *et al.*, 2019; Truchy *et al.*, 2020). Contrary to previous drought studies on perennial streams, channel fragmentation (riffles dewatering) did not drastically reduced taxonomic richness (Boulton, 2003; Aspin, 2018; Datry *et al.*, 2022), indicating that migration to more suitable habitats rather than direct mortality and increased competition might be the cause of richness reductions previously observed (Sarremejane, Messager and Datry, 2022).

As shown in previous studies, biofilm functioning is highly modified by flow reduction and cessation (Acuña *et al.*, 2005; Sabater *et al.*, 2016; Arroita *et al.*, 2017). Given the asymmetric distribution of autotrophic and heterotrophic biofilm (hyporheic zone) and lower resistance to dewatering of photosynthetic pigments (Ponsatí *et al.*, 2015; Colls *et al.*, 2021), drought and dewatering events generate greater reduction on the autotrophic biofilm resulting in a heterotrophy increase (chapter 2 and chapter 4). During river intermittency, oxygenation of the hyporheic zone contributes further to this heterotrophy increase (Arias-Real *et al.*, 2020). Furthermore, possible interactions with heatwaves might exacerbated this heterotrophic stimulation (chapter 4), with stronger effects for recalcitrant material (Brown, 2004; Arrhenius, 1915; Van't Hoff, 1896). Nevertheless, this heterotrophic stimulation might be modulated during the different stages of drying (habitat contraction, riffle dewatering, surface water loss and dewatering of hyporheic zone) and the resilience and resistance of biofilm modified depending on the desiccation severity and duration (Sabater *et al.*, 2016; Arias-Real *et al.*, 2020).

5.3. Implications for management and conservation

By improving our understanding of flow-ecology relationships this study provides new insights into how environmental flows can be best managed to sustain river ecosystem biodiversity and functioning in the present and under future climate change scenarios. Most significantly, this study highlighted the need for the protection of refugia under drought conditions (pools and hyporheic zone) to ensure the maintenance of ecosystem functioning and macroinvertebrate communities (chapter 2 and 3). Given the similarity in macroinvertebrate between the drought treatment and the homogenised flow, this study showed that neglecting inter-annual variability in dam releases (i.e % of annual flow management approach) can lead to the development of lentic communities below dams, even where water quality and biofilm functioning are maintained (chapter 2 and 3). Furthermore, flow alteration effects might interact with additional dam effects, as thermal regime and nutrients alterations exacerbate ecosystem deterioration (Sabater *et al.*, 2008; Ellis and Jones, 2013). For example, a change in the timing of high and low flows (i.e. flow reversal, irrigation) led to bed greening (extensive algae growth), with growth likely to further increased under high-nutrient concentration releases (Ponsatí *et al.*, 2015).

Furthermore, it is essential to consider to what extent these flow alterations persist along the river continuum. Findings from this study demonstrated that drought and reduction of flow magnitude generated a reduction in biofilm functionality, and a shift to heterotrophy with major shifts in macroinvertebrate community structure apparent (chapter 2, 3, and 4). Questions remain, however, as to how far OM and nutrient concentrations effects persist downstream of dams, and whether effects are diluted by inputs from unregulated tributaries downstream (Westhorpe *et al.*, 2015). For instance, where damming causes low flows downstream, reaches receiving tributary inputs might display reduced flow magnitude and width, but can recover annual variability thereby minimizing the negative effects on biofilm functioning and community structure (Growns *et al.*, 2014). We found homogenised flow and flow reversal generated milder effects but might have longer longitudinal persistence since tributaries downstream might not be able to compensate alteration on the flow patterns. Both flow alterations have the potential to increase low flows and/or reduce annual flow variability (Poff *et al.*, 2007), with their persistence depending on the relative discharge of downstream tributaries (i.e. contribution to the flow of the regulated channel). Under these circumstances, the bed greening observed in the flow reversal might persist and expand downstream due to reduced flow velocity and sustained homogenization (Piqué *et al.*, 2016; Mineeva, Semadeny and Makarova, 2020). Previous studies have found water quality changes to persist up to 100 km below the dam (Preece and Jones, 2002) and toxic algae as far as 150 km (Baldwin, Wilson, Gigney, 2009).

Future climate scenarios will potentially affect carbon and organic matter cycling (chapter 4). During heatwaves, increased processing rates (i.e. decomposition rates, ER) can increase both DOM inputs and greenhouse gasses emissions. Subsequent changes in DOC quality and processing efficiencies of carbon may further accelerate the respiration of recalcitrant and slow decomposing carbon. This is especially relevant in ecosystems containing high levels of recalcitrant OM in the riverbed where biological processes are often constrained by low temperature, such as in peatlands or tundra. Generally, warming does not generate microbial community diversity changes but stimulates or decreases metabolism and process rates depending on community tolerance temperature optimum, which are 18-25 °C in soil microbes (Yvon-Durocher *et al.*, 2010; Carey *et al.*, 2016; Woodward *et al.*, 2016). In temperate regions, water temperature is typically below these values, hence, heatwaves during early spring, autumn, or at high latitudes may promote a marked increase in process rates. Hence, there is need for river managers to consider both heatwave timing and geographical location to determine CO_2 release to the atmosphere, particularly given climate extremes are becoming more frequent and of greater magnitude.

Climate change and rising population are increasing the pressures for freshwater provisioning globally, and will be one of the biggest challenges of the 21st century (Dudgeon, 2010; Solomon, 2010). Given the deterioration of rivers worldwide and the need to protect the ecosystem services they provide, efficient environmental flows need to be developed to sustain biodiversity and ecosystem functioning. Instead of current approaches such as releasing a proportion of the natural flow regime or high pulse floods (Chen and Olden, 2017; Gillespie, Kay and Brown, 2020), environmental flows need to target certain biotic processes, trigger geomorphological activity or enhanced connectivity (Acreman and Dunbar, 2004; Poff *et al.*, 2010). High flows downstream of dams and lateral connectivity of river segments could be enhanced by rehabilitating floodplain areas (Baldwin and Mitchell, 2000; Aristi *et al.*, 2014). River widening is increasingly being used for flood protection in lower stretches of river basins (Poff, 2002; Aerts, 2018; Klijn, Asselman and Wagenaar, 2018). Similar approaches could be used below dam reaches to permit river lateral connectivity and increase flow lamination areas during flood events. Similarly, future restoration measures should target changes in flow patterns to achieve improvement in ecosystem biodiversity (chapter 3) and controlled dam releases is the most practicable way to achieve it (Lu *et al.*, 2019). Dam releases can also be used to mitigate the effects of future climate change scenarios. Cold hypolimnetic releases could be used to lower water temperatures during heatwaves to minimise greenhouse emissions (chapter 4) and reduce fish thermal stress; and generally prevent the expansion of eurythermic invasive species (Olden and Naiman, 2010; Radinger and García-Berthou, 2020). Environmental flow management could be improve by synchronising releases with tributaries increase discharge (rainfall events) which would promote sediment transport and riparian connection (Consoli *et al.*, 2022). Currently, dams are one of the major threats for river ecosystems, but if properly managed they also offer an unforeseen potential to mitigate current degradation status of rivers(Dudgeon, 2010).

5.4. Future directions

This section provides an overview of future research needed in the field to further advance flow-ecology relationships and management of environmental flows:

i. Given the variability of hydrological alterations generated by flow regulation, quantification of the deviation from the natural flow regime (or naturalised, which is flow without withdrawals and flow regulations) could provide fresh insights on what variation will cause ecological impacts. As the natural flow regime determines life-cycle adaptations of taxa (Bunn and Arthington, 2002; Arthington *et al.*, 2018), the regulations on different flow components (magnitude, timing, duration, frequency and rate of change) could lead to differing outcomes for specific species and the biological community (e.g. biofilm, macroinvertebrates and fishes, Tonkin *et al.*, 2021). Furthermore, physicochemical alterations (dissolved oxygen, nutrients and water temperature) generated by dams have been widely studied (Olden and Naiman, 2010; Poff, 2018; Aspin *et al.*, 2020), but, specific interactions of these additional stressors with flow alterations should be further examined in depth.

ii. Given the increase pressure in water resources and climate change, reservoirs water levels are more prone to fluctuate and reach lower levels in the future. In the south of Europe, some reservoirs are below 10% of their capacity after the 2022 drought and the frequency and duration of these events are expected to increase in the future under climate change (Ministerio para la Transición Ecológica and AEMET, 2022; Satoh *et al.*, 2022). Water quality in reservoirs during low flow periods can be altered by concentration effects, but also by biochemical reactions inside the reservoir (Wang *et al.*, 2020). During drought, reduced flow magnitude downstream might further intensity the effects of water quality alterations (Pereira *et al.*, 2017). Elucidating the effect of these combine stressors during droughts need to be investigated to improve management of running waters in the future and unravel possible drought long-term effects.

iii. Although functional traits have been described for a sizeable fraction of European macroinvertebrates (Tachet *et al.*, 2010; Serra *et al.*, 2016), these are focused on biological and ecological traits and provide limited information on timing of emergence and synchronization of life-cycles and resistance and resilience of species to disturbance. Hence, there is an urgent need to develop studies to inform dispersal potential, seasonality patterns on reproduction and better constrain phenotypical plasticity of reproduction traits, particularly for Diptera (Poff *et al.*, 2006; Sarremejane *et al.*, 2020; Twardochleb *et al.*, 2021). While some studies are being developed, there is a strong focus on aerial adult life stage which is relevant from a recolonization perspective (Twardochleb *et al.*, 2021), but neglect persistence of taxa in the aquatic phases of their life cycles, which is especially relevant for reduced mobility phases as eggs and nymph .

iv. As this study did not examine extreme drought, which can generate pool dewatering and stream bed drying, the findings describe resistance and resilience to moderate drought. An increase in frequency and duration of drought is predicted in future climate change scenarios (Satoh *et al.*, 2022) which could lead to more extensive dewatering of perennials streams and increased river fragmentation (Cañedo-Argüelles *et al.*, 2015; Tonkin *et al.*, 2018; Datry *et al.*, 2022). Thus, there is an urgency to research how more intense drought severity might affect aquatic communities, while accounting for temporal variability of rivers systems (i.e. supra-seasonal drought, characterised by reduced flow magnitude and more severe dewatering in hottest months). Also, post-drought recovery trajectories might be affected by river fragmentation as habitats become more isolated from recolonization pools (Ellis and Jones, 2013; Mellado-Díaz *et al.*, 2019), which might make the effects of drought after flow resumption more persistent. Manipulative mesocosm experimentation and large-scale studies are necessary to elucidate drought resistance and resilience mechanism and how those interact with different drought severity.

v. As this study investigated the functioning and structure alteration of communities generated by flow regulation, the results represent community succession under a press disturbance (continuous flow discharge alteration)(Lake, 2000). Below dams, flow regulation simplifies and modifies the communities structure and functioning (Ellis and Jones, 2013; Krajenbrink *et al.*, 2019), which could be further modified by additional flow pulse disturbances. In the future, the occurrence of extreme events, such as drought, floods or heatwaves, are expected to increase (Ledger *et al.*, 2013; Ledger and Milner, 2015) which would act as pulse disturbances on an already affected ecosystem. Mesocosm and manipulation experiments could bring new insight to understand the ecological responses of regulated streams to shifting climate patterns and increase occurrence of extreme events.

vi. As this study used a mesocosm approach, the role of the hyporheic zone and riparian areas could not be fully incorporated into the experimental design. Nevertheless, recent studies have highlighted the high processing rates within the hyporheic zone during low periods (Arias-Real *et al.*, 2020). Given the high accumulation of sediment below dams, the oxygenation of the hyporheic zone during low flows might lead to high emissions of greenhouse gasses (Benítez-Mora and Camargo, 2014). Furthermore, nutrient releases by dams might booster processing rates downstream (priming effect) (Guenet *et al.*, 2010). During low flow, heatwaves might have similar consequences on hyporheic zone processing rates as superficial layers might prevent temperature tolerance being cross and UV protection, while maintaining increased temperatures. Field studies and manipulative experiments could bring some insights into the mechanisms boosting processing rates in the hyporheic zone during extreme events.

vii. Flow reduction and/or increases in depth below dams reinforce nutrient and pollution intake (Covino, 2017; Hannah *et al.*, 2022), which could alter the food-web by bottom-up control (Pereira *et al.*, 2017; de Guzman *et al.*, 2022). As the presence of pharmaceuticals and microplastics is increasing globally (Mohamad Ibrahim *et al.*, 2019), interactions between novel pollutants and flow regulation (and intermittency) need to be investigated as these may alter ecosystem function across a range of spatiotemporal scales (Stubbington *et al.*, 2022).

viii. As this study explored the impact of heatwaves and low flow only on biofilm communities, further research is necessary to predict the effects of heatwave effects and compound events (heatwaves plus additional stressors) on the whole stream ecosystems. Experiments capturing higher trophic levels are necessary to assess species interactions (i.e. bottom-up or top-down effects), species specific temperature tolerances, colonisation and drift effects (Ledger *et al.*, 2013). Similarly, understanding responses of top predators (fishes and Odonates) and terrestrial interactions effects (birds and spiders) to flow regulations could bring new insight on environmental flow management (Lu *et al.*, 2016; Radinger and García-Berthou, 2020; Dahlin *et al.*, 2021; Steward, Datry and Langhans, 2022). Furthermore, exploring the impacts of different frequencies, magnitude and duration of heatwaves might be crucial to understand the management practises needed to sustain freshwater systems in future climate change scenarios.

5.6. Reference

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6. Appendices

Table S2.1. One-way ANOVA results on hydrological metrics: annual mean Q (Q_{Annual}), coefficient of variation (Q_{CV}), Q_{95}, Q_5, summer mean Q (Q_{Summer}), winter mean Q (Q_{Winter}) and annual mean depth (D_{Annual}). Hydrological indices were calculated in each flume in a given period

variable	treat	Df	F_	p_adj	sig
Q_{Annual}	Flow	3	82.837	<0.001	***
Q_{CV}	Flow	3	126.360	<0.001	***
Q_{95}	Flow	3	58.623	<0.001	***
Q_5	Flow	3	59.483	<0.001	***
Q_{Summer}	Flow	3	70.183	<0.001	***
Q_{Winter}	Flow	3	106.888	<0.001	***
D_{Annual}	Flow	3	66.009	<0.001	***

Table S2.2. Pairwise comparison for the control and three flow treatments. Hydrological index presented are: annual mean Q (Q_{Annual}), coefficient of variation (Q_{CV}), Q_{95}, Q_5, summer mean Q (Q_{Summer}), winter mean Q (Q_{Winter}) and annual mean depth (D_{Annual}).

variable	group1	group2	p.adj	p.adj.signif
Q_{Annual}	H	N	<0.001	****
	D	N	<0.001	****
	I	N	0.439	ns
Q_{CV}	H	N	0.01	**
	D	N	<0.001	****
	I	N	0.674	ns
Q_{95}	H	N	<0.001	****
	D	N	<0.001	****
	I	N	0.756	ns
Q_5	H	N	<0.001	****
	D	N	<0.001	****
	I	N	0.866	ns
Q_{Summer}	H	N	<0.001	****
	D	N	<0.001	****
	I	N	0.031	*
Q_{Winter}	H	N	<0.001	****
	D	N	<0.001	****
	I	N	<0.001	***
D_{Annual}	H	N	0.015	*
	D	N	<0.001	****
	I	N	0.186	ns

Table S2.3. Marginal mean differences between mean Q for each treatment and season (summer and winter). Values are the difference between the column names group to the row group (i.e. MM N-Summer – MM N-Winter). Bolded values are significant comparisons (P<0.05). Values are mean differences of the transformed data as marginal means depend on a model. Data assess differences between flow treatments (natural control, N; flow reversal, R; homogenised, H; and drought, D) within season (lighter grey shaded cells), seasonal differences within treatments (darker shaded cells) and differences between treatments and seasons (clear cells).

		Summer			Winter			
Season	Treatment	D	R	N	H	D	R	N
Summer	H	0.7333	**-5.7718**	**-4.2614**	0.0296	0.1563	**-3.6374**	**-5.6538**
	D		**-6.5051**	**-4.9947**	-0.7037	-0.5770	**-4.3707**	**-6.3871**
	R			**1.5104**	**5.8014**	**5.9280**	**2.1343**	0.1180
	N				**4.2910**	**4.4177**	0.6240	**-1.3924**
Winter	H					0.1266	**-3.6671**	**-5.6834**
	D						**-3.7937**	**-5.8100**
	R							**-2.0163**

Fig. S2.1. Biofilm decomposition efficiencies: decomposition rate of leaf (degree^{-1} d^{-1}, left) and wood (degree^{-1} d^{-1}, middle) substrates and ratio of k_{wood} and k_{leaf}(right). Means and CI_{95} are presented for the three treatments (flow reversal, green; Homogenised, yellow; and drought, orange) and the control (natural, blue) during the summer (Sum) and autumn sampling (Aut) in 2020. Dots represent the values of each sample.

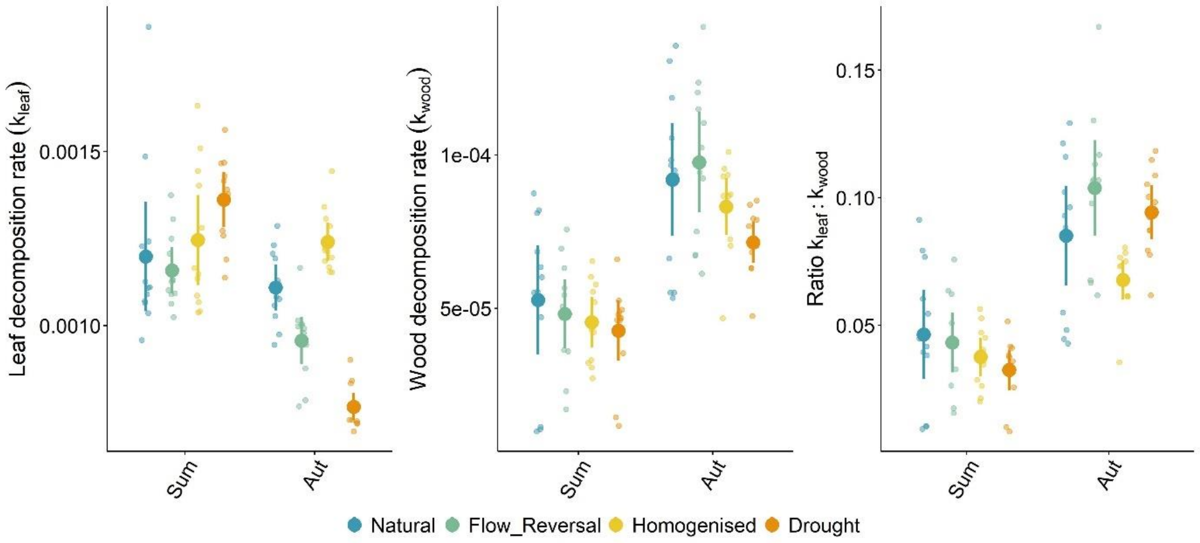

Table S2.4. Biofilm functional diversity index (Ecoplates). Mean and SD are shown for richness (S), Shannon index (H) and evenness (ev).

Flow	S	H	Ev
Natural	24.75±5.56	3.09±0.24	0.97±0.01
Flow Reversal	29.75±0.96	3.31±0.07	0.97±0.02
Homogenised	28.75±0.96	3.23±0.02	0.96±0.01
Drought	28±1.41	3.25±0.11	0.98±0.02

Table S2.5. One-way ANOVA results for the each of the 31 substrates present in the Ecolplates.

subs	variable	Df	F_	p_adj	sig
Itaconic Acid	**itaAc**	**3**	**7.010**	**0.006**	**
α-Cyclodextrin	**aCy**	**3**	**6.548**	**0.007**	**
i-Erythritol	**Ery**	**3**	**6.099**	**0.009**	**
D-Xylose	**Dxyl**	**3**	**4.916**	**0.019**	*
Glycogen	**Gly**	**3**	**3.895**	**0.037**	*
L-Arginine	Larg	3	3.483	0.050	ns
Phenylethyl amine	PheA	3	3.491	0.050	*
Tween-40	t40	3	3.325	0.057	ns
L-Asparagine	Lasp	3	3.233	0.061	ns
D-galacturonic Acid	DgalaAc	3	3.023	0.071	ns
Tween-80	t80	3	2.745	0.089	ns
Putrescine	Putr	3	2.698	0.093	ns
D-Cellobiose	Dcel	3	2.560	0.104	ns
D-Galactonic Acid γ-Lactone	DgalAcLac	3	2.385	0.120	ns
N-Acetyl-D-Glucosamine	DgluA	3	2.285	0.131	ns
L-Serine	Lser	3	2.216	0.139	ns
γ-Amino-Butyric Acid	aaButAc	3	2.136	0.149	ns
α_D-Lactose	aDlact	3	2.037	0.162	ns
D-Mannitol	Man	3	2.011	0.166	ns
4-Hydroxy-Benzoic_Acid	Ben4Ac	3	1.548	0.253	ns
β-Methyl-D-Glucoside	bDGlu	3	1.481	0.269	ns
Glycyl-L-glutamic Acid	GlyL_glu	3	1.360	0.302	ns
Glucose-1-Phosphate	GluP	3	1.033	0.413	ns
D-L-α-Glycerol-Phosphate	DLaglyP	3	0.964	0.441	ns
Pyruvic-Acid-Methyl-Ester	pyr	3	0.926	0.458	ns
L-Threonine	Lthr	3	0.891	0.474	ns
D-Glucosaminic Acid	DgluAAc	3	0.774	0.530	ns
D-Malic Acid	MalAc	3	0.699	0.571	ns
L-Phenylalanine	Lphe	3	0.639	0.604	ns
α-Keto-Butyric Acid	aKeButAc	3	0.615	0.618	ns
2-Hydroxy-Benzoic Acid	Ben2Ac	3	0.029	0.993	ns

Table S3.6. Functional trait considered based on Tachet *et al.* (2010).

Trait type	Functional trait	Functional trait category	Definition
Biological	Maximal potential size	small	≤ 0.5 cm
		medium	> .5-1 cm
		large	> 1-2 cm
		vlarge	> 2-4 cm
		vvlarge	> 4 cm
	Life cycle duration	short	≤ 1 year
		long	> 1 year
	Voltinism	semivoltin	< 1
		univoltin	1
		multivoltin	> 1
	Aquatic stages	egg	egg
		larva	larva
		nymph	nymph
		adult	adult
	Reproduction	ovoviviparity	ovoviviparity
		isolated_eggs_free	isolated eggs, free
		isolated_eggs_fixed	isolated eggs, cemented
		clutches_fixed	clutches, cemented or fixed
		clutches_free	clutches, free
		clutches_vegetation	clutches, in vegetation
		clutches_terrestrial	clutches, terrestrial
		asexual_reproduction	asexual reproduction
	Dispersal	aquatic_passive	aquatic passive
		aquatic_active	aquatic active
		aerial_passive	aerial passive
		aerial_active	aerial active
	Resistance forms	eggs_statoblasts	eggs, statoblasts
		cocoons	cocoons
		housings	housings against desiccation
		diapause	diapause or dormancy
		no_resistance	none
	Drought resistance	resistant	Presenting resistance forms
		susceptible	No resistance form
	Respiration	tegument	tegument
		gill	gill
		plastron	plastron
		spiracle	spiracle
	Locomotion	flier	flier
		surface_swimmer	surface swimmer
		swimmer	full water swimmer
		crawler	crawler
		burrower	burrower
		interstitial	interstitial
		temporarily_attached	temporarily attached
		permanently_attached	permanently attached

	Food	microorganisms	microorganisms
		FPOM	detritus (< 1mm)
		CPOM	dead plant (>= 1mm)
		microphytes	living microphytes
		macrophytes	living macrophytes
		dead_animal	dead animal (>= 1mm)
		microinvertebrates	living microinvertebrates
		macroinvertebrates	living macroinvertebrates
	Generalist diet	generalist	Generalist diet
		specialist	Specialist diet
	Feeding habits	absorber	absorber
		deposit_feeder	deposit feeder
		shredder	shredder
		scraper	scraper
		filter_feeder	filter-feeder
		piercer	piercer
		predator	predator
		parasite	parasite
	Thermal preference	cold	cold (< 15°C)
		warm (> 15°C)	warm (> 15°C)
		eurythermic	eurythermic
Ecological	Transversal distribution	river_channel	river channel
		banks	banks, connected side-arms
		pools	ponds, pools, disconnected side-arms
		marshes	marshes, peat bogs
		temporary_waters	temporary waters
		lakes	lakes
		groundwaters	groundwaters
	Substrate preference	boulders	flags/boulders/cobbles/pebbles
		gravel	gravel
		sand	sand
		silt	silt
		macrophytes	macrophytes
		microphytes	microphytes
		twigs_roots	twigs/roots
		detritus	organic detritus/litter
		mud	mud
	Flow velocity preference	null	null
		slow	slow (< 25 cm/s)
		medium	medium (25-50 cm</s)
		fast	fast (> 50 cm/s)

Table S3.7. Total densities of the recorded taxa in each of the flow treatments (flow reversal, homogenised and drought) and the control (natural). Data is presented as mean and SD for total densities for each season. Taxa is order based on the mean total density for all the treatments.

	Natural		Flow Reversal		Homogenised		Drought	
	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn
Asellus aquaticus	3129.6±2216.6	1116.7±795.1	1566.7±1233.9	505.6±387.9	5048.1±4689.5	2363±2447.9	8381.5±4147.3	2629.6±1562.3
Potamopyrgus antipodarum	6488.9±7161.7	15527.8±12837.4	163±274.3	438.9±518.1	394.4±627.4	307.4±421.8	22.2±21	168.5±219.3
Gammarus pulex	2796.3±1601.1	1535.2±994.7	1068.5±1081.1	2603.7±1643.5	1135.2±453	2361.1±2437.9	459.3±216.8	159.3±168.1
Crangonyx pseudogracilis	953.7±939.8	381.5±427.2	659.3±544.6	563±618.8	2463±1546.1	1111.1±589.8	2761.1±2095.8	2909.3±2394.7
Oligochaeta	351.9±230.2	324.1±347.8	894.4±1447.6	207.4±200	511.1±262.9	763±425.9	720.4±428.8	1209.3±1234.8
Ancylus fluviatilis	822.2±768.8	831.5±1128.6	892.6±1648.7	24.1±25.2	88.9±111	335.2±406.4	46.3±54.9	137±254.7
Radix peregra	63±58.5	90.7±114.3	1203.7±1851.4	335.2±240	127.8±255.6	288.9±322.1	98.1±153.4	7.4±10.5
Orthocladiinae	53.7±102.5	0±0	98.1±139.7	0±0	846.3±1551	0±0	59.3±80	0±0
Polycelis nigra tenuis	51.9±46.1	203.7±203.7	14.8±20.1	13±21.3	109.3±160.7	231.5±453.1	340.7±450.9	20.4±22.9
Baetis rhodani	274.1±212.4	190.7±189	285.2±463.6	55.6±70.9	3.7±4.3	11.1±17.6	0±0	0±0
Tanytarsini	35.2±33.9	0±0	24.1±43.4	0±0	407.4±698.4	1.9±3.7	198.1±201.6	0±0
Physa fontinalis	338.9±396.4	164.8±319.8	0±0	0±0	57.4±109.9	87±174.1	0±0	1.9±3.7
Plectrocnemia conspersa	118.5±114.1	46.3±66.4	164.8±110.4	94.4±89.8	38.9±26.6	63±62.4	13±7.1	0±0
Polycentropus flavomaculatus	74.1±63.7	27.8±25.2	155.6±108.5	18.5±22.2	38.9±40.3	66.7±104.9	7.4±6	0±0
Diamesinae	70.4±79	0±0	27.8±22.1	0±0	81.5±129	0±0	35.2±24.5	0±0
Planorbarius corneus	57.4±47	7.4±14.8	0±0	1.9±3.7	16.7±16.4	53.7±97.8	18.5±32.3	3.7±7.4
Glossiphonia	109.3±199.3	0±0	13±16.4	1.9±3.7	9.3±7.1	3.7±4.3	3.7±4.3	1.9±3.7
Hydroptila	55.6±65.3	0±0	51.9±59.9	0±0	5.6±11.1	0±0	18.5±37	0±0
Dugesia polychroa	1.9±3.7	0±0	35.2±51.5	22.2±44.4	13±25.9	37±49.1	5.6±11.1	0±0
Chironomini	11.1±17.6	0±0	0±0	1.9±3.7	75.9±92.6	1.9±3.7	5.6±3.7	0±0
Limoniidae	1.9±3.7	0±0	0±0	0±0	1.9±3.7	0±0	0±0	16.7±23.7
Dendrocoelum lacteum	0±0	3.7±7.4	0±0	0±0	5.6±7.1	1.9±3.7	1.9±3.7	0±0
Empididae	0±0	0±0	0±0	0±0	0±0	0±0	0±0	3.7±7.4
Sympetrum	0±0	0±0	0±0	0±0	3.7±7.4	0±0	0±0	0±0
Psychodidae	0±0	0±0	0±0	0±0	0±0	0±0	3.7±7.4	0±0
Batracobdella paludosa	0±0	0±0	0±0	1.9±3.7	0±0	0±0	0±0	0±0
Stratiomyidae	0±0	0±0	0±0	0±0	1.9±3.7	0±0	0±0	0±0

Table S3.8. RM-ANOVA pairwise comparison for macroinvertebrate structural metrics: taxon richness (S), taxonomic diversity (Shannon index, H), evenness (ev) and total density (den). Marginal mean differences (MMD) are presented for each treatment and season (summer and autumn). Values are the difference between the column names group to the row group (i.e. MM N-Summer – MM N-Autumn). Bolded values are significant comparisons (P<0.05). Values are mean differences of the transformed data as marginal means depend on a model. Data assess differences between flow treatments (natural control, N; flow reversal, R; homogenised, H; and drought, D) within season (lighter grey shaded cells), seasonal differences within treatments (darker shaded cells) and differences between treatments and seasons (clear cells).

			Summer			Autumn			
variable	Season	Flow	R	H	D	N	R	H	D
S	sum	N	-0.09	0.07	-0.04	**-0.37**	**-0.41**	-0.24	**-0.63**
		R		0.16	0.05	-0.28	**-0.32**	-0.16	**-0.54**
		H			-0.12	**-0.44**	**-0.48**	**-0.32**	**-0.70**
		D				**-0.33**	**-0.37**	-0.20	**-0.59**
	aut	N					-0.04	0.13	-0.26
		R						0.17	-0.22
		H							**-0.39**
H	sum	N	0.23	-0.06	-0.43	-0.50	-0.19	0.07	-0.46
		R		-0.29	-0.66	-0.74	-0.42	-0.16	-0.69
		H			-0.37	-0.45	-0.13	0.13	-0.40
		D				-0.08	0.24	0.50	-0.03
	aut	N					0.32	0.58	0.04
		R						0.26	-0.27
		H							-0.53
den	sum	N	-0.82	-0.24	-0.18	0.27	-1.11	-0.63	-0.75
		R		0.58	0.64	1.08	-0.30	0.19	0.07
		H			0.06	0.50	-0.88	-0.39	-0.51
		D				0.45	-0.93	-0.44	-0.56
	aut	N					-1.38	-0.89	-1.01
		R						0.49	0.37
		H							-0.12
ev	sum	N	0.11	-0.04	-0.15	-0.12	0.02	0.09	-0.03
		R		-0.14	-0.26	-0.23	-0.09	-0.02	-0.14
		H			-0.11	-0.09	0.06	0.13	0.00
		D				0.03	0.17	0.24	0.12
	aut	N					0.14	0.22	0.09
		R						0.07	-0.05
		H							-0.12

Fig. S3.2. Sheppard plot from the macroinvertebrates NDMS. Plot indicates the scatter around the distances between each pair of communities (samples) against their original dissimilarities.

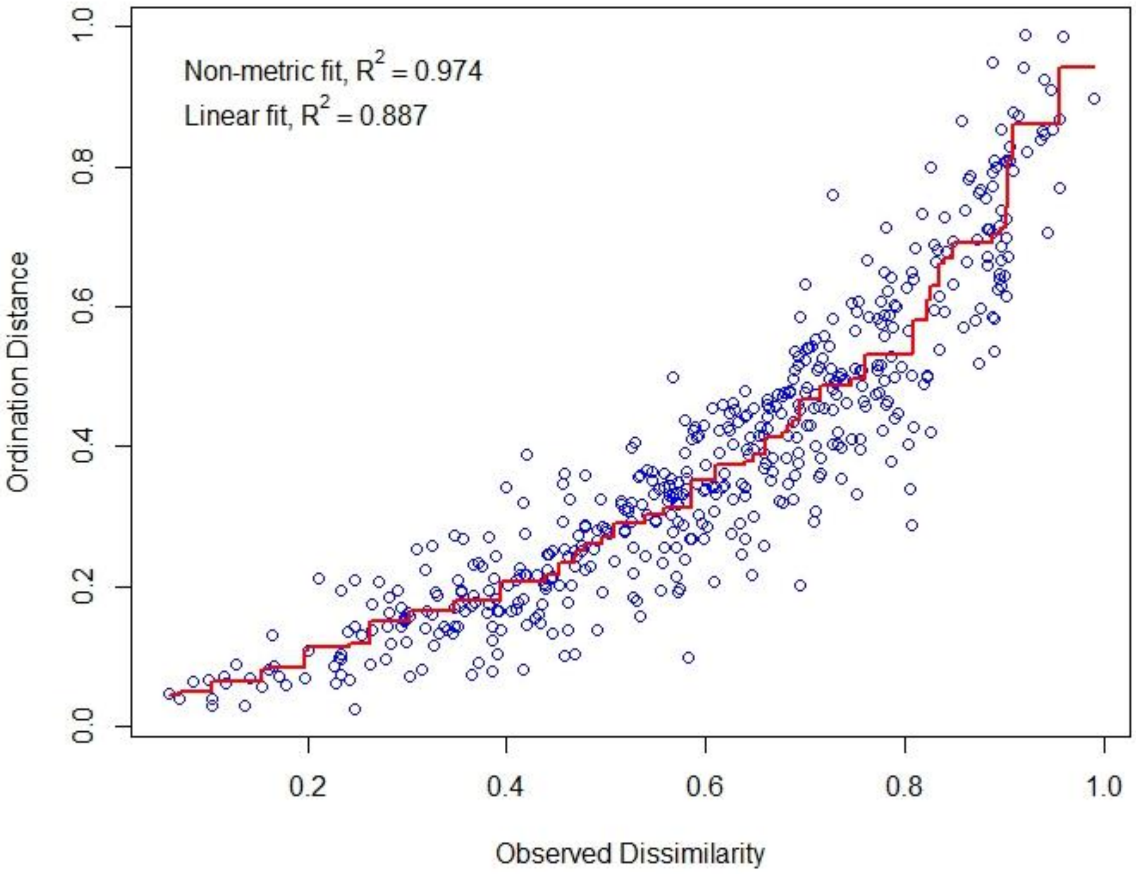

Table S3.9. Pairwise PERMANOVA comparison of the Bray-Curtis distance matrix on structural composition of macroinvertebrate communities between treatments. Results presented are dissimilarity R^2 with significance values in bold. Data assess differences between flow treatments (natural control, N; flow reversal, R; homogenised, H; and drought, D).

Flow	R	H	D
N	**0.277**	**0.285**	**0.494**
R		0.117	**0.380**
H			**0.146**

Table S3.10. Indicator Species Analysis for individual treatments. Only significant indicator species are presented showing their correspondent treatment group, point-biserial correlation coefficient (r_{pb}, R) and p-values (P).

Flow	Species	R	P
Natural	*Gammarus pulex*	0.48	0.045
	Physa fontinalis	0.49	0.030
	Potamopyrgus antipodarum	0.68	0.005
	Plectrocnemia conspersa	0.47	0.045
	Baetis rhodani	0.48	0.025
Flow Reversal	*Gammarus pulex*	0.48	0.045
	Radix peregra	0.41	0.030
	Plectrocnemia conspersa	0.47	0.045
	Baetis rhodani	0.48	0.025
Homogenised	*Crangonyx pseudogracilis*	0.55	0.020
	Gammarus pulex	0.48	0.045
	Chironomini	0.41	0.045
Drought	*Crangonyx pseudogracilis*	0.55	0.020

Table S3.11. Indicator Species Analysis for individual treatments and seasons. Only significant indicator species are presented showing their correspondent treatment-season group, point-biserial correlation coefficient (r_{pb}, R) and p-values (P).

Flow Season	Species	R	P
Natural Autumn	*Potamopyrgus antipodarum*	0.69	0.005
Homogenised Summer	*Asellus aquaticus*	0.64	0.025
	Crangonyx pseudogracilis	0.63	0.025
	Chironomini	0.64	0.015
Drought Summer	*Asellus aquaticus*	0.64	0.025
	Crangonyx pseudogracilis	0.63	0.025
Drought Autumn	*Crangonyx pseudogracilis*	0.63	0.025
	Limoniidae	0.58	0.040

Table S3.12. Indicator Species Analysis for each season. Only significant indicator species are presented showing point-biserial correlation coefficient (r_{pb}, R) and p-values (P).

Season	Species	R	P
Summer	*Asellus aquaticus*	0.44	0.010
	Hydroptila	0.44	0.020
	Orthocladiinae	0.24	0.005
	Tanytarsini	0.32	0.005
	Chironomini	0.29	0.015
	Diamesinae	0.47	0.005
	Glossiphonia sps.	0.23	0.020

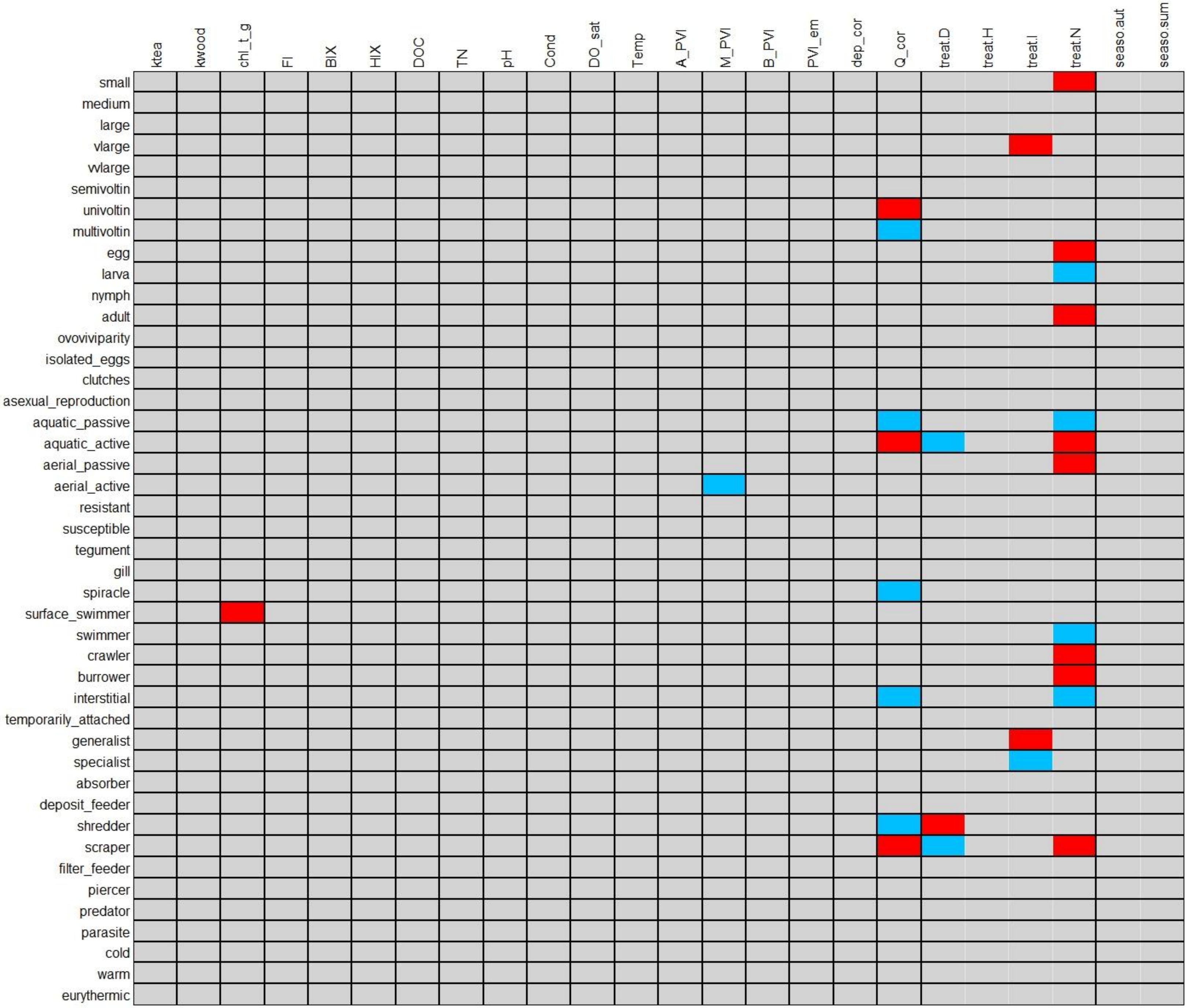

Fig. S3.3. Results of the fourth-corner test (model 2). Significant positive associations are presented with red cells, significant negative ones with blue ones and non-significant in grey. Environmental variables (columns) presented are: kleaf (ktea), kwood (kwood), chltot (chl_t_g), FI, BIX, DOC, TN, pH, conductivity (Cond), DO saturation (DO_sat), temperature (Temp), PVIA (A_PVI), PVIM (M_PVI), PVIW (W_PVI), PVIem (PVI_em), depth (dep_cor), Q (Q_cor), flow treatment (natural, treat.N; drought, treat.D; flow reversal, treat.I; and homogenised, treat.H) and season (summer, seaso.sum; and autumn, season.aut).

Table S3.13. Results of the RM-ANOVA with REML estimation of the macroinvertebrates biological functional trait categories. Note, that only significant trait categories are shown. Parameters showed: degrees of freedom (Df), chis-square (Chi-sq), adjust p-value (p-adj) and level of significance (sig). Levels of significance presented are: non-significant (ns), <0.05 (*), <0.01 (**), and <0.001 (***). Significant results are showed in bold.

Trait	Trait category	treat	Df	Chi-sq	p-adj	sig
Size	small	**Flow**	**3**	**8.224**	**0.042**	*
		Season	**1**	**9.662**	**0.003**	**
		Flow_Season	**3**	**16.058**	**0.003**	**
	vlarge	**Flow**	**3**	**60.446**	**<0.001**	***
		Season	1	0.204	0.651	ns
		Flow_Season	**3**	**15.822**	**0.002**	**
Aquatic stages	egg	**Flow**	**3**	**52.540**	**<0.001**	***
		Season	**1**	**7.384**	**0.007**	**
		Flow_Season	**3**	**12.377**	**0.007**	**
	larva	**Flow**	**3**	**35.621**	**<0.001**	***
		Season	**1**	**6.920**	**0.009**	**
		Flow_Season	**3**	**29.155**	**<0.001**	***
	adult	**Flow**	**3**	**29.725**	**<0.001**	***
		Season	**1**	**13.294**	**<0.001**	***
		Flow_Season	3	0.991	0.803	ns
Dispersal	aquatic passive	**Flow**	**3**	**41.019**	**<0.001**	***
		Season	1	2.242	0.201	ns
		Flow_Season	3	4.593	0.204	ns
	aquatic active	**Flow**	**3**	**39.520**	**<0.001**	***
		Season	**1**	**8.924**	**0.004**	**
		Flow_Season	**3**	**8.986**	**0.029**	*
	aerial passive	**Flow**	**3**	**31.998**	**<0.001**	***
		Season	1	1.198	0.274	ns
		Flow_Season	3	5.969	0.17	ns
Locomotion	swimmer	**Flow**	**3**	**17.073**	**0.002**	**
		Season	**1**	**4.971**	**0.026**	*
		Flow_Season	**3**	**13.040**	**0.007**	**
	crawler	**Flow**	**3**	**11.517**	**0.014**	*
		Season	1	1.626	0.202	ns
		Flow_Season	**3**	**18.389**	**0.001**	**
	burrower	**Flow**	**3**	**12.783**	**0.008**	**
		Season	**1**	**10.777**	**0.003**	**
		Flow_Season	**3**	**9.296**	**0.026**	*
	interstitial	**Flow**	**3**	**29.641**	**<0.001**	***
		Season	**1**	**11.063**	**0.001**	**
		Flow_Season	**3**	**9.014**	**0.029**	*
Generalist diet	generalist	**Flow**	**3**	**25.047**	**<0.001**	***
		Season	1	0.817	0.366	ns
		Flow_Season	**3**	**19.674**	**<0.001**	***
	specialist	**Flow**	**3**	**25.047**	**<0.001**	***
		Season	1	0.817	0.366	ns
		Flow_Season	**3**	**19.674**	**<0.001**	***

Trait	Trait category	treat	Df	Chi-sq	p-adj	sig
Feeding	shredder	**Flow**	**3**	**14.535**	**0.005**	**
		Season	1	0.000	0.995	ns
		Flow_Season	**3**	**13.776**	**0.005**	**
	scraper	**Flow**	**3**	**52.205**	**<0.001**	***
		Season	1	0.342	0.559	ns
		Flow_Season	**3**	**9.684**	**0.032**	*

Table S3.14. RM-ANOVA pairwise comparison for macroinvertebrate biological functional traits categories: maximum potential size (small and vlarge), aquatic stages (egg, larva and adult), dispersal (aquatic passive, aquatic active and aerial passive), resistance forms (diapause), locomotion (swimmer, crawler, burrower and interstitial), generalist diet (generalist and specialist) and feeding habits (shredder and scraper). Notice that categorical traits detected by the fourth-corner analysis are shown. Marginal mean differences (MMD) are presented for each treatment and season (summer and autumn). Values are the difference between the column names group to the row group (i.e. MM N-Summer – MM N-Autumn). Bolded values are significant comparisons (P<0.05). Values are mean differences of the transformed data as marginal means depend on a model. Data assess differences between flow treatments (natural control, N; flow reversal, R; homogenised, H; and drought, D) within season (lighter grey shaded cells), seasonal differences within treatments (darker shaded cells) and differences between treatments and seasons (clear cells).

				Summer			Autumn			
Trait	Trait category	Season	Flow	R	H	D	N	R	H	D
Maximal potential size	small	sum	N	-0.14	-0.07	-0.12	**0.16**	-0.13	-0.11	0.00
			R		0.07	0.02	**0.30**	0.01	0.03	0.14
			H			-0.06	0.23	-0.06	-0.04	0.06
			D				**0.29**	0.00	0.01	0.12
		aut	N					**-0.29**	**-0.27**	-0.17
			R						0.02	0.12
			H							0.10
	vlarge	sum	N	0.72	-0.24	**-1.04**	-0.82	**1.32**	0.55	**-1.31**
			R		**-0.97**	**-1.77**	**-1.55**	0.59	-0.18	**-2.04**
			H			-0.80	-0.58	**1.56**	0.79	**-1.07**
			D				0.22	**2.36**	**1.59**	-0.27
		aut	N					**2.14**	**1.37**	-0.49
			R						-0.77	**-2.63**
			H							**-1.86**
Aquatic stage	egg	sum	N	-0.24	**-1.75**	**-1.91**	0.78	-0.80	-0.64	**-1.32**
			R		**-1.51**	**-1.67**	**1.03**	-0.55	-0.39	**-1.07**
			H			-0.16	**2.54**	**0.96**	**1.12**	0.44
			D				**2.70**	**1.11**	**1.28**	0.59
		aut	N					**-1.58**	**-1.42**	**-2.10**
			R						0.16	-0.52
			H							-0.68
	larva	sum	N	0.05	**0.13**	**0.14**	**-0.10**	0.10	0.09	**0.13**
			R		0.07	0.09	**-0.15**	0.04	0.04	0.08
			H			0.01	**-0.23**	-0.03	-0.03	0.00
			D				**-0.24**	-0.04	-0.05	-0.01
		aut	N					**0.20**	**0.19**	**0.23**
			R						0.00	0.04
			H							0.04
	adult	sum	N	**-1.33**	**-1.69**	**-1.63**	0.78	-0.64	-0.55	-1.08
			R		-0.35	-0.30	**2.12**	0.69	0.79	0.26
			H			0.06	**2.47**	1.04	1.14	0.61
			D				**2.41**	0.99	1.08	0.55
		aut	N					**-1.42**	**-1.33**	**-1.86**
			R						0.09	-0.44

					Summer			Autumn		
Trait	Trait category	Season	Flow	R	H	D	N	R	H	D
			H							-0.53
Dispersal	aquatic_passive	sum	N	0.04	0.16	**0.29**	-0.12	0.04	0.13	**0.30**
			R		0.12	**0.25**	-0.16	0.00	0.09	**0.26**
			H			0.13	**-0.28**	-0.12	-0.03	0.14
			D				**-0.41**	**-0.25**	-0.16	0.01
		aut	N					0.16	**0.25**	**0.42**
			R						0.09	**0.26**
			H							0.17
	aquatic_active	sum	N	-0.12	-0.86	**-1.46**	0.50	0.75	-0.09	**-1.76**
			R		-0.74	**-1.34**	0.62	**0.87**	0.02	**-1.65**
			H			-0.60	**1.36**	**1.61**	0.77	-0.90
			D				**1.96**	**2.21**	**1.37**	-0.30
		aut	N					0.25	-0.59	**-2.27**
			R						-0.84	**-2.51**
			H							**-1.67**
	aerial_passive	sum	N	-0.46	-0.63	**-1.45**	0.69	-1.20	-1.10	**-1.92**
			R		-0.17	-0.99	1.15	-0.74	-0.64	**-1.46**
			H			-0.82	**1.32**	-0.57	-0.47	**-1.29**
			D				**2.14**	0.25	0.35	-0.47
		aut	N					**-1.89**	**-1.79**	**-2.61**
			R						0.10	-0.72
			H							-0.82
Locomotion	swimmer	sum	N	0.04	0.04	-0.01	-0.05	**0.12**	0.07	0.06
			R		0.00	-0.05	-0.09	0.09	0.03	0.03
			H			-0.05	-0.09	0.09	0.03	0.03
			D				-0.04	**0.14**	0.08	0.07
		aut	N					**0.18**	**0.12**	**0.12**
			R						-0.06	-0.06
			H							0.00
	crawler	sum	N	-1.08	-0.81	-0.42	0.74	-0.99	-1.47	-1.41
			R		0.27	0.66	**1.83**	0.10	-0.38	-0.32
			H			0.39	1.56	-0.18	-0.65	-0.59
			D				1.17	-0.57	-1.04	-0.98
		aut	N					-1.73	**-2.21**	**-2.15**
			R						-0.48	-0.42
			H							0.06
	burrower	sum	N	-0.05	-0.05	-0.07	**0.08**	-0.05	-0.04	-0.04
			R		0.00	-0.02	**0.13**	0.00	0.02	0.01
			H			-0.02	**0.13**	0.00	0.02	0.01
			D				**0.15**	0.02	0.04	0.03
		aut	N					**-0.13**	**-0.11**	**-0.12**
			R						0.02	0.01
			H							0.00
	interstitial	sum	N	0.01	**0.11**	**0.18**	**-0.08**	0.03	0.07	**0.13**
			R		0.10	**0.16**	-0.10	0.01	0.06	**0.12**
			H			0.06	**-0.20**	-0.08	-0.04	0.02
			D				**-0.26**	**-0.15**	-0.10	-0.04
		aut	N					**0.11**	**0.15**	**0.21**

				Summer			Autumn			
Trait	Trait category	Season	Flow	R	H	D	N	R	H	D
			R						0.04	0.10
			H							0.06
Generalist diet	generalist	sum	N	0.04	-0.02	-0.04	-0.06	**0.12**	0.04	-0.08
			R		-0.06	-0.08	**-0.10**	**0.09**	0.00	**-0.11**
			H			-0.02	-0.04	**0.14**	0.06	-0.06
			D				-0.02	**0.16**	0.08	-0.04
		aut	N					**0.19**	**0.10**	-0.01
			R						-0.08	**-0.20**
			H							**-0.12**
	specialist	sum	N	-0.04	0.02	0.04	0.06	**-0.12**	-0.04	0.08
			R		0.06	0.08	**0.10**	**-0.09**	0.00	**0.11**
			H			0.02	0.04	**-0.14**	-0.06	0.06
			D				0.02	**-0.16**	-0.08	0.04
		aut	N					**-0.19**	**-0.10**	0.01
			R						0.08	**0.20**
			H							**0.12**
Feeding habits	shredder	sum	N	-0.17	0.10	0.24	-0.08	0.05	0.01	0.19
			R		0.27	**0.41**	0.09	0.21	0.18	**0.36**
			H			0.14	-0.17	-0.05	-0.09	0.09
			D				**-0.31**	-0.19	-0.23	-0.05
		aut	N					0.12	0.08	0.27
			R						-0.04	0.14
			H							0.18
	scraper	sum	N	0.26	-0.90	**-1.71**	0.78	-0.37	-0.56	**-1.81**
			R		**-1.16**	**-1.97**	0.52	-0.63	-0.82	**-2.07**
			H			-0.80	**1.68**	0.54	0.34	-0.91
			D				**2.49**	**1.34**	**1.15**	-0.10
		aut	N					**-1.15**	**-1.34**	**-2.59**
			R						-0.19	**-1.44**
			H							**-1.25**

Table S3.15. RM-ANOVA pairwise comparison for macroinvertebrate ecological functional trait categories. Marginal mean differences (MMD) are presented for each treatment and season (summer and autumn). Values are the difference between the column names group to the row group (i.e. MM N-Summer – MM N-Autumn). Bolded values are significant comparisons (P<0.05). Values are mean differences of the transformed data as marginal means depend on a model. Data assess differences between flow treatments (natural control, N; flow reversal, R; homogenised, H; and drought, D) within season (lighter grey shaded cells), seasonal differences within treatments (darker shaded cells) and differences between treatments and seasons (clear cells).

				Summer			Autumn			
Trait	Trait category	Season	Flow	R	H	D	N	R	H	D
Transversal distribution	river channel	sum	N	-0.99	-1.39	-1.30	0.47	-0.94	-1.27	-1.15
			R		-0.40	-0.32	1.45	0.04	-0.28	-0.16
			H			0.09	1.85	0.45	0.12	0.24
			D				1.77	0.36	0.03	0.16
		aut	N					-1.41	-1.74	-1.61
			R						-0.33	-0.21
			H							0.12
	banks	sum	N	-0.01	-0.05	-0.04	0.03	-0.01	-0.03	**-0.07**
			R		-0.04	-0.02	0.04	0.00	-0.02	**-0.06**
			H			0.02	**0.08**	0.04	0.02	-0.02
			D				**0.06**	0.02	0.00	**-0.04**
		aut	N					-0.04	**-0.06**	**-0.10**
			R						-0.02	**-0.06**
			H							-0.04
	pools	sum	N	0.02	0.06	0.07	-0.01	0.00	0.04	**0.07**
			R		0.04	0.05	-0.03	-0.02	0.02	0.06
			H			0.01	-0.07	-0.06	-0.02	0.02
			D				**-0.08**	**-0.07**	-0.03	0.00
		aut	N					0.01	0.05	**0.09**
			R						0.04	**0.08**
			H							0.04
	temporary waters	sum	N	1.04	0.18	-0.84	-0.58	0.61	0.28	**-1.51**
			R		-0.86	**-1.88**	**-1.62**	-0.43	-0.76	**-2.55**
			H			-1.02	-0.76	0.44	0.10	**-1.69**
			D				0.26	**1.46**	1.12	-0.67
		aut	N					1.20	0.86	-0.93
			R						-0.33	**-2.12**
			H							**-1.79**
	lakes	sum	N	0.00	**0.04**	0.02	-0.01	0.02	0.02	**0.05**
			R		**0.04**	0.02	-0.01	0.02	0.02	**0.05**
			H			-0.01	**-0.05**	-0.02	-0.01	0.02
			D				**-0.04**	-0.01	0.00	**0.03**
		aut	N					0.03	**0.03**	**0.06**

				Summer			Autumn			
Trait	Trait category	Season	Flow	R	H	D	N	R	H	D
			R						0.00	**0.04**
			H							**0.03**
	boulders	sum	N	0.01	**-0.06**	**-0.10**	0.02	0.00	-0.04	**-0.11**
			R		**-0.07**	**-0.11**	0.01	-0.01	-0.05	**-0.12**
			H			-0.04	**0.08**	**0.06**	0.03	**-0.05**
			D				**0.11**	**0.10**	**0.06**	-0.02
		aut	N					-0.02	**-0.05**	**-0.13**
			R						-0.03	**-0.11**
			H							**-0.08**
Substrate preference	gravel	sum	N	-0.01	**-0.04**	**-0.06**	0.01	-0.01	-0.03	**-0.06**
			R		-0.03	**-0.05**	0.02	-0.01	-0.02	**-0.05**
			H			-0.01	**0.05**	0.03	0.01	-0.02
			D				**0.07**	**0.04**	0.03	0.00
		aut	N					-0.02	-0.04	**-0.07**
			R						-0.01	**-0.05**
			H							-0.03
	silt	sum	N	0.00	0.01	0.00	0.01	0.01	0.01	0.03
			R		0.00	-0.01	0.00	0.00	0.01	0.03
			H			-0.01	0.00	0.00	0.01	0.02
			D				0.01	0.01	0.02	**0.04**
		aut	N					0.00	0.01	0.02
			R						0.00	0.02
			H							0.02
	macrophytes liv	sum	N	0.04	**0.06**	**0.09**	-0.02	-0.01	0.04	**0.11**
			R		0.03	0.05	**-0.06**	**-0.05**	0.00	**0.07**
			H			0.02	**-0.09**	**-0.07**	-0.03	0.04
			D				**-0.11**	**-0.10**	-0.05	0.02
		aut	N					0.01	0.06	**0.13**
			R						0.04	**0.12**
			H							**0.07**
	microphytes liv	sum	N	-0.05	-0.03	-0.01	0.02	-0.04	-0.04	-0.04
			R		0.02	0.04	**0.07**	0.00	0.00	0.00
			H			0.02	0.05	-0.01	-0.01	-0.02
			D				0.03	-0.04	-0.04	**-0.04**
		aut	N					**-0.06**	**-0.06**	**-0.06**
			R						0.00	0.00
			H							0.00
	twigs roots	sum	N	0.00	0.00	0.02	-0.03	0.04	0.01	-0.01
			R		0.00	0.02	-0.04	0.03	0.01	-0.02
			H			0.02	-0.04	0.03	0.01	-0.02
			D				-0.06	0.01	-0.01	-0.04
		aut	N					0.07	0.05	0.02
			R						-0.02	-0.05
			H							-0.03

				Summer			Autumn			
Trait	Trait category	Season	Flow	R	H	D	N	R	H	D
	detritus	sum	N	0.02	**0.09**	**0.11**	**-0.06**	0.06	0.07	**0.15**
			R		**0.07**	**0.09**	**-0.08**	0.04	0.05	**0.13**
			H			0.01	**-0.15**	-0.04	-0.02	0.06
			D				**-0.17**	-0.05	-0.04	0.04
		aut	N					**0.12**	**0.13**	**0.21**
			R						0.01	**0.09**
			H							**0.08**
	mud	sum	N	-0.03	-0.05	-0.05	**0.06**	-0.05	-0.04	-0.05
			R		-0.02	-0.02	**0.09**	-0.02	-0.01	-0.02
			H			0.00	**0.11**	0.00	0.01	0.00
			D				**0.11**	0.00	0.01	0.00
		aut	N					**-0.11**	**-0.10**	**-0.11**
			R						0.01	0.00
			H							-0.01
Flow velocity preference	null	sum	N	0.04	0.10	**0.16**	-0.04	-0.03	0.06	**0.16**
			R		0.06	**0.12**	-0.07	-0.06	0.03	**0.12**
			H			0.06	**-0.14**	**-0.13**	-0.04	0.05
			D				**-0.20**	**-0.19**	-0.10	-0.01
		aut	N					0.01	0.10	**0.19**
			R						0.09	**0.18**
			H							0.09
	slow	sum	N	0.03	0.06	**0.10**	-0.01	0.00	0.03	**0.10**
			R		0.03	0.08	-0.04	-0.03	0.01	0.07
			H			0.04	-0.07	-0.06	-0.03	0.04
			D				**-0.12**	**-0.11**	-0.07	-0.01
		aut	N					0.01	0.04	**0.11**
			R						0.04	**0.10**
			H							0.06
	medium	sum	N	-0.08	**-0.15**	**-0.22**	0.06	0.01	-0.09	**-0.21**
			R		-0.07	**-0.14**	**0.15**	0.09	-0.01	**-0.13**
			H			-0.07	**0.22**	**0.16**	0.06	-0.06
			D				**0.28**	**0.23**	**0.13**	0.01
		aut	N					-0.06	**-0.16**	**-0.28**
			R						-0.10	**-0.22**
			H							**-0.12**
	fast	sum	N	0.15	-0.31	-1.32	-0.78	0.37	-0.02	-1.50
			R		-0.46	-1.47	-0.93	0.22	-0.17	-1.65
			H			-1.01	-0.47	0.69	0.29	-1.19
			D				0.54	1.70	1.30	-0.18
		aut	N					1.15	0.76	-0.72
			R						-0.39	-1.88
			H							-1.48

Equation S4.1. Cohen's d effect size for single stressors (i.e. low flow, heatwaves) was calculated as:

$$d = \frac{m_{treat} - m_{control}}{s_{pooled}} \qquad \text{Equation 1}$$

Where m $_{\text{treat}}$ is the mean of the treatment (i.e. low flow, heatwaves) response variable, m $_{\text{control}}$ is the mean value for the control. The effect size for the interaction between heatwaves and low flow was calculated as:

$$d_{HW:LF} = \frac{(m_{HW:LF} - m_{HW}) - (m_{LF} - m_{control})}{2 \times s_{pooled}} \qquad \text{Equation 2}$$

Where m $_{\text{HW:LF}}$ is the mean of the low flow : heatwaves treatment. The s$_{\text{pooled}}$ in Equation 1 and 2 is the pooled standard deviation calculated as:

$$s_{pooled} = \sqrt{\frac{(n_{LF}-1)s_{LF}^2 + (n_{HW}-1)s_{HW}^2 + (n_{HW:LF}-1)s_{HW:LF}^2 + (n_{control}-1)s_{control}^2}{n_{control} + n_{LF} + n_{HW} + n_{LF:HW} - 2}} \qquad \text{Equation 2}$$

Where n $_{\text{LF}}$, n $_{\text{HW}}$, n $_{\text{HW:LF}}$ and n $_{\text{control}}$ is the number of replicates in the low flow, heatwaves and low flow and heatwave treatment and in the control group, respectively. T the standard deviation of each treatment and control is denoted by s. To correct the bias for a small sample size, we calculated as:

$$d_{unbiased} = d_{biased}\left[1 - \frac{3}{4(\,n_{control} + n_{treat} - 2) - 1}\right] \qquad \text{Equation 3}$$

Where d $_{\text{biased}}$ is the biased Cohen's d calculated using equation 1. Effect size confidence intervals were calculated as:

$$95\%CI = d - t_{23}^{0.975} \times se_d;\; d + \; + t_{23}^{0.975} \times se_d \qquad \text{Equation 4}$$

Where d is the unbiased Cohen's d, $t_{23}^{0.975}$ is the t distribution with appropriate degrees of freedom and the se was calculated as:

$$se_d = \sqrt{\frac{n_{treat} + n_{control}}{n_{treat} \times n_{control}} + \frac{d^2}{2\,(n_{treat} + n_{control} - 2)}} \qquad \text{Equation 5}$$

Table S4.16. Results of the mixed-effect ANOVA type II to test the effect of the treatments on physiochemical variables: mean water temperature, electrical conductivity (EC), pH and DO saturation. Standardized effect size (Cohen's d) is presented for each factor and categorized following Cohen (1988). All p-values were calculated using Chi-squared likelihood ratio tests and were adjusted to control for type 1 errors (see methods).

Response	Treatment	Cohen's d	±CI	Categories	p-adj
Temperature	**Heatwave**	**15.289**	**70.743**	**Large**	**<0.001**
	Lowflow	-0.975	1.277	Large	0.200
	HW:LF	**1.472**	**6.915**	**Large**	**<0.001**
EC	**Heatwave**	**0.637**	**3.179**	**Moderate**	**0.020**
	Lowflow	-1.024	1.285	Large	0.108
	HW:LF	0.362	2.056	Weak	0.326
pH	Heatwave	-0.192	1.488	Trivial	0.446
	Lowflow	-0.185	1.198	Trivial	0.446
	HW:LF	0.491	2.567	Weak	0.446
DO saturation	Heatwave	-0.627	3.139	Moderate	0.444
	Lowflow	-0.902	1.265	Large	0.222
	HW:LF	0.241	1.633	Weak	0.515

Fig. S4.4. Degree-day decomposition rates (k) excluding direct temperature effect. Leaf k (a), wood k (b) and leaf k: wood k ratio (c) were calculated using cumulative degree-days. Heatwaves significantly decreased the efficiency of leaf degree-day decomposition rates but did not impact the other decomposition measurements. Notice that scales are different for the all the plots.