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Individual and combined impacts of carbon dioxide enrichment, heatwaves, flow velocity variability, and fine sediment deposition on stream invertebrate communities

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Abstract

Climate change and land-use change are widely altering freshwater ecosystem functioning and there is an urgent need to understand how these broad stressor categories may interact in future. While much research has focused on mean temperature increases, climate change also involves increasing variability of both water temperature and flow regimes and increasing concentrations of atmospheric CO_2, all with potential to alter stream invertebrate communities. Deposited fine sediment is a pervasive land-use stressor with widespread impacts on stream invertebrates. Sedimentation may be managed at the catchment scale; thus, uncovering interactions with these three key climate stressors may assist mitigation of future threats. This is the first experiment to investigate the individual and combined effects of enriched CO_2, heatwaves, flow velocity variability, and fine sediment on realistic stream invertebrate communities. Using 128 mesocosms simulating small stony-bottomed streams in a 7-week experiment, we manipulated dissolved CO_2 (ambient; enriched), fine sediment (no sediment; 300 g dry sediment), temperature (ambient; two 7-day heatwaves), and flow velocity (constant; variable). All treatments changed community composition. CO_2 enrichment reduced abundances of Orthocladiinae and Chironominae and increased Copepoda abundance. Variable flow velocity had only positive effects on invertebrate abundances (7 of 13 common taxa and total abundance), in contrast to previous experiments showing negative impacts of reduced velocity. CO_2 was implicated in most stressor interactions found, with $CO_2 \times$ sediment interactions being most common. Communities forming under enriched CO_2 conditions in sediment-impacted mesocosms had ~20% fewer total invertebrates than those with either treatment alone. Copepoda abundances doubled in CO_2-enriched mesocosms without sediment, whereas no CO_2 effect occurred in mesocosms with sediment. Our findings provide new insights into potential future impacts of climate change and land use in running freshwaters, in particular highlighting the potential for elevated CO_2 to interact with fine sediment deposition in unpredictable ways.

1 | INTRODUCTION

Climate change is altering biodiversity and ecosystem functioning across all biomes in most regions of the world (IPCC, 2022), with ~3°C global surface warming expected by 2100 under current greenhouse gas emissions (IPCC, 2021a). Alongside this underlying mean temperature shift is an increase in the frequency and intensity of heatwaves and hot days (IPCC, 2021a). While climate-change experiments have predominantly focused on mean temperature, this variability is crucial to consider as it plays a different and potentially more important role in restructuring communities (Katz & Brown, 1992; Lloret et al., 2012; Thompson et al., 2013). Freshwater ecosystem functioning is vulnerable to warming as water temperature controls, or strongly influences, many biological and ecological processes such as metabolism, decomposition, production of biomass, and energy flux through food webs (Woodward et al., 2010). Alongside water temperature alterations, elevated atmospheric CO_2 and changing hydrological regimes are two further key climate-change stressors warranting consideration in freshwater ecological research (Woodward et al., 2010).

For running freshwater systems, land-use change and climate change are predicted to be the most important threats to biodiversity this century (Sala et al., 2000). Future climate-change effects on a given freshwater system will likely depend on the degree of local land-use impact (Bayramoglu et al., 2020; Zhai et al., 2023). Therefore, there is an urgent need to understand how these broad stressor categories interact and to identify key stressors within them (Jackson et al., 2020). Experiments elucidating individual and combined climate-change and land-use effects will be crucial for predicting future impacts, as these often reveal effects not otherwise considered by models (Ormerod et al., 2010). While such research has increased in recent years, community-level experiments are rare (Mantyka-Pringle et al., 2014). This is problematic because stressor effects found in single-taxon experiments cannot be extrapolated to real-world outcomes as they fail to account for species interactions, which can alter the observed response to a stressor or stressor combinations (Bruder et al., 2017; Schuwirth et al., 2016; Thompson et al., 2018).

Atmospheric CO_2 will increase to ~600–1100 ppm by 2100 under moderate-to-high emission scenarios (IPCC, 2021b). Predicting implications of this change for running freshwaters is inherently difficult due to high variability both within and between catchments, particularly with respect to pH, alkalinity, and dissolved inorganic carbon (DIC) (Cole et al., 2007). Therefore, streams will likely vary greatly in their sensitivity to rising atmospheric CO_2, in both water chemistry alterations and ecological effects. Well-buffered streams that are not already CO_2-supersaturated, thus potentially sensitive to elevated CO_2, will likely be affected by increased CO_2 uptake by CO_2-limited autotrophs (Song et al., 2014). A recent synthesis of 22 predominantly laboratory-based CO_2-enrichment studies on freshwater microalgae found that elevated CO_2 generally lowers pH and increases DIC, algal productivity and nutrient uptake, but growth responses vary by algal type and sometimes between species of the same type (Brown et al., 2020; Hu & Gao, 2008). CO_2-enrichment studies on freshwater invertebrates have primarily focused on direct effects (e.g., behavior, acidification effects on calcification), mostly via single-species laboratory experiments (e.g., Hasler et al., 2017; Ninokawa & Ries, 2022; Ramaekers et al., 2022). In some cases, invertebrates have been studied in laboratory microcosms containing algae (or leaf litter; see Alto et al., 2005), allowing assessment of indirect invertebrate responses. In one such study, CO_2 stimulated growth of the alga *Scenedesmus acutus* and decreased its P:C ratio (and thus nutritional quality), which in turn reduced growth of the cladoceran *Daphnia pulicaria* (Urabe et al., 2003). Ecological modelling has suggested that lowered nutritional quality in primary producers can lead to increased consumption rates and negative energetic effects on primary consumers (Emmerson et al., 2005). This could occur via autochthonous and/or allochthonous energy pathways (Stiling & Cornelissen, 2007), with leaf litter also containing increasing carbon-to-nutrient ratios (Norby et al., 2001; Perkins et al., 2010). However, there is currently insufficient evidence for such effects from field experiments. In one field-based study, aqueous CO_2 was elevated by ~1100 ppm in artificial stream channels with water diverted from a relatively pristine sandy-bottomed temperate stream, with no effects on periphyton biomass, elemental composition or community composition (Brown et al., 2017). In the only known CO_2-enrichment mesocosm experiment investigating community-level benthic invertebrate responses, Hargrave et al. (2009) found that raised atmospheric CO_2 appeared to increase bottom-up autochthonous energy transfer, increasing Chironomidae larvae abundance despite lower nutritional quality than at ambient CO_2. While logistically challenging, similar field-based, community-level studies will improve our understanding of potentially complex CO_2 effects that likely differ dramatically between and within lotic systems (Brown et al., 2020).

Climate change is causing an increasingly intensified hydrological cycle, leading to more frequent extreme events (floods or droughts) and increased within-season flow variability (IPCC, 2021a; Jackson et al., 2001; Schneider et al., 2013). These changing hydrological regimes have implications for lotic invertebrate community assemblages (Larsen et al., 2023; Mouton et al., 2022; Pyne & Poff, 2017), particularly in smaller streams which are most vulnerable to changes in water depth, temperature, and velocity (Mustonen et al., 2018). Flow velocity is a key physical feature of discharge alteration in

streams that can influence invertebrate community composition via several pathways (Dewson et al., 2007). Velocity controls both the rate of food delivery for filter and deposit feeders (Nowell & Jumars, 1984) and nutrient delivery for the algal food source of grazers (Dewson et al., 2007). Moreover, invertebrates preferring either fast- or slow-flowing habitats may respond to velocity alterations by changing active drift rates (Poff & Ward, 1991). The effects of continuously applying slow flow velocity in mesocosm experiments have usually reflected these mechanisms (e.g., Blöcher et al., 2020; Elbrecht et al., 2016; Matthaei et al., 2010). However, human interference with flow regimes (e.g., flood control, agricultural water abstraction) can lead to a reduction in natural variability and consequent deleterious effects on communities (Ledger & Milner, 2015; Poff et al., 1997). While the negative impacts of continuous slow flow velocity are clear, it has not been established whether applying intermittent fast-, medium- and slow-velocity periods will maintain these negative effects, or if such velocity variability will benefit the community.

Loads of fine sediment (commonly defined as particles <2 mm) exceeding natural variability due to land-use activities, and most commonly associated with intensive agricultural practices (Wood & Armitage, 1997), are a pervasive stressor frequently linked to biological change in running freshwaters (Fanelli et al., 2022; Kemp et al., 2011; Vörösmarty et al., 2010). Sediment settling on a stony-bottomed streambed fills interstitial spaces, resulting in reduction of habitat for many benthic invertebrates, while benefitting those that utilize fine sediment (Lenat et al., 1981), for example, by burrowing or constructing casings (Dudgeon, 1994). When applied to stream mesocosms, experiments have revealed generally negative effects of fine sediment on pollution-sensitive Ephemeroptera, Plecoptera and Trichoptera (EPT) taxa (mayflies, stoneflies and caddisflies) and positive effects on worms and midge larvae (e.g., Beermann et al., 2018; Blöcher et al., 2020; Piggott, Townsend, & Matthaei, 2015a). As a more localized stressor, agricultural fine sediment loading in streams can be managed at the regional or catchment scale. Therefore, knowledge of sediment interactions with global stressors (those with a global cause) such as heatwaves, increasing CO_2 and changing flow regimes may allow mitigating potentially harmful effects of these global stressors that generally cannot be influenced by local management (Brown et al., 2013).

The present experiment employed 128 flow-through mesocosms to assess, for the first time, the individual and combined effects of CO_2 enrichment, heatwaves, flow velocity variability, and fine sediment deposition (using a $2 \times 2 \times 2 \times 2$ factorial design) on stream invertebrate communities. We made the following predictions:

1. CO_2 enrichment changes invertebrate community composition by reducing algal grazing taxa. This effect is likely due to indirect negative effects imposed on primary consumers via lowered nutritional quality (Emmerson et al., 2005; Stiling & Cornelissen, 2007; Urabe et al., 2003).
2. Heatwaves reduce invertebrate diversity and change community structure as heat-sensitive taxa leave the mesocosms

(Macaulay et al., 2021; Piggott, Townsend, & Matthaei, 2015a), with smaller effects than for continuous temperature increases as temperature-sensitive taxa re-establish between heatwaves.
3. Variable flow velocities allow less sensitive non-drifting taxa that prefer slow velocity to dominate, shifting invertebrate community composition (e.g., Beermann et al., 2018; Blöcher et al., 2020), with less negative effects than for continuous slow velocity as sensitive drifting taxa (particularly EPT) re-establish in fast- and medium-velocity periods.
4. CO_2 enrichment effects on invertebrate communities depend on the fine sediment level, as sediment deposition changes periphyton community composition (Magbanua, Townsend, Hageman, Lange, et al., 2013; Passy, 2007), with stronger negative effects on invertebrates in whichever algal community responds more strongly to CO_2 enrichment.
5. Negative CO_2 effects on invertebrates are stronger when combined with heatwaves, as these amplify CO_2 effects on algal growth and food quality (Li et al., 2016).
6. Heatwave effects are stronger when combined with flow velocity variability as the heatwaves occur simultaneously with the experimental slow-velocity periods (simulating hot, dry conditions), leading to an overall harsher habitat for sensitive taxa.

2 | MATERIALS AND METHODS

2.1 | Study site

From October 21 to December 12, 2019 (Austral spring/early summer), 128 circular stream mesocosms (volume 3.5 L) were continuously supplied water from the adjacent Kauru River, a fifth-order stream in the Otago region of New Zealand (45°06′22″ S, 170°44′51″ E; 98 m a.s.l.). The Kauru catchment ranges from 55 to 1273 m a.s.l. and covers 124 km^2. The geology of the upper catchment comprises quartzofeldspathic sandstone interbedded with schist and mudstone, volcanic basalt, and non-marine sedimentary rock (Otago Regional Council, 2010). Upstream of the experimental site, the river passes through native tussock grassland and exotic pasture grazed by low-density sheep and cattle. The river has a gravel/cobble bed and contains diverse periphyton (Lange et al., 2011; Piggott, Salis, et al., 2015) and invertebrate communities (Herrmann et al., 2012; Piggott, Townsend, & Matthaei, 2015a; Wagenhoff et al., 2012). The river water is nutrient-poor (means ± SD across two dates during the experiment; $NO_3/NO_2 = 13.8 \pm 3 \mu g/L$, $NH_3 = 15.7 \pm 1.5 \mu g/L$, $PO_4 = 4.3 \pm 1 \mu g/L$; $n = 128$).

2.2 | Experimental setup

The experimental system (*ExStream System*; Figure 1; see Piggott, Salis, et al., 2015) is a tightly controlled, statistically powerful yet highly field-realistic setup that has been used previously in >20 experiments in New Zealand, Germany, Ireland, China, and Brazil. Our
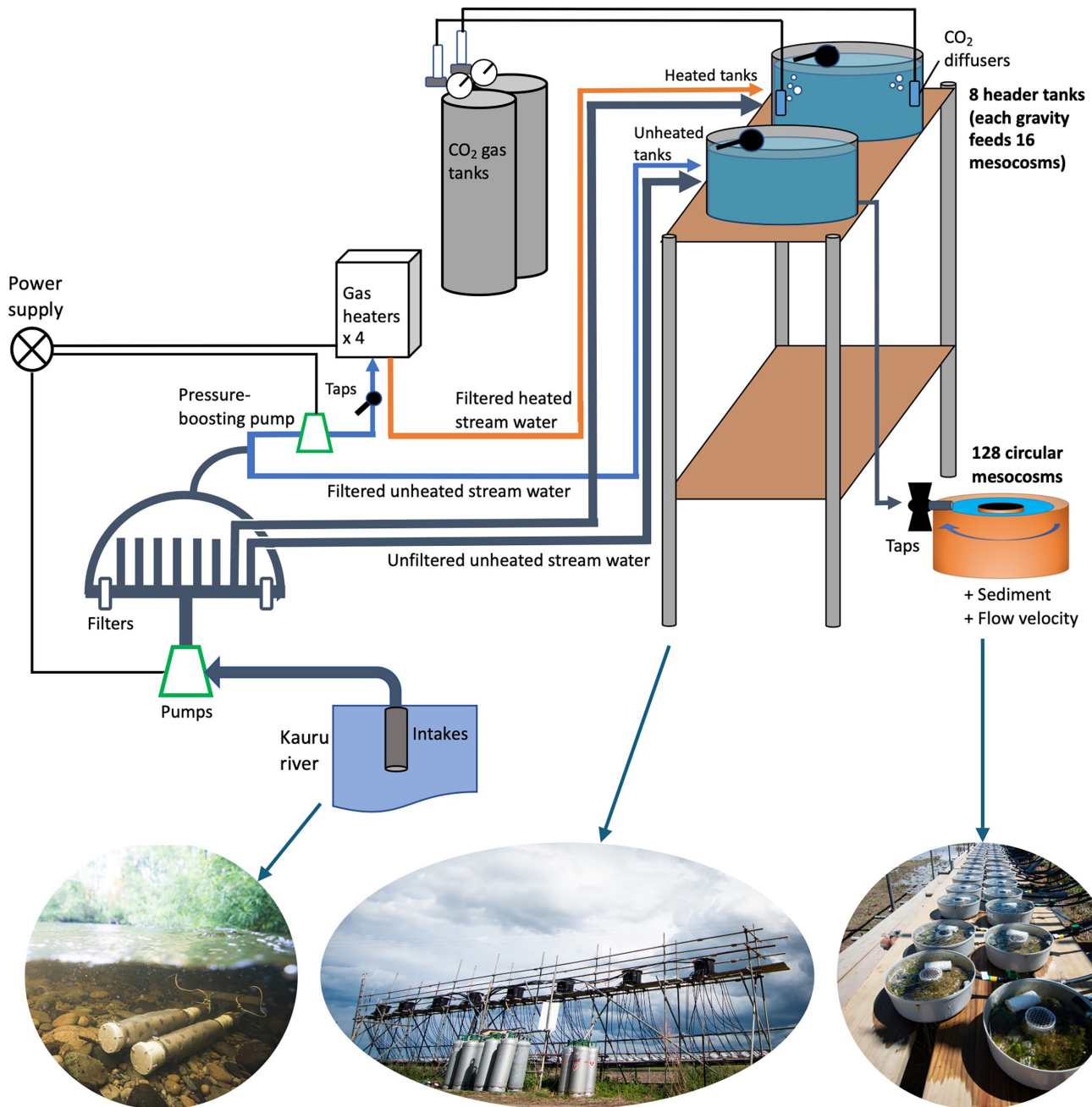
FIGURE 1 Schematic of the experimental system (adapted from Lange et al., 2011): Stream water was pumped continuously to the eight header tanks. During heating treatment periods, heated header tanks received an even mix of water from all heaters, and when heaters were turned on, unheated (control) tanks received filtered unheated water. CO_2 was also manipulated at the header tank level and each CO_2-enriched header tank received an even mix of gas from two CO_2 tanks. Fine sediment and flow velocity variability (by altering inflow jets) were manipulated directly at the mesocosm level (see text for details).

basic setup was identical to that of a recent related study also using the Kauru River *ExStream System* (see Macaulay et al., 2021), except for the adaptations pertaining to the experimental manipulations described below.

For the heating system, two 25-mm polythene pipes fed stream water through two separate inline filters (0.5mm) before joining to a single 25-mm pipe, which split into two pipes. One of these supplied water to four separate 13-mm PVC pipes each fitted with a tap

regulator to four continuous flow gas water heaters (model VT26; Rinnai, Germany). Each heater had an outflow pipe leading to a manifold before separating again into four 13-mm PVC pipes of 4m length, connecting to regulating inflow taps which provided filtered heated water to the four heated header tanks. The second pipe split into four 13-mm PVC pipes which provided filtered, unheated water to the four unheated header tanks at the same volume as the heated header tanks received heated, filtered water.

For CO_2 treatments, two 33-kg liquefied CO_2 gas tanks were fitted with a dual-stage CO_2 regulator with four manifold blocks, each with a bubble counter and valve (Pro-Elite Series Dual Stage CO_2 Regulator; CO_2 Art Technologies Limited, Dublin). The inside walls of each CO_2-treated header tank were fitted with two in-tank diffusers (In-Tank Bazooka Flux_ CO2 Diffuser; CO_2 Art Technologies Limited, Dublin), supplied by CO_2-resistant 4-mm polyurethane aquarium tubing. Each regulator was connected to one diffuser in each of the four CO_2-enriched header tanks (two in each spatial block) so that each header tank received CO_2 from both tanks. Regulators were checked and adjusted daily (~180 bubbles/min, 40 psi working pressure) so that each header tank received the same constant rate of CO_2 bubbles.

Each of the eight 135-L header tanks gravity-fed stream water to 16 mesocosms at a constant discharge of 2 L/min, measured at the start of the colonization period (Day 16) and recalibrated daily, via 4-m length of 13-mm polythene pipe controlled by a tap regulator. To create a bed substratum emulating small New Zealand streams (Matthaei et al., 2006), 500 mL of small-to-medium-sized gravel was collected from the river floodplain, sieved to remove fine sediment (particles <2 mm; Zweig & Rabeni, 2001), and added to each mesocosm with 14 randomly selected 40- to 50-mm flat cobbles placed on top. On Day 0, a piece of PVC pipe (80 mm length, diameter 40 mm) was placed in the remaining space to act as a fish shelter, and a 1-cm stainless steel mesh covering was placed over the outflow to prevent fish escaping, scrubbed daily with filtered stream water to remove any trapped organic material.

2.3 | Experimental design and procedures

CO_2, fine sediment, flow velocity variability, and temperature were manipulated in a full-factorial $2 \times 2 \times 2 \times 2$ design with eight replicates of each treatment combination. Flow to the mesocosms began on October 21, 2019 (Day 17), the start of a 17-day colonization period. During this, the CO_2 (from Day 17) and sediment (from Day 14) manipulations were already implemented. A 35-day "experimental" period (beginning on Day 0) followed, during which temperature and flow velocity were manipulated, as well (Figure 2).

CO_2 treatments were randomly assigned at the header tank level, with one CO_2-enriched header tank in each of four spatial blocks

of two tanks per block. CO_2 was bubbled into CO_2-enriched header tanks continuously from the start of the colonization period (Day 17). On Days 14 and 28, 1-L water samples taken from 16 randomly selected channels (eight ambient and eight CO_2-enriched) were stored in sealed glass bottles and preserved with mercuric chloride for DIC analysis. Within 5 min of sampling, pH and temperature were also measured in these channels using a handheld pH meter (HI-98128; Hanna, Rhode Island).

Total DIC was measured by coulometric analysis of the CO_2 evolved from samples acidified with phosphoric acid. Accuracy of this method, described in Dickson et al. (2007), is estimated at $\pm 1 \mu mol/kg$. DIC was higher in CO_2-enriched than ambient channels on both sampling dates, unaffected by heating treatments (Figure S1). On Day 14, average DIC concentrations were $361 \pm 1 \mu mol/kg$ in ambient and $456 \pm 15 \mu mol/kg$ in enriched channels. On Day 28, both DIC and alkalinity were higher overall and the difference in DIC between treatments was smaller. Average DIC concentrations on Day 28 were 463 ± 3 and $508 \pm 2 \mu mol/kg$ in ambient and enriched channels, respectively. The pH was lowered in CO_2-enriched channels, from 7.9 ± 0.1 to 6.9 ± 0.1 on Day 14 and from 8.3 ± 0.2 to 7.4 ± 0.1 on Day 28.

DIC, pH, and water temperature were used to calculate the partial pressure of CO_2 (pCO_2) using the package seacarb (Gattuso et al., 2022) in R 4.2.0 (R Core Team, 2022). While calculations were adjusted for low salinity, this program is optimized for seawater analysis, thus these values are approximations. Estimated mean pCO_2 in ambient channels was low compared to the atmosphere (ca. 418 ppm; NOAA, 2023). Mean pCO_2 was 218 µatm in ambient and 2212 µatm in enriched channels on Day 14, and 119 µatm in ambient and 981 µatm in enriched channels on Day 28.

On Day -14, flow was interrupted (~5 min) and 300 g dry fine sediment (average grain size 0.2 mm, Spectrachem Analytical; Clyde Scree Supplies Ltd., New Zealand) pretested for low nutrients (Blöcher et al., 2020) was added to 64 mesocosms, randomized within eight header tank blocks of 16 mesocosms each. This amount, a "moderate" level of sediment impact, has previously been shown to create ~90% sediment cover and ~5 mm sediment depth in near-identical mesocosms (Wagenhoff et al., 2012).

During colonization, drifting invertebrates, algae and microbes were able to enter the mesocosms via the unfiltered stream water pipes and establish realistic, diverse communities (Lange et al., 2011;
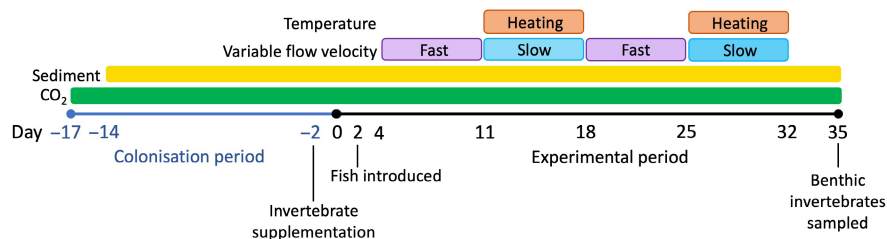
FIGURE 2 Timeline of the experiment. CO_2 and sediment were applied from the beginning of the colonization period before invertebrate supplementation and fish introduction. The entire benthic invertebrate community in each mesocosm was sampled following a 35-day period during which (in addition to CO_2 and sediment) temperature and flow velocity were manipulated in 7-day presses. See text for details.

Wagenhoff et al., 2012). Throughout the experiment, invertebrates were also able to drift out of the mesocosms via the central outflow with 1-cm mesh covering. On Day 2, natural invertebrate colonization was supplemented with taxa underrepresented in the drift by adding one standard sample of the Kauru River benthic invertebrate community to each mesocosm, following the methods described in Piggott, Townsend, and Matthaei (2015a).

Leaf packs, cotton strips, and fish were also added to each mesocosm on Day 0. While key components of the experimental stream ecosystem, their inclusion was primarily for the study of additional ecosystem components. Three grams (air-dried weight) of rehydrated mahoe (*Melicytus ramiflorus*) leaves were placed beneath the surface stones in each mesocosm where they remained until Day 35. One juvenile upland bully (*Gobiomorphus breviceps*), a locally abundant fish endemic to New Zealand (Herrmann et al., 2012), was added to each mesocosm (mean fork length 36.3 ± 3.5 mm). All mesocosms were fitted with polyester emergence hoods (mesh size $50 \mu m$) for the entire experimental period from Day 0 onward to prevent fish escaping from the mesocosms. This meant that aerial colonization by emerged insects was possible during the colonization period, while colonization via stream drift was the only source of insect larvae from Day 0 onward.

Two flow velocity treatments were created: "constant" velocity (medium velocity throughout) and "variable" velocity (alternating fast and slow periods). Discharge (2 L/min) remained constant among these treatments. "Constant" velocity channels were fitted with a "medium" inflow jet (diameter 5 mm) installed from the start of colonization and throughout the experiment. "Variable" velocity channels were also fitted with a medium jet throughout colonization and replaced on Day 4 with a "fast" jet (diameter 3 mm) for 1 week. On Day 11, the jet was removed altogether for the "slow" velocity period for 1 week, leaving a 10-mm diameter inflow. This process was repeated from Day 18, creating four alternating fast and slow periods, and on Day 32 "variable" channels were fitted with a medium jet for 3 days before the final sampling day. Flow velocities were measured in all channels on Days −12, −1, 4, and 11 using a precision flow meter (MiniWater20; Schiltknecht, Gossau, Switzerland). Average near-bed velocity was 20 ± 1.1 cm/s on Day -12, with velocity gradually decreasing over time as prolific benthic algal communities (including filamentous taxa) formed in the channels. Mean velocity prior to beginning flow treatments (Day 1) was 17.9 ± 1.4 cm/s. On Day 4 (the first "fast" period), average velocities were 7.0 ± 2.8 cm/s in "constant" channels and 14.9 ± 3.2 cm/s in "variable" channels. On Day 11 (the first "slow" period), average velocities were 9.3 ± 2.6 and 2.0 ± 1.5 in "constant" and "variable" channels, respectively. Based on these two dates, "variable" channels experienced a mean velocity of 8.5 cm/s across the "fast" and "slow" periods. For "constant" channels, the corresponding mean velocity was 8.2 cm/s.

Temperature treatments were assigned randomly at the header tank level within two spatial blocks, each consisting of four header tanks. Heating was applied in two 7-day pulses starting on Days 11 and 25, the same periods as the two slow-velocity periods of the "variable" velocity treatments (to simulate hot, dry conditions).

Water temperatures in heated header tanks were measured at 5-min intervals from Days 0 to 35 with loggers on the bottom of the tanks (HOBO® Pendant Temperature/Light Data Loggers; Onset Computer Corporation). Temperature was increased gradually to a target of 5°C above ambient over the first 24 h of heating, and reduced gradually during the final 24 h. During the 35-day experimental period, temperatures reached a maximum of 23.4 in "ambient" and 28.2°C in "heated" header tanks (Figure S2). The latter maximum is close to the highest temperature measured in the Kauru River during a 10-day heatwave in December 2017 (29.8°C; see Macaulay et al., 2021). Average achieved temperature increase between treatments was 4.8 ± 0.7°C during the first 7-day heating period and 4.3 ± 1.5°C during the second. These increases are on the conservative end of experimental heatwaves in existing research, but are arguably more realistic than many experiments where maximum temperatures were raised by 8–10°C (see review of heatwave multiple-stressor effects on freshwater systems by Polazzo et al., 2022), given that river-water temperature increases of 5°C or more during heatwaves are rare worldwide (Graham et al., 2024). Average temperatures across the entire 35-day period were 16.4 ± 3.2°C (ambient) and 18.1 ± 2.5°C (heated).

2.4 | Benthic invertebrate sampling

On the final experimental day (Day 35), the entire benthic community of each mesocosm was sampled by elutriating the whole substratum into a 250-μm sieve using filtered water. Invertebrates and associated organic material were preserved in 90% ethanol on site. These samples were later divided into quarter subsamples using an automated rotating sample divider (which produced eight subsamples, of which two were randomly combined to create one quarter of a sample), one of which was fully processed with all invertebrates identified and counted. Another quarter was scanned for rare taxa not found in the first quarter, which were added to taxon richness counts but not to abundances. Invertebrates were identified to the lowest practicable taxonomic level and body length measured to the nearest 1 mm using a dissecting microscope (8-409; Olympus SZ51, Tokyo, Japan). Length was not recorded for Annelida, which fragment, or Nematoda, which often became coiled and could not be measured accurately.

2.5 | Algal cover, fish survival, and fish gut contents

To help understand treatment effects on invertebrate habitat, algal cover was estimated visually by the same person (J.G.H.) based on photographs of all mesocosms on six occasions (Days 0, 9, 13, 19, 26, and 32; to cover all stages of the experiment including all fast-flow and slow-flow + heating periods). This method was carried out as in previous *ExStream* experiments (e.g., Magbanua, Townsend, Hageman, Lange, et al., 2013; Wagenhoff et al., 2013), except that

one quarter of each mesocosm (without leaf packs or fish shelter) was consistently used for estimations.

A range of upland bully responses were examined primarily for an additional companion study. However, as fish survival and feeding habits may be linked to some invertebrate community responses, upland bully survival and key prey items (and the methods used to obtain these) can be found in the Supporting Information.

2.6 | Data analysis

Statistical analyses were carried out in R 4.2.0 (R Core Team, 2022). The four experimental factors were fixed categorical factors in the (M)ANOVAs used, each with two levels, and a block factor was included to account for background variation across the two spatial blocks comprising four header tanks each. The resulting model was intercept (d.f. 1)+temperature (1)+CO_2 (1)+flow (1)+sediment (1)+block (1)+temperature×CO_2 (1)+temperature×flow (1)+CO_2×flow (1)+temperature×sediment (1)+CO_2×sediment (1)+flow×sediment (1)+temperature×CO_2×flow (1)+temperature×CO_2×sediment (1)+temperature×flow×sediment (1)+CO_2×flow×sediment (1)+temperature×CO_2×flow×sediment (1) (d.f. [residual]=111, n=128).

In total, 36 freshwater invertebrate taxa were identified in the experiment (Table S2). This complete dataset was used to examine six community-level metrics: total abundance (number of invertebrates per channel), total taxon richness, Simpson's diversity, EPT abundance, EPT taxon richness and mean body size. Individual four-way ANOVAs were carried out for the six community-level invertebrate responses investigated and for algal cover (separately for each day), with no data transformations needed (as determined during exploratory data analysis). The 13 most common taxa were chosen to investigate invertebrate community composition and effects on individual taxa, each contributing at least 0.5% of the total invertebrate count (adding to 98.5% of the total count). For the chironomid subfamily Orthocladiinae, we also analyzed effects on body size as this taxon occurred in every sample, comprised a large proportion of the dataset (17.3%), and its body size was normally distributed. Treatment effects on community composition and individual taxon abundances (square-root-transformed) were examined by four-way MANOVA using the manova function (package car) (Fox et al., 2019). Non-metric multidimensional scaling plots based on Bray–Curtis dissimilarities, using the same 13 taxa as in the MANOVA, were created as graphical approximations of changes to multivariate invertebrate community composition (main effects of each factor, exclusive of interactions). Fish survival (# days) and success of the heatwave, CO_2, and flow velocity experimental treatments were also tested with ANOVAs.

Significance level α for all tests was .05. Effect sizes (partial η^2 values, range 0–1) were calculated using the package heplots (Friendly et al., 2022) where $p < .10$, to allow readers to evaluate the biological relevance of results (Nakagawa & Cuthill, 2007). When interpreting partial η^2 effect sizes, the following benchmarks were adopted: small

≥0.01 ($f = 0.10$), medium ≥0.06 ($f = 0.25$), and large ≥0.14 ($f = 0.40$) (Cohen, 1988; Richardson, 2011). For multivariate MANOVA results, partial η^2 values are equal to Pillai's trace.

Significant factor main effects were classified as positive (+) or negative (−) based on the direction of each response to manipulated versus control (ambient) levels. Significant interactive effects were classified directionally as antagonistic (A) or synergistic (S) according to a classification system where directions of individual stressor effects in relation to controls are compared to combined effect directions (Piggott, Townsend, & Matthaei, 2015b). Where significant interactions were present, the recommendations of Quinn and Keough (2002) were followed: lower order interactions and main effects interpreted only where the effect size of the higher order interaction was smaller than that of the lower order interactions or main effects. Otherwise, the higher order interaction overrides the lower order interactions or main effects.

3 | RESULTS

3.1 | Success of the experimental treatments

Dissolved inorganic carbon and temperature were significantly raised in treatment groups relative to controls during the heatwave periods, with no significant effects of other experimental treatments (Table S3). Flow velocity was affected by all experimental factors; however, most of the variation during fast- and slow-velocity periods was attributed to the flow velocity treatments (Figure S3; Table S4).

3.2 | Algal cover

CO_2 enrichment strongly increased algal cover across all dates (Table S5; Figure S4). Sediment addition had an initial strong negative effect on algal cover on Day 0, but a positive effect across three later dates (13, 19, and 26). Temperature had an initial negative effect following the first heatwave on Day 19, after which it had a positive effect by Day 26. CO_2 and temperature interacted on Day 32; the positive effect of CO_2 on algal cover was stronger in channels exposed to the heatwaves. Flow velocity variability had one moderate positive effect on Day 26, during the second slow-velocity period.

3.3 | Fish survival and key invertebrate prey items

In total, 98 of 128 bullies survived until Day 33 (when they were sampled). Fish survival was reduced by CO_2 enrichment, an effect that was largely driven by an interaction between CO_2 and sediment. Survival was decreased by CO_2 enrichment in channels without sediment, whereas CO_2 had no negative effect in channels with sediment (Table S6; Figure S5). Orthocladiinae (Chironomidae) were the taxon most commonly consumed (55.4%), followed by Chydoridae (Cladocera) (14.5%), Chironomidae pupae (8.6%), *Austrosimulium*

spp. larvae (5.3%), Chironominae larvae (4.7%), and emerged insects (4.6%; all others 6.9%).

3.4 | Community-level responses

Heating had a negative main effect on five of the six community-level invertebrate metrics (total taxon richness, Simpson's diversity, EPT abundance, EPT taxon richness, mean body size), with the strongest effect occurring for body size (Table 1; Figure 3). CO_2 enrichment also had a moderate negative effect on body size. Variable flow velocity increased total invertebrate abundance compared with constant flow channels. Sediment addition affected all community-level metrics, with four negative effects and positive effects on diversity and body size. CO_2 enrichment had no main effect on total abundance but interacted synergistically with sediment (Figure 4a). In channels without sediment, CO_2 increased total abundance, whereas the combined effect of CO_2 and sediment became more strongly negative than that of sediment alone. Furthermore, flow velocity and sediment interacted antagonistically when affecting invertebrate body size (Figure 5a). While sediment alone increased body size, this effect was much weaker when combined with variable velocity.

3.5 | Orthocladiinae body size

No factor main effects were detected for Orthocladiinae body size; however, temperature interacted separately with both CO_2 and sediment. An antagonistic interaction occurred where CO_2 reduced body size in ambient-temperature channels, while no CO_2 effect was seen in heated channels, where body size was already somewhat reduced (Figure 5b). Temperature and sediment interacted synergistically to affect Orthocladiinae body size, with sediment having a positive effect in ambient-temperature channels, whereas in channels with sediment at increased temperature body size was smaller than in channels with neither stressor (Figure 5c).

3.6 | Benthic invertebrate community composition

Sediment addition had the strongest influence on benthic community composition in the MANOVA, followed by heating and CO_2 addition, while variable flow velocity had the smallest effect (Table 1; Figure 6). No significant interactions occurred.

3.7 | Individual taxon responses

Abundances of all 13 taxa were affected by at least one experimental factor (Table 1; Figures S6–S8). Heating negatively affected seven taxa, especially the chironomid subfamilies Orthocladiinae and Chironominae. Heating had a positive effect on Chydoridae

(Cladocera), the most common taxon (28.5% of total count). CO_2 enrichment decreased abundances of Orthocladiinae, Chironominae, and the snail *Potamopyrgus antipodarum* and increased the abundance of Copepoda. Variable flow velocity had only positive effects and increased the abundances of seven taxa. Sediment addition negatively affected seven taxa, with strong effects occurring for Copepoda, Chydoridae, and Leptophlebiidae. Sediment also had a strong positive effect on Nematoda abundance.

Annelida were affected by an interaction between CO_2 and sediment (Figure 4b). In channels without added sediment, CO_2 enrichment increased Annelida numbers, whereas the opposite pattern was seen in channels with added sediment. Furthermore, CO_2 enrichment had a positive effect on Copepoda in the absence of sediment, but this effect was cancelled out by sediment addition, which reduced Copepoda to similar numbers in both CO_2 groups (Figure 4c). Leptophlebiidae were affected by an interaction between CO_2 and flow velocity (Figure 4d). While CO_2 enrichment had no effect at constant velocity, it reduced Leptophlebiidae abundance at variable velocity. Finally, a complex three-way interaction was detected for the caddisfly *Oxyethira* spp. (Figure 4e). At ambient CO_2, variable velocity increased *Oxyethira* abundance in both temperature treatments, whereas at enriched CO_2 variable velocity reduced abundance when combined with heating.

4 | DISCUSSION

4.1 | Stressor main effects

4.1.1 | CO_2 enrichment

Our study is one of the first to examine raised CO_2 effects on invertebrate communities in outdoor stream mesocosms, with only one other experiment known (Hargrave et al., 2009). We predicted that continuous enrichment of CO_2 throughout our 52-day experiment would change invertebrate community composition by reducing algal grazing taxa. As ambient aqueous CO_2 was undersaturated compared to the atmosphere, and CO_2 enrichment strongly increased algal cover, at least some algae were apparently experiencing CO_2 limitation. Negative effects on grazing invertebrates including *P. antipodarum* (Broekhuizen et al., 2001) and many members of Orthocladinae and Chironominae (although these midge subfamilies contain several feeding groups and many are generalists; Serra et al., 2016) appear to at least partly support our prediction. Nevertheless, these responses could reflect indirect effects (e.g., lowered algal nutritional quality, reduction in habitat due to excessive algal growth), direct effects of weak acidification (e.g., increased metabolic demand, changes in feeding rates or predator avoidance behavior; see review of marine invertebrate taxa by Thomas et al., 2020), or a combination of direct and indirect effects. However, raised CO_2 clearly did not increase bottom-up autochthonous energy transfer via periphyton, as seen in Hargrave et al. (2009). In this North American experiment, CO_2 concentration was doubled in

TABLE 1 Summary of M(ANOVA)s comparing benthic invertebrate responses between experimental treatments.

Response	%	Temperature	C	CO_2	C	Flow	C	Sediment	C	Temp.×CO_2	C	Temp.×flow	C	CO_2×flow	C
Total abundance		.09 (0.03)		.61		.002 (0.08)	+	<.001 (0.11)	−	.50		.80		.62	
Taxon richness		.002 (0.09)	−	.06 (0.03)		.42		.01 (0.06)	−	.09 (0.03)		.89		.06 (0.03)	
Simpson's Diversity Index		<.001 (0.13)	−	.32		.12		<.001 (0.18)	+	.68		.16		.45	
Total EPT abundance		<.001 (0.12)	−	1.00		.36		.001 (0.09)	−	.47		.23		.39	
EPT taxon richness		.04 (0.04)	−	.29		.72		.04 (0.04)	−	.83		.72		.62	
Invertebrate body size		<.001 (0.23)	−	<.001 (0.11)	−	.07 (0.03)		.001 (0.09)	+	.28		.10		.52	
Orthocladiinae body size	28.5	0.47		.08 (0.03)		.67		.76		.03 (0.04)	−A	.09 (0.03)		.39	
Chydoridae (Cladocera)	17.3	.01 (0.05)	+	.22		.002 (0.08)	+	<.001 (0.26)	−	.63		.37		.89	
Orthocladiinae (Chironomidae)	17.2	<.001 (0.40)	−	<.001 (0.15)	−	.04 (0.04)	+	.002 (0.08)	−	.20		.87		.4	
Annelida	9.2	.80		.50		<.001 (0.11)	+	.01 (0.06)	+	.94		.34		.76	
Potamopyrgus antipodarum	7.0	.20		<.001 (0.11)	−	.02 (0.06)	+	.52		.08 (0.03)		.85		.98	
Copepoda	5.3	.35		<.001 (0.10)	+	.93		<.001 (0.37)	−	.61		.93		.56	
Nematoda	4.3	.01 (0.07)	−	.09 (0.03)		.06 (0.03)		<.001 (0.43)	+	.15		.75		.96	
Ostracoda	3.9	.06 (0.03)		.81		.04 (0.04)	+	.50		.72		.66		.76	
Tanypodinae (Chironomidae)	2.9	<.001 (0.06)	−	.11		.03 (0.04)	+	.005 (0.07)	−	.26		.39		.54	
Chironominae (Chironomidae)	1.0	<.001 (0.42)	−	<.001 (0.11)	−	.08 (0.03)		<.001 (0.10)	−	.41		.17		.43	
Pycnocentrodes spp.	0.8	.04 (0.04)	−	.81		.54		.80		.86		.84		.77	
Oxyethira spp.	0.6	<.001 (0.12)	−	.79		.02 (0.05)	+	.08 (0.03)		.35		.22		.20	
Hydrobiosidae	0.5	.004 (0.07)	−	.63		.39		.04 (0.04)	−	.31		.14		.29	
Leptophlebiidae		.82		.10		.67		<.001 (0.23)	−	.33		.49		.04 (0.04)	+A
Community composition (MANOVA—13 taxa)	98.5	<.001 (0.62)		<.001 (0.48)	−S	.001 (0.27)		<.001 (0.79)		.41		.78		.71	

Response	%	Temp.× sediment	CO_2× sediment	C	Flow× sediment	Temp.×CO_2× flow	Temp.×CO_2× sediment	Temp.×flow× sediment	CO_2×flow× sediment	Temp.×CO_2×flow× sediment
Total abundance		.14	.01 (0.06)		.81	.69	.42	.86	.70	.35
Taxon richness		.27	.42		.47	.53	.14	.67	.96	.74
Simpson's Diversity Index		.06 (0.03)	.79		.90	.25	.85	.60	.65	.65
Total EPT abundance		.66	.29		.42	.39	.34	.36	.42	.23
EPT taxon richness		.29	.62		.44	.36	.23	.94	.29	.94

(Continues)

TABLE 1 (Continued)

| Response	%	Temp.× sediment	C	CO₂× sediment	C	Flow× sediment	C	Temp.× CO₂× flow	Temp.× CO₂× sediment	Temp.× flow× sediment	CO₂× flow× sediment	Temp.× CO₂× flow× sediment
Invertebrate body size		.96		.39		**.04** (0.04)	+A	.99	.33	.62	.81	.69
Orthocladiinae body size		**.04** (0.03)	−S	.98		.33		.15	.18	.29	.23	.53
Chydoridae (Cladocera)	28.5	.40		.40		.96		.78	.60	.92	.98	.32
Orthocladiinae (Chironomidae)	17.3	.65		.14		.06 (0.03)		.92	.09 (0.03)	.69	.88	.69
Annelida	17.2	.08 (0.03)		**.003** (0.08)	+A	.86		.73	.10	.40	.42	.70
Potamopyrgus antipodarum	9.2	.59		.14		.42		.70	.42	.38	.13	.87
Copepoda	7.0	.36		**.02** (0.05)	+A	.33		.73	.52	.74	.52	.86
Nematoda	5.3	.06 (0.03)		.55		.90		.98	.98	.11	.19	.56
Ostracoda	4.3	.79		.09		.61		.60	.10	.12	.99	.90
Tanypodinae (Chironomidae)	3.9	.85		.81		.63		.90	.45	.88	.29	.48
Chironominae (Chironomidae)	2.9	.38		.35		.61		.27	.25	.13	.32	.35
Pycnocentrodes spp.	1.0	.69		.54		.17		.84	.51	.93	.89	.70
Oxyethira spp.	0.8	.51		.95		.42		**.04** (0.04)	.31	.12	.47	.36
Hydrobiosidae	0.6	.18		.45		.08 (0.03)		.79	.73	.78	.10	.74
Leptophlebiidae	0.5	.37		.41		.90		.49	.33	.62	.97	.11
Community composition (MANOVA—13 taxa)	98.5	.58		.14		.30		.62	.23	.24	.45	.88

Note: Relative percentage (%) of the total invertebrates counted are given for each taxon. *p*-Values are bolded where $p < .05$ and effect sizes [η^2_p values; range 0–1; effect size categories: small ≥ 0.01, medium ≥ 0.06, large ≥ 0.14 (Cohen, 1988; Richardson, 2011)] are printed in parentheses where $p < .10$. Direction (+ or −) of main factor effects (increased temperature, enriched CO_2; variable flow velocity, and sediment addition) relative to controls are given where $p < .05$. Combined (C) two-way interactive effects are given where $p < .05$ and classified directionally (+ or −) as antagonistic (A) or synergistic (S).

(a) Benthic Invertebrate Abundance

(b) Benthic EPT Abundance

(c) Benthic Invertebrate Taxon Richness

(d) Benthic EPT Taxon Richness

(e) Benthic Invertebrate Diversity (Simpson's)

(f) Benthic Invertebrate Body Size
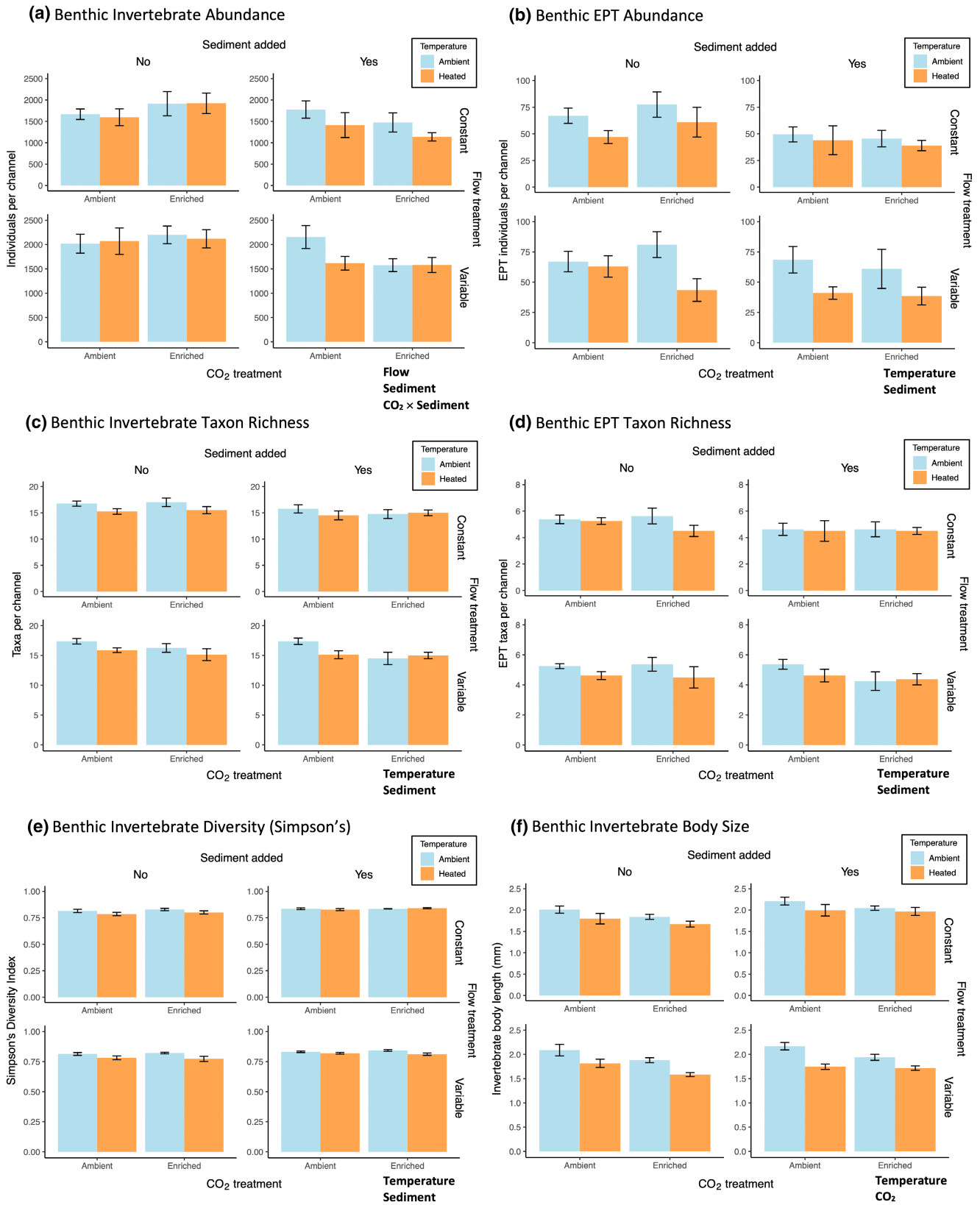
FIGURE 3 Benthic invertebrate community-level responses (a–f) to the experimental manipulations at the end of the experiment (Day 35). Bars represent means ± SE ($n = 8$ replicates per treatment combination). Significant factor main effects or interactions are indicated at bottom right for each response.
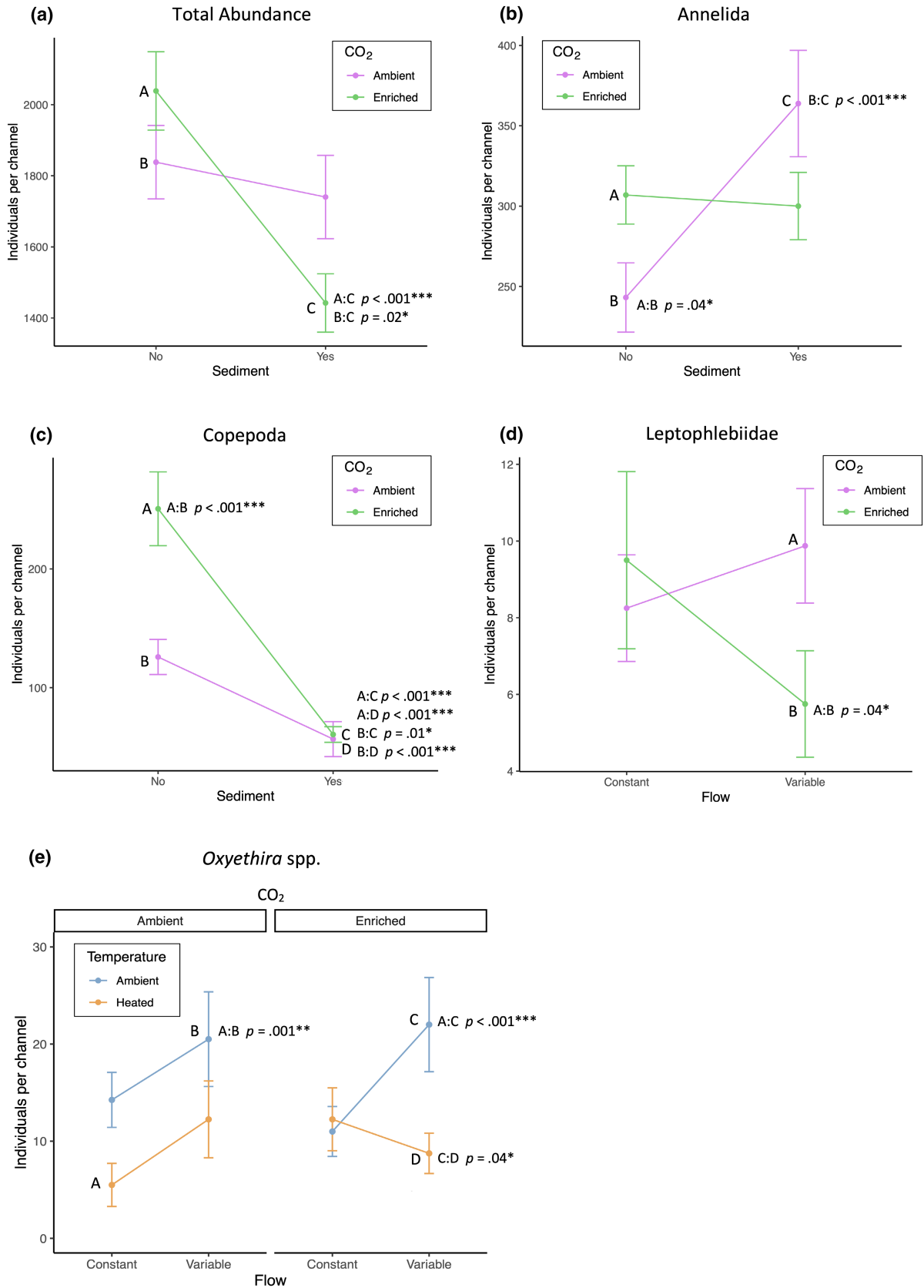
FIGURE 4 Two-way (a–d) and three-way (e) interactive effects on benthic invertebrate abundance responses. Points represent means ± SE. $N = 32$ replicates per treatment combination for two-way interactions; $n = 16$ per combination for the three-way interaction. Group mean comparisons based on post hoc testing are given with adjusted p-values and significance level (< .05*; < .01**; < .001***) where $p < .05$.
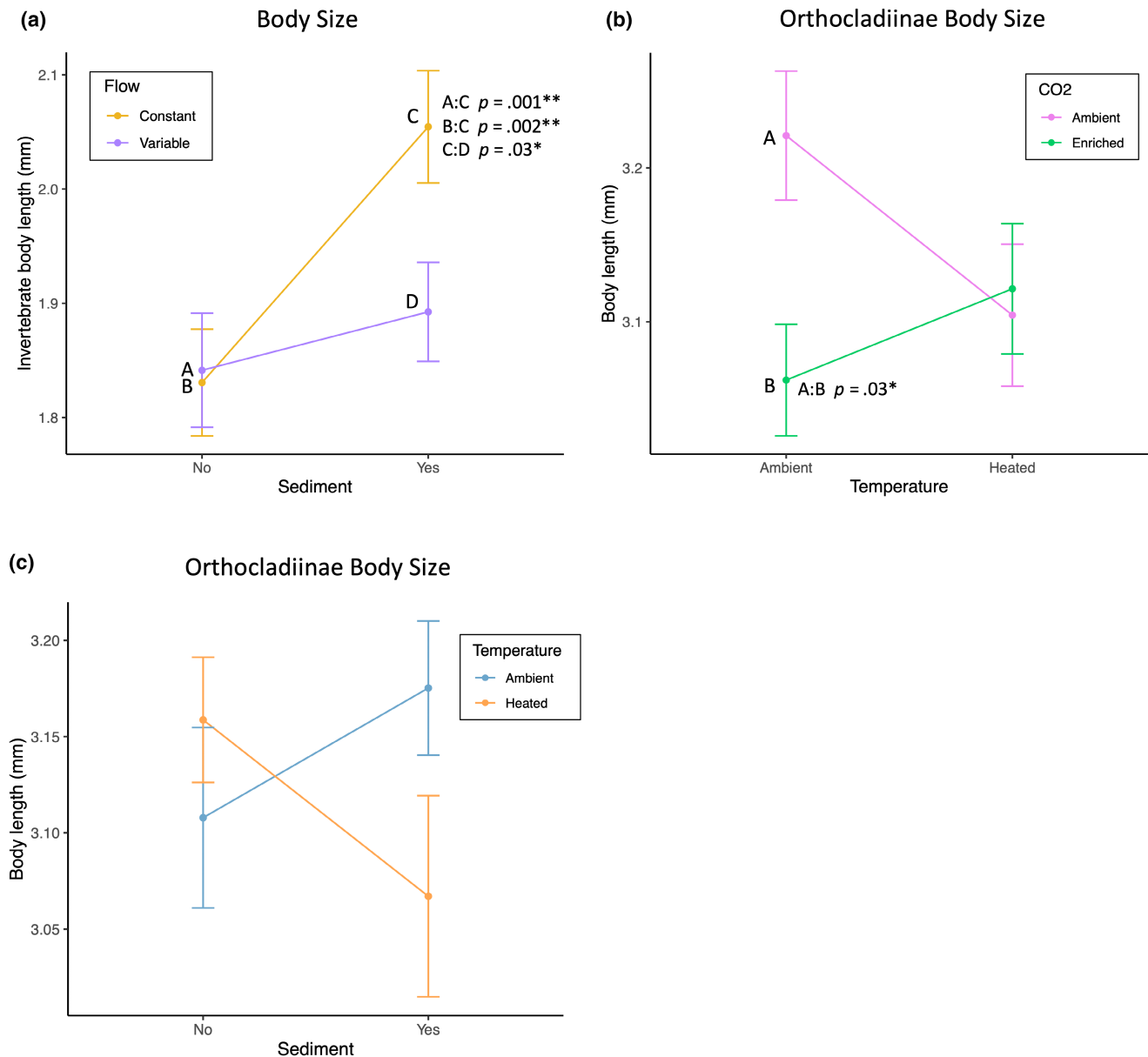
FIGURE 5 Two-way interactive effects on average body size of all invertebrates (excluding Annelida) (a) and Orthocladiinae (b, c). Points represent means ± SE. $N = 32$ replicates per treatment combination for two-way interactions; $n = 16$ per combination for the three-way interaction. Group mean comparisons based on post hoc testing are given with adjusted p-values and significance level (< .05*; < .01**; < .001***) where $p < .05$.

the air above large, sandy-bottomed stream mesocosms. The positive effects on chironomids in this earlier study contrast with the negative effects on two of the three chironomid subfamilies in our own study. One explanation could be differences in experimental substrata and the algal communities formed. Hargrave et al. (2009) noted that the unstable sand substratum in their experiment prevented growth of filamentous diatoms. In our experiment simulating stony-bottom streams, the increased algal cover in CO_2-enriched channels was mainly due to long filamentous algae (J.G.H., personal observations; Figure S4). This algal type may have become the dominant food source in CO_2-enriched channels for grazing invertebrates, with negative effects for Chironomidae via reduced habitat

or lowered food quality. As freshwater invertebrates have been understudied with respect to rising atmospheric CO_2 compared with primary producers and vertebrates (Hasler et al., 2016), future studies may consider examining raised CO_2 effects on algal grazing taxa and filamentous algae in simplified experimental food webs, disentangling the direct and indirect effects wherever possible.

4.1.2 | Heatwaves

Two week-long heatwaves (~4.8 and ~4.3°C increase in water temperature), with a mean overall increase of 1.7°C across 35 days,
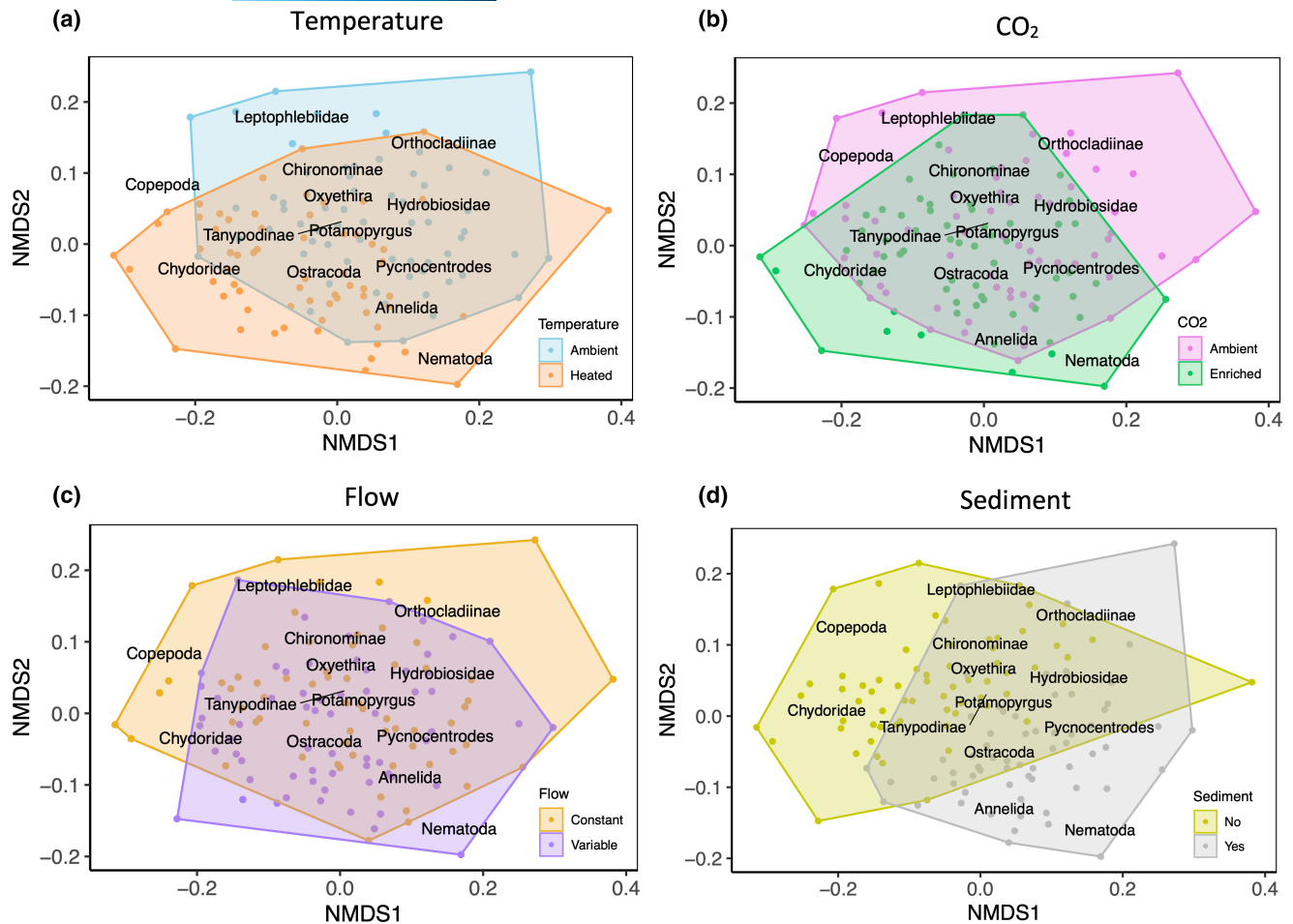
FIGURE 6 Non-metric multidimensional scaling (2D, 999 permutations) of relative abundances of the 13 most common taxa based on Bray–Curtis dissimilarities. Stress = 0.163. These plots are graphical approximations of the differences in multivariate invertebrate community composition detected in the MANOVA (main effects of each factor, exclusive of interactions between factors)—see Section 2 for details. Plots highlight each main factor (heatwaves [a], CO_2 enrichment [b], flow velocity variability [c], and fine sediment deposition [d]) separately, with colored points representing individual channels belonging to each treatment group and convex hulls showing the outer limits of treatment groups.

resulted in the highest number of negative effects on the benthic invertebrate community (12 of 20 possible responses) and the second strongest change in community composition of our four stressors. As we predicted, there was a loss in diversity alongside reductions in several taxa; yet in comparison to a recent experiment in the same experimental system (in the same river and season) in which mesocosms were warmed by 3°C constantly for 24 days (Macaulay et al., 2021), the effects of our heating treatments were somewhat weaker and not quite as pervasive. For example, eight individual taxon abundances were strongly reduced by this constant warming, whereas four of these same taxa showed no evidence of a response (Annelida, Copepoda, and Leptophlebiidae) or a positive response (Chydoridae). One possible explanation for this difference is that a natural heatwave during Macaulay et al. (2021) raised ambient water temperature to ~30°C leading to a maximum temperature of ~33°C, almost 5°C higher than the maximum temperature reached in our heatwave treatments. Evidence from existing constant-warming stream mesocosm experiments suggest that the degree to which

heating can reconfigure invertebrate community structure depends on whether experimental temperatures reach or exceed the local natural upper limit (Dossena et al., 2012; Macaulay et al., 2021; Piggott, Townsend, & Matthaei, 2015a; Yvon-Durocher et al., 2011) or fall within the natural temperature range (Nelson et al., 2021). Another possible explanation is the intermittent nature of our heating treatment, which appeared to affect the outcome for some sensitive taxa. For example, the mayfly taxon Leptophlebiidae (mostly represented by the nationally common genus *Deleatidium*) was unaffected by heatwaves in our experiment, despite being among the most temperature-sensitive groups in New Zealand streams (Quinn et al., 1994) and showing a clear negative response to constant warming in previous experiments (Macaulay et al., 2021; Piggott, Townsend, & Matthaei, 2015a). Evidently, the 3-day period between our second heatwave and invertebrate sampling at the end of our experiment was sufficient for Leptophlebiidae to repopulate the heat-treated mesocosms via drift. However, extrapolation of this result to real-world heatwaves should be done with care. As mayflies

drift downstream, their fast recovery from a heatwave would depend on conditions upstream (discussed below in Section 4.1.7).

Heatwaves had the strongest impact on invertebrate body size of the four stressors tested, although body size of Orthocladiinae (Chironomidae) was unaffected. Due to the continuous immigration and emigration of drifting invertebrates, our results are unlikely to reflect the temperature-size rule that ectotherms developing at higher temperatures will develop to be smaller (Atkinson, 1994; Kingsolver & Huey, 2008). Instead, heatwaves more likely affected average invertebrate size in the mesocosms by reducing habitat suitability and increasing drift or emergence rates in larger taxa (all three Chironomidae subfamilies and all three Trichoptera taxa) and having a positive effect, or no effect, on smaller taxa (~1–2 mm Chydoridae [Cladocera], Copepoda, *P. antipodarum*, and Ostracoda). Particularly during the second heatwave when ambient temperatures were higher, temperatures frequently reached or exceeded the average upper thermal limits of several frequently drifting insect taxa for which data are available: the three Chironomidae subfamilies (McKie et al., 2004) and Coenosucidae (containing *Pycnocentrodes*; Quinn et al., 1994). In contrast, temperatures never reached the thermal limit of *P. antipodarum* snails, which do not actively drift (Quinn et al., 1994). However, higher-than-ambient temperatures during the first heatwave could still have elicited a drift response or early emergence response in sensitive insect taxa; these drift and emergence responses we assessed in a related companion study.

4.1.3 | Flow velocity

We predicted that variable flow velocities would allow less sensitive, non-drifting taxa that prefer slow velocity to dominate, shifting invertebrate community composition, but that these effects would be less negative than continuous slow velocity. Our prediction was largely supported, except that velocity variability changed invertebrate community composition only by increasing taxon abundances, despite involving 2-week-long periods of slow velocity. Related previous experiments have shown that continuous application of slow velocity can decrease abundances of pollution-sensitive EPT taxa (Elbrecht et al., 2016; Matthaei et al., 2010) and that fewer EPT individuals drift at fast flow velocity (Blöcher et al., 2020). In our experiment, the benthic community was sampled after 3 days of medium flow velocity following a slow-velocity period, suggesting that any negative effects on EPT taxa during slow velocity were temporary and these taxa were able to re-establish in the community once flow returned to medium velocity. In several past experiments, Copepoda increased in abundance at constant slow velocity (Beermann et al., 2018; Blöcher et al., 2020; Matthaei et al., 2010). Copepoda were unaffected by our flow variability treatment, suggesting that any positive effect of slow-velocity periods on this taxon were counteracted by negative effects of faster-velocity periods. These responses combined with the previously discussed experimental evidence suggest that shorter slow-velocity periods due to climate-related variability in within-season precipitation may be

of less concern for invertebrate communities than long-term continuous reduced velocity in streams caused by climate change or direct human interference.

4.1.4 | Fine sediment

Addition of 300 g dry fine sediment (equivalent to a high but not extreme sediment load; Wagenhoff et al., 2012) 7 weeks prior to sampling resulted in the strongest change in invertebrate community composition of our four stressors and pervasive negative effects on the community, with a positive effect on Nematoda. Negative effects of sediment on taxon richness, EPT richness, and EPT abundance are consistent with previous related research (e.g., Blöcher et al., 2020; Piggott, Townsend, & Matthaei, 2015a) and likely reflect increased propensity of sensitive invertebrate taxa to drift when sediment is present (Larsen & Ormerod, 2010; Magbanua et al., 2016; Suren & Jowett, 2001). For several responses measured, including total invertebrate abundance and abundances of the midge subfamilies Orthocladiinae, Tanypodinae, and Chironominae, effects of 7 weeks of sediment deposition were frequently more negative than in previous experiments of shorter durations (e.g., Blöcher et al., 2020; Magbanua, Townsend, Hageman, & Matthaei, 2013; Piggott, Townsend, & Matthaei, 2015a). Thus, our findings suggest that longer term sediment deposition in stony-bottom streams may be more harmful than shorter term deposition or any temporary effects of suspended sediment.

4.1.5 | $CO_2 \times$ sediment interactions

Non-additive interactive effects between experimental treatments were mostly antagonistic, the interaction type most commonly found in studies on freshwater invertebrates (Jackson et al., 2016). While CO_2 enrichment had the fewest factor main effects on community and individual taxon responses, it was involved in the highest number of interactions (6 total) of the four experimental factors, with $CO_2 \times$ sediment interactions most common. We predicted that CO_2 enrichment effects on invertebrate communities would depend on the fine sediment level. While CO_2 and sediment did not interact to affect overall community composition, the effect of enriched CO_2 on total invertebrate abundance and Copepoda abundance depended strongly on the sediment treatment, partially supporting our prediction. Communities forming under enriched CO_2 conditions in sediment-impacted mesocosms had ~20% fewer total invertebrates than those with either treatment alone. For Copepoda, positive effects of CO_2 occurred only in channels without sediment. The latter pattern could reflect the reduction in fish survival in channels with this treatment combination; however, this appears unlikely considering that Copepoda were not a common prey item for *G. breviceps*, and this pattern did not occur for any of their common prey taxa. Copepods in our mesocosms comprised the orders Cyclopoida and Harpacticoida (Hunn, 2023), which play a key role in aquatic

food webs by linking higher consumers with phytoplankton, heterotrophic bacteria, protists and smaller zooplankton (Frangoulis et al., 2005; Roff et al., 1995). Freshwater phytoplankton growth is not generally believed to be CO_2-limited (Low-Décarie et al., 2011), partly due to the CO_2 supersaturation found in many freshwaters (Raymond et al., 2013), but also because most phytoplankton taxa have active carbon concentration mechanisms allowing uptake of HCO_3^- and conversion to CO_2 for photosynthesis (Giordano et al., 2005). For these reasons, perhaps, potential effects of CO_2 enrichment on these meiofauna taxa have not been established. In running freshwaters, however, Cyclopoida and Harpacticoida mainly have a benthic lifestyle, found in high abundances in the hyporheic zone (Boulton & Foster, 1998; Kowarc, 1991), grazing on microbial biofilms in interstitial spaces (O'Doherty, 1985), and also scraping the outer surfaces of filamentous algae (Decho & Moriarty, 1990). In our experiment simulating a stream environment where periphyton dominates primary production, ambient CO_2 was undersaturated and elevated CO_2 might have boosted production of periphyton taxa with high CO_2 affinity. While algal carbon-to-nutrient ratios are often increased by elevated CO_2 (Finkel et al., 2010), effects of elevated CO_2 on polyunsaturated fatty acids (essential macronutrients for copepod growth and reproduction) are sometimes negative but commonly neutral (see McLaskey et al., 2019). Although little research exists on elevated CO_2 effects on copepods in freshwaters, there is some evidence that raised CO_2 may reduce fatty acid concentrations in marine phytoplankton, resulting in negative effects on copepod growth and reproduction (Rossoll et al., 2012). Conversely, in marine mesocosms, both phytoplankton and copepod abundance increased at elevated CO_2 (Algueró-Muñiz et al., 2017). These contrasting outcomes of simplified laboratory experiments and mesocosm studies may be attributed to the complexity and adaptability of natural systems (Rossoll et al., 2012): if some, but not all, phytoplankton decrease in nutritional quality, copepods actively choose high-quality food that meets their nutritional demands (Meunier et al., 2016). While the restricted time frame of our stream mesocosm experiment did not allow studying effects across multiple generations, it appears that food quantity was sufficiently raised by CO_2 enrichment to create favorable conditions for benthic copepods, with no impact of lowered food quality. However, this effect was limited to channels without deposited fine sediment. Sediment strongly reduced copepod abundance, likely due to a reduction in suitable habitat. Harpacticoida and Cyclopoida use interstitial spaces to enter diapause (Santer, 1998) and/or to avoid predation by fish (Gliwicz, 1986) or macroinvertebrates (Neill, 1990). Sediment deposition may reduce the size of these interstices, lowering their capacity for organic matter retention (Maridet et al., 1995; Ward et al., 1998). It is therefore plausible that sediment deposition reduced both habitat size and quality and predation refugia for benthic copepods in our experiment, and any positive CO_2-enrichment effect was offset by increased predation. Besides their important role in food webs, copepods are now recognized as a significant contributor to carbon flux to the ocean (Steinberg & Landry, 2017). Therefore, any CO_2 effects on freshwater copepods and interactions with land-use stressors should be areas of interest to climate-change research warranting further study.

4.1.6 | Other stressor interactions

Negative CO_2 effects were not generally worsened by heatwaves, despite algal cover showing a positive synergistic interaction between CO_2 and temperature after the second heatwave. Thus, our prediction that negative CO_2 effects on invertebrates would be stronger when combined with heatwaves was not supported. Only one 2-way interaction was found for invertebrates, where a slightly negative effect of CO_2 on Orthocladiinae body size occurred solely in ambient-temperature channels. In the single detected three-way interaction, variable flow velocity had generally positive effects on the larvae of the cased caddis *Oxyethira* spp., except when combined with CO_2 enrichment and heatwaves, when the variable-flow-velocity effect turned negative. This pattern suggests some negative impact of the combined slow-flow and heating periods on *Oxyethira*; however, their abundance was similarly low at constant flow velocity in heated channels with no CO_2 enrichment. Furthermore, no two-way interactions occurred between flow variability and heatwaves, despite low-flow periods occurring simultaneously with heatwaves. Overall, these results did not support our final prediction that heatwave effects would be stronger when combined with flow-velocity variability.

4.1.7 | Realism, limitations, and management implications

In general, interactive effects (particularly higher order interactions) were relatively scarce in our experiment. This could lead to the interpretation that only factor main effects were important, but detection of potentially important interactions may have been limited by our choice of study design (two levels of each factor tested). While it was not practical in our experiment to explore further levels of our four stressors of interest, interactive effects found in large-scale field studies are frequently nonlinear (D'Amario et al., 2019; Kefford et al., 2021). Although our study found little or no evidence of interactive effects for many stressor combinations, results may vary with the frequency and intensity of stressors applied or depend on thresholds along gradients of further variables, which our experiment could not capture. We therefore encourage future climate-change stream mesocosm experiments to explore a range of levels of each of these variables wherever possible.

An inherent limitation of our study is the small size of the mesocosms and its restricted duration due to the sustained effort involved. Consequently, our results cannot be generalized to larger lotic systems, other seasons or longer time periods without further complementary research at various scales (these limitations have been discussed several times in previous related studies; e.g., Beermann et al., 2018; Elbrecht et al., 2016).

Connectivity between the source river and our stream meso-cosms allows a considerable level of environmental realism due to the natural colonization and continuous immigration and emigration of drifting invertebrates, plus several other reasons discussed in depth in the >30 publications based on previous *ExStream System* experiments. However, as we cannot apply treatments to the entire catchment, natural colonization also means invertebrates entering the mesocosms freely throughout the experiment arrive from a source community experiencing ambient conditions. As previously discussed, negative heatwave treatment effects on the invertebrate community were not as large or pervasive as in some previous constant warming experiments. Flow velocity variability had no negative effects, despite including slow-velocity periods which had negative impacts in previous studies. These patterns can likely be attributed in part to the adaptability of the benthos, particularly the ability to recolonize areas quickly through passive and active drift from upstream (Mackay, 1992; Williams & Hynes, 1976). As global stressors, some degree of climate-mediated change in flow regimes and water temperatures is inevitable. Nevertheless, our findings highlight the importance of careful management or restoration practices for mitigating potential negative impacts of increasing climactic variability. River regulation has led to channelized rivers that lack mobility across the landscape and lose connectivity with floodplains, leading to a loss of wider retention zones that often contain higher water volumes, increasing vulnerability of communities to climactic events (Wohl et al., 2018). River restoration measures, such as adding physical structures such as artificial meanders, boulders or wood, can be insufficient if flow regimes are degraded and upstream refuge areas are lost (Palmer et al., 2010). If the goal is improving resilience to climate change, retaining or restoring natural flow regimes that maintain stream morphology heterogeneity (Milner et al., 2018) and connectivity (Rivers-Moore & Dallas, 2022; Urban et al., 2006) are measures likely to yield larger benefits, by allowing recolonization by remnant populations in areas of refuge following extreme events.

Our achieved aqueous CO_2 concentrations changed considerably across the experiment, although the amount added was constant. Ambient total DIC was higher later in the experiment, but ambient CO_2 was lower despite higher alkalinity, possibly due to increased benthic primary production (Talling, 1976) or CO_2 outgassing upstream (Demars & Manson, 2013; Wallin et al., 2011). Ambient CO_2 levels in our mesocosms were low compared to the global average of ~2400 µatm for streams and rivers of orders 3–12 (Lauerwald et al., 2015), but less unusual for New Zealand streams which (based on limited existing data) do not appear to be super-saturated with CO_2 (Raymond et al., 2013). Global patterns of CO_2 concentrations in streams are unpredictable based on geographic location alone and reflect a complex combination of factors including soil respiration rates, habitat degradation, local geology, and primary productivity (Raymond et al., 2013; Stets et al., 2017). During times of high productivity, autotrophs can deplete freshwater systems of CO_2 (Talling, 1976). However, well-buffered streams with

high alkalinity can hold much higher CO_2 concentrations than the atmosphere if CO_2 inputs from soil respiration, diffusion from the atmosphere, decomposition, or weathering are high, as the shift to equilibrium is delayed (Stets et al., 2017). Moreover, high variability also exists within catchments (Johnson et al., 2008; Jones & Mulholland, 1997). The Kauru River, as a fifth-order stream, is neither a small headwater stream nor a large river. Thus, it likely receives relatively small CO_2 inputs from groundwater (as in headwater streams) or in-stream organic carbon degradation (as in higher order rivers) (Marx et al., 2017). Therefore, our achieved CO_2 concentrations (~2212 µatm on Day 14, ~981 µatm on Day 28) were possibly higher than one might expect in similar fifth-order streams through gaseous exchange with a future atmosphere of 600–1100 ppm CO_2 by 2100 under moderate-to-high emissions (IPCC, 2021b), even if the other sources mentioned above also increased in future. Our pH change of ~1.0 in CO_2-enriched channels was also ~0.5 larger than in the abovementioned study by Hargrave et al. (2009), where CO_2 in the air above stream mesocosms was doubled. Nevertheless, our achieved concentrations are by no means globally unrealistic for running waters (Lauerwald et al., 2015; Raymond et al., 2013) and could occur in New Zealand's higher order rivers or headwater streams in future. Furthermore, pH values in our CO_2-enriched channels (6.9 on Day 14, 7.4 on Day 28) were within the naturally variable pH range of the Kauru River. During a previous study in the same mesocosm setup, Bruder et al. (2017) measured a mean in-stream pH of 7.1.

Given the complexity of CO_2 dynamics in streams, it is impossible to manipulate aqueous CO_2 in field-based studies to an exact desired concentration. Nevertheless, to our knowledge, our study is the first worldwide to manipulate CO_2 in outdoor stream mesocosms in a multiple-stressor context. Thus, detecting any individual and combined CO_2 effects were important for improving knowledge in this area, and detecting such effects might not have been possible at a lower CO_2 concentration during our 7-week study. Our experiment takes a step toward broadening the understanding of indirect freshwater CO_2 effects to trophic levels beyond primary producers, as recommended by Brown et al. (2020). Effects of enriched CO_2 on the invertebrate community in our study largely contrasted with those of Hargrave et al. (2009), the only other directly comparable study, emphasizing the importance of conducting similar multiple stressor experiments in many regions of the world with varying carbonate chemistry, substratum type, and biological communities. Future research efforts would also benefit from longer durations, across multiple seasons where possible, covering a range of conservative CO_2 enrichment levels.

AUTHOR CONTRIBUTIONS

J. G. Hunn: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; visualization; writing – original draft. **J. A. Orr:** Investigation; methodology; visualization; writing – review and editing. **A.-M. Kelly:** Investigation; methodology; resources. **J. J. Piggott:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; writing – review

CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data and code that support the findings of this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.11064543.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.