

Combined effects of nutrient enrichment and inorganic sedimentation on benthic biota in an experimental stream system

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ABSTRACT

Sedimentation and nutrient loading are among the most prevalent threats to fluvial ecosystem integrity. This study employed artificial streams (*mesocosms*) to simulate individual and combined impacts of nutrient enrichment and deposited fine sediment on benthic biota. Ninety-six circular mesocosms were used in a 21-day crossed experiment that measured the impact of three substrate compositions (0, 25, and 50% fines <2 mm) and four nitrogen concentrations (17, 22, 43, and 94 $\mu\text{g L}^{-1}$ (soluble inorganic nitrogen)) on periphyton and benthic macroinvertebrate assemblages. Permutational multivariate analysis of variance (PERMANOVA) of macroinvertebrate assemblages indicated substantial shifts in structural composition, while univariate models for Lepidostomatidae and total Ephemeroptera, Plecoptera and Trichoptera revealed that nutrient and sediment subsidies related to single factors were suppressed by an additional stressor. Stressor mechanism overlap was evident at higher treatment levels, as moderate nutrient enrichment increased nutritional resources but high nitrogen concentrations lead to substrate smothering by periphyton, contributing to habitat degradation originating from inorganic sedimentation. Our study is consistent with research showing that nutrient loading and sedimentation interact to deteriorate lotic systems beyond levels attributable to either individual stressor. Management practices and pollution standards need to incorporate relationships between stressors tightly co-vary in natural settings.

Key words | agriculture, benthic macroinvertebrates, experimental streams, multiple stressors, nutrients, sediment

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INTRODUCTION

Excess nutrients and sediment are pervasive, non-point source pollutants of fluvial environments. Many studies have centered on multifactorial explanations of fluvial ecosystem decline (see review by Adams (2003)). Ecosystem degradation can result from a variety of mechanisms and individual contributions and interactions of stressors can vary considerably (Townsend *et al.* 2008; Fausch *et al.* 2010). Anthropogenic inputs are considered stressors when they exceed naturally occurring in-stream variance (Wagenhoff *et al.* 2011). Furthermore, 'multiple-stressor effect' refers to any case where several stressors are both present and active

(Townsend *et al.* 2008). Previous investigation of the relationship between nutrients and sediment in multiple-stressor contexts has raised concern about stressor interactions, suggesting that ecological consequences not predictable from knowledge of single-stressor effects alone can occur (Lemly 1982; Townsend *et al.* 2008; Wagenhoff *et al.* 2011). However, in a three-way manipulative experiment including nutrients, fine sediment and flow velocity, it was found that single-stressor effects could predict multiple-stressor responses mainly because statistical interactions between stressors were rare (Elbrecht *et al.* 2016).

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Anthropogenic increases in nutrient availability in streams and rivers can trigger rapid proliferation of algae, which would otherwise be limited by phosphorus and/or nitrogen (Biggs 2000; Dodds 2006). Densities of pollution-sensitive taxa such as Ephemeroptera, Plecoptera and Trichoptera (EPT) tend to decrease with nutrient enrichment, often across relatively short ranges in nutrient concentration (Chambers *et al.* 2012). However, many studies have shown that over broad nutrient gradients, subsidy-stress responses are common for many invertebrate taxa, including several EPT families (e.g. Niyogi *et al.* 2007; Wagenhoff *et al.* 2011, 2012). Though eutrophication is often associated with higher productivity of whole food webs, competition for habitat and oxygen can result in decreased abundance and diversity of indigenous, sensitive invertebrates and fishes (Mainstone & Parr 2002).

The implications of sediment loading can be loosely partitioned into two components: increased turbidity due to suspended particulate matter and increased sediment deposition and subsequent modification of streambed composition (Henley *et al.* 2000; Jones *et al.* 2011). For most cobble-bottomed streams in temperate biomes, sedimentation acts primarily as a stressor (Niyogi *et al.* 2007), which can affect habitat structure and availability, and water quality (Wood & Armitage 1997; Jones *et al.* 2015). It can also lead to declines in EPT (Benoy *et al.* 2012) and allow pollution-tolerant taxa, such as Oligochaeta, to represent a more substantial proportion of benthic assemblages (Jones *et al.* 2011).

Because nonpoint source stressors in streams tend to co-vary with land use gradients (Quinn & Stroud 2002), traditional field-based studies that involve modelling relationships between biological data and environmental factors are often unable to adequately describe stressor relationships. *In situ* gradients of specific stressors functioning in both single and multiple stressor conditions occur infrequently and thus experimental manipulations of real streams or artificial habitats have become vital diagnostic tools for the determination of ecological thresholds (Culp *et al.* 2000; Townsend *et al.* 2008; Wagenhoff *et al.* 2012). Our primary objective was to assess the individual and combined impacts of nutrients and sediments on benthic biota. This was accomplished by examining the response of benthic algae, benthic macroinvertebrates, and emergent

adult insects to the simultaneous manipulation of nutrient (soluble inorganic nitrogen (SIN)) concentration (four levels) and fine sediment (three levels) in replicate artificial streams, hereafter referred to as mesocosms. The ranges of nutrient and sediment levels used in this study come from field surveys of streams in agricultural regions across Canada (Benoy *et al.* 2012; Chambers *et al.* 2012).

Many multiple stressor studies define stressor relationships according to a null model of additivity, where combined effects less than or greater than the *sum* of the individual effects (i.e. analysis of variance (ANOVA) interactions) are considered antagonisms or synergisms (e.g. Townsend *et al.* 2008; Matthaei *et al.* 2010). Synergism and antagonism can also be defined based on a multiplicative model, which tests against the null hypothesis that stressors in combination elicit an effect equivalent to the *product* of the individual effects (Folt *et al.* 1999). Within ecology, a universal framework by which to describe stressor relationships does not yet exist (Piggott *et al.* 2015). In this study, we formed our central hypothesis around the comparative effects model proposed by Folt *et al.* (1999), which tests against the null hypothesis that stressors in concert will elicit an effect comparable to that of the single worst stressor. We addressed the following question: are the combined effects of these stressors stronger than that of either individual stressor? To answer this question, we tested two main hypotheses sequentially. First, we used permutational multivariate analysis of variance (PERMANOVA) to determine whether nutrients and deposited sediment were both influential to benthic invertebrate assemblage composition. Then we tested whether final abundances (i.e. survivorship) of pollution-sensitive taxa were lower in mesocosms treated with both stressors than in those given comparable doses of either single factor.

MATERIALS AND METHODS

Experimental design

The 21-day experiment was carried out from July 20 to August 10, 2011 at Environment and Climate Change Canada's (ECCC) mesocosm facility located at Agriculture and

Agri-Food Canada's Fredericton Research and Development Centre in Fredericton, New Brunswick. The facility is exposed to ambient environmental temperature, light, and meteorological conditions. Multiple years of water chemistry tests of groundwater available from the on-site well show consistently high levels of phosphorus and relatively low levels of nitrogen, with constant concentrations of approximately $80 \mu\text{g L}^{-1}$ orthophosphate (as P) and $10\text{--}20 \mu\text{g L}^{-1}$ SIN, determined as the sum of $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, and $\text{NH}_3\text{-N}$ (J. Culp, ECCC, unpublished data). Therefore, primary productivity of the mesocosms is limited by nitrogen and all nutrient treatments involved manipulation of nitrogen concentrations only. Three sediment levels (0, 25, and 50% fines (<2 mm)) were replicated within each of three 'tables' (Culp & Baird 2006) for each of four target nutrient concentrations (20, 40, 80, and $150 \mu\text{g L}^{-1}$ [SIN]; Figure 1). For each of the four nutrient levels, there were nine replicates at the 0 and 25% fine sediment treatment levels and six replicates for the 50% fine sediment treatment level. A total of 96 mesocosms were evenly distributed across 12 tables.

The flow-through mesocosms are closed to recruitment or escape of macroinvertebrates. Thus, experimental treatment effects can be examined without confounding factors such as community shifts due to immigration or emigration via drift. Furthermore, given the size of the mesocosms and the duration of experiments, sexual reproduction is inhibited except for early development of larvae from eggs/gravid females that entered the mesocosms during the initial seeding process. Therefore, the mechanisms of treatment effects on organism densities reflect survivorship and loss to emergence. High emergence rates of EPT were not anticipated

because adults in these orders tend to emerge during spring and early summer (Merritt et al. 2008).

Establishment of treatments

Individual mesocosms consisted of 10 L polyethylene, open-top cylinders. Each mesocosm was equipped with a motorized plastic stirrer, which was regulated by a DC power supply (Circuit-Test, PS-3330), allowing current velocity to be maintained at approximately 7 cm s^{-1} . Untreated groundwater was pumped to large (~700 L) polyethylene head tanks, where nitrogen (NaNO_3) was constantly added according to the desired treatment. Pulsar[®] diaphragm metering pumps were used to deliver water to reservoir tanks beneath each table, from which it was evenly distributed to each mesocosm via a manifold fed by a small centrifugal pump (March: LC-3CP-MD). The mesocosms constantly overflowed through $400 \mu\text{m}$ Nitex[®] mesh covers into a polyethylene collection tray such that water within individual mesocosms was fully exchanged every 2–3 min. Water drained back to the reservoir tank and was recirculated to the mesocosms. Excess water was constantly drained from the system to produce a 2 h hydraulic residence time at each table. Shade cloth was installed over each table to prevent excessive water temperatures during the day. The shade cloth reduced light levels by approximately 30–35%; the emergence traps caused a further reduction of about 5%, as determined using a LI-COR[®] LI 1400 data logger. Further information including photographs of the artificial stream systems can be found in Culp & Baird (2006) and Alexander et al. (2008).

Mesocosm substrate material consisted of 250 mL of very fine gravel ranging in size from 2 to 4 mm mixed with 750 mL of coarser gravel ranging in size from 4 to 30 mm. Five relatively flat, ~10 cm cobbles were placed above the gravel substrate to simulate natural armoring and provide a source of colonization and a sampling surface for algae. Sediment-treated mesocosms received fines ranging in size from 0 to 2 mm, mixed evenly into the substrate matrix; moderate and high sediment treatments contained 260 and 520 mL of fine sediment, respectively. Visual estimations of streambed surface coverage by fines <2 mm in each sediment treatment were 0, 10–20, and 40–50%. By visual inspection, substrate composition and arrangement did not

		Single Nutrient Treatment Example: $20 \mu\text{g L}^{-1}$		
		Table 1	Table 2	Table 3
Sediment	0% Fines	n = 3	n = 3	n = 3
	25% Fines	n = 3	n = 3	n = 3
	50% Fines	n = 2	n = 2	n = 2

Figure 1 | Schematic representation of a single nutrient treatment ($20 \mu\text{g L}^{-1}$ [SIN]), depicting nesting of mesocosms on a set of three tables, and sediment treatments replicated (i.e. 'n') within each table. The other nine tables were allocated to the other three nutrient treatments (i.e. three tables for each of 40, 80 and $150 \mu\text{g L}^{-1}$ [SIN]).

change over the course of the experiment and following initial deposition, the water cleared rapidly and no turbidity or motile sediment was observed. All substrate material was collected from the Nashwaak River (N 46.18609; W 66.61061 (WGS84)), sieved on site, and transported to the mesocosm facility in large coolers where it was kept moist until use. Since the organic content of the fine sediment, based on ash-free dry mass, was less than 1% (mean = 0.8%), and material was sourced from a low nutrient river, it was assumed that the nutrient content of the fines is negligible. Material was set in place and well water circulation through the mesocosm system was initiated 5 days prior to the beginning of the experiment to facilitate algal colonization. Within the temperature ranges of our mesocosms, 5 days is an adequate time to expect at least one cell division in most benthic algae (Bothwell 1988).

Benthic invertebrates were collected just prior to the beginning of the experiment from a site approximately 8 km downstream from the sediment source on the Nashwaak river using U-nets (area = 0.06 m², mesh size = 250 µm). Multiple samples were collected from a riffle, homogenized in a pail, and subdivided into four equal portions for every five U-net samples (i.e. approximately 20% extra to account for potential mortality incurred during capture and transport). Mesocosms, which are comparable in planar area (0.07 m²) to a U-net sample, were inoculated with one subsample within 5 hours of collection. Subsamples from each round of collection were allocated evenly across tables and among nutrient and sediment treatments.

On three occasions during the experiment, chemical analysis of water supplying one table per nutrient treatment confirmed that a strong nitrogen gradient was maintained,

although actual nitrogen concentrations achieved were generally lower than anticipated (Table 1). Water samples were analyzed by ECCC's National Laboratory for Environmental Testing (NLET) in Burlington, Ontario. NLET is accredited by the Canadian Association for Laboratory Accreditation (CALA) to the standard ISO/IEC 17025. Nitrogen parameters analyzed were total Kjeldahl nitrogen, ammonia, nitrate, and nitrite (all unfiltered). Total phosphorus (TP) was determined for filtered (47 mm Whatman[®] glass microfiber filter) and unfiltered samples. For TP measurements, only 5–10% of the phosphorus was particulate. Water samples were preserved on ice in a cooler and shipped on the day of collection by courier. Preservatives were not used. Water temperature, recorded every 15 minutes at each table using HOBO[®] temperature loggers, remained similar among tables (Table 1).

Biological response variables

Periphyton biomass was approximated based on chlorophyll-*a* concentration from three combined 9.3 cm² samples scraped from cobbles within each mesocosm stream. Samples were stored in distilled water and analyzed in our lab in Fredericton. Following ethanol extraction, chlorophyll-*a* of non-acidified samples was quantified using a fluorometer (as described in Culp *et al.* 2003). Additionally, species-level identification and enumeration of algae was completed for 36 mesocosms, based on two combined 9.3 cm² samples scraped from cobbles within one mesocosm per sediment treatment per table (Bio-Limno Research & Consulting Inc., Halifax, Nova Scotia). Bio-Limno also provided biomass estimates for each taxon by measuring length and breadth of each, which was used

Table 1 | Mean temperature and water chemistry parameters in nutrient treatments measured during the mesocosm experiment

Nominal nutrient treatment [SIN] (µg L ⁻¹)	Temperature °C	Measured water chemistry (µg L ⁻¹)			
		Total P	Total N	NO ₃ + NO ₂	NH ₃
20	15.1 (11–23)	85.4 (83–88)	55.7 (50–66)	5.3 (0–10)	11.7 (0–23)
40	15.1 (11–22)	85.5 (84–87)	65.3 (55–72)	17.0 (9–21)	4.7 (0–14)
80	15.3 (11–22)	85.5 (84–88)	106.7 (99–112)	39.0 (30–51)	4.3 (0–13)
150	15.2 (11–21)	85.8 (84–86)	143.0 (138–148)	94.5 (93–96)	0.0 (0–0)

[SIN] refers to soluble inorganic nitrogen determined as the sum of NO₃-N, NO₂-N, and NH₃-N. All nutrient values from unfiltered samples. Temperature was recorded constantly throughout experiment and one water chemistry sample per nutrient treatment was collected on three separate occasions.

to approximate biovolume and then converted to biomass (mg cm^{-2}).

Benthic invertebrates were collected at the end of the experiment by repeatedly swirling the contents of each stream in a large pail and pouring water and suspended organic material through 500 μm mesh sieves. Remaining sediment from five mesocosms was checked for residual invertebrates to ensure that more than 99% of macroinvertebrates were captured during the swirling process. Samples were preserved in 10% formalin for a minimum of 3 days and then transferred to 70% ethanol. Invertebrates were enumerated and identified to family or order (oligochaete and nematode worms) at 16 \times magnification with the aid of a Leica[®] M80 dissection microscope and standard keys (Wiggins 1996; Merritt *et al.* 2008) and expressed as absolute abundances (i.e. individuals per mesocosm).

Emergent adult insects were captured by the mesh covers and collected every 2 days using a Bug-Vac[®] #2 insect aspirator, a suction device that removes organisms from the headspace overlying mesocosms. Specimens were preserved immediately in ethanol and later identified to family (Wiggins 1996; Merritt *et al.* 2008). Body length (excluding eyes, antennae, and cerci) of abundant taxa was measured using a dissection microscope equipped with an ocular micrometer.

Analytical approach

Multivariate and univariate ANOVA was used to test the significance of experimental treatment effects on algae and invertebrate response variables. A split-plot design (Winer *et al.* 1991) was used because nutrient treatments were delivered to whole tables ('plots'), and sediment treatments were replicated within tables. Nutrient, sediment, and their interaction were analyzed as fixed factors and all other factors were considered random. Typical linear models for split-plot designs include an interaction between the sub-plot factor (sediment in this case) and plot as follows:

$$y = \mu + \text{Nut}_i + \text{Sed}_j + \text{Nut} \times \text{Sed}_{ij} + \text{Table}_k(\text{Nut}_i) + \text{Sed} \times \text{Table}(\text{Nut}_i)_{jk} + \varepsilon_{l(ijk)} \quad (1)$$

To increase the probability of detecting fixed effects on macroinvertebrate assemblages, the Sed \times Table(Nut) interaction term was excluded when P was greater than 0.25, following Quinn & Keough (2002), effectively pooling subplot error with residual variance:

$$y = \mu + \text{Nut}_i + \text{Sed}_j + \text{Nut} \times \text{Sed}_{ij} + \text{Table}_k(\text{Nut}_i) + \varepsilon_{l(ijk)} \quad (2)$$

The reduced model (Equation (2)) was also used for all periphyton analyses (except chlorophyll-*a*) because periphyton was only sampled from one stream per sediment treatment within each table; thus the Sed \times Table(Nut) effect could not be adequately measured. Furthermore, the effects of substrate composition on algae were not characterized because periphyton was sampled from the top of the cobbles.

To test the significance of nutrient and sediment treatments on macroinvertebrate and algae assemblage structure, we conducted PERMANOVA using the PERMANOVA+ add-on (Anderson *et al.* 2008) for the software package PRIMER version 6.0 (Primer-E, Plymouth, UK). Densities (individuals cm^{-2}) were fourth root transformed to down-weight the influence of highly abundant taxa (Thorne *et al.* 1999), and analysis was performed on Bray-Curtis dissimilarities between samples. The PERMDISP routine (Anderson 2006) in PRIMER was used prior to PERMANOVA to detect violations of the assumption of homogeneity of dispersion of samples within levels of each experimental factor (p -values derived by permuting residuals). Although our design had one less high sediment treatment per table, PERMANOVA is robust to unequal replication. A random subset of 4999 permutations was used for the generation of pseudo F distributions of the null model for each PERMANOVA and PERMDISP significance test. Due to the hierarchical nature of the experimental design, residuals were permuted under a reduced model according to Anderson (2001). Type II (conditional) sums-of-squares was selected so as not to prioritize either experimental treatment. For macroinvertebrates only, pairwise inter-centroid distances between groups of assemblages were calculated and simple main effects multiple comparison tests (Quinn & Keough 2002) for nutrient and sediment treatments were performed using the PERMANOVA+ add-on.

Following the detection of significant treatment effects on whole assemblages, densities of abundant taxa as well as selected compositional metrics were analyzed with univariate ANOVA. Macroinvertebrate metrics analyzed include densities of taxa present in at least 50% of samples; total combined density of EPT; number of EPT families (EPT Richness); and total number of taxa (Total Richness). Univariate algae metrics examined include three major taxonomic divisions: diatoms (Bacillariophyceae), green algae (Chlorophyta) and 'other' (trace amounts of cyanobacteria, Chrysophyceae, and Euglenophyceae), chlorophyll-*a* concentration, as well as the proportions of three ecological diatom 'guilds' proposed by Passy (2007) – low profile, high profile, and motile – which have been shown to respond strongly to various environmental gradients (Lange et al. 2011; Schneck et al. 2011). Since several taxa found were not included in Passy (2007) or the supplementary table associated with Passy & Larson (2011), several species were assigned to guilds based on the supplementary table associated with Lange et al. (2011). All univariate models for algae and macroinvertebrate metrics were fit with restricted maximum likelihood estimation (REML; Bolker et al. 2009) using the nlme package (Pinheiro et al. 2012) in R (version 2.14.0; R Development Core Team, Vienna, Austria). Type II ANOVA tables with Wald's Chi-Square significance tests for fixed effects were then generated for each model using the car R package (Fox & Weisberg 2011). Anderson–Darling tests of residuals were performed using the nortest R package (Gross 2012) to confirm that errors were normally distributed. Square root and fourth root transformations (adding a constant for values <1) were applied as necessary to normalize residual distributions. Simple main effects were assessed with Tukey's honestly significant difference (HSD) multiple comparisons procedure in the stats R package (R Development Core Team 2012) for all univariate metrics that significantly responded to both nutrients and sediments. The Student–Newman–Keuls (SNK) test was also conducted, using the mutoss R package (MuToss Coding Team 2012), for cases where Tukey's HSD was considered overly conservative.

For adult insects, densities, sex ratios, and total body length of abundant families were analyzed using the ANOVA procedure described previously. Sex ratios were analyzed by family as a continuous response variable

approximated by dividing the number of males + 1 by females + 1. Because mayflies exhibit strong sexual dimorphism, body length was analyzed for each sex independently.

RESULTS

Periphytic algae

Chlorophyll-*a* concentrations of periphyton showed a strong positive response to nutrient enrichment (Figure 2) with no significant response to fine sediment addition (Table 2). PERMANOVA of algal densities confirmed that nutrient enrichment was also influential to the overall structure of the periphyton community while sediment was not (Table 2). Without augmentation of background nitrogen concentrations, periphytic algal communities were dominated primarily by low profile diatoms (Figures 2 and 3); 30–75% of which were *Achnantheidium minutissimum*. Slight nutrient enrichment ($22 \mu\text{g L}^{-1}$ [SIN]) resulted in relatively even densities of green algae (Chlorophyta) and diatoms, with diatom assemblages consisting of similar proportions of low and high growth forms. High nutrient enrichment (43 and $94 \mu\text{g L}^{-1}$ [SIN]) allowed green algae to proliferate, and, in the most enriched mesocosms ($94 \mu\text{g L}^{-1}$ [SIN]), high profile taxa, predominantly *Synedra rumpens*, to dominate diatom assemblages. Green algal biomass in nutrient enriched mesocosms consisted almost entirely of filamentous taxa such as *Mougeotia* sp. and *Stigeoclonium* sp.

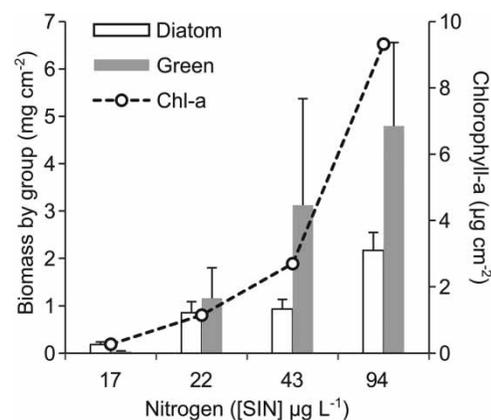


Figure 2 | Biomass (mg cm^{-2}) of diatoms and Chlorophyta (mean \pm 1 SE) in nutrient treatments ($n = 9$); total chlorophyll-*a* represented by a dashed line (other algae groups not shown).

Table 2 | PERMANOVA and permutational analysis of multivariate dispersions (PERMDISP) results evaluating the effects of nutrient enrichment (through manipulation of SIN) and fine sediment addition on Bray-Curtis dissimilarities of periphyton communities

	Transformation	Nutrient P	Sediment P	Interaction P
Whole community				
PermDISP	$^4\sqrt{\quad}$	0.970	0.751	–
PerMANOVA	$^4\sqrt{\quad}$	0.012	0.892	0.483
Biomass				
Chl-A ($\mu\text{g cm}^{-2}$)	$^4\sqrt{\quad}$	< 0.001	0.436	0.255
Greens (mg cm^{-2})	$^4\sqrt{\quad}$	< 0.001	0.938	0.771
Diatoms (mg cm^{-2})	$^4\sqrt{\quad}$	< 0.001	0.369	0.531
Others (mg cm^{-2})	$^4\sqrt{\quad}$	0.561	0.836	0.153
Ecological guilds				
% Low profile	none	0.035	0.663	0.630
% High profile	none	0.040	0.387	0.334
% Motile	none	0.152	0.872	0.799

Effects of treatments on biomass metrics and ecological guilds tested with univariate ANOVA. Significant treatment effects indicated by bold font. $n = 3$ mesocosms sampled per nutrient-sediment combination.

Trace amounts of other algal groups, including cyanobacteria, Chrysophyceae, and Euglenophyceae, were present in several mesocosms but followed no discernible pattern related to nutrient or sediment conditions. Motile taxa represented roughly 10–15% of diatoms and did not vary significantly across experimental treatments ($p = 0.152$).

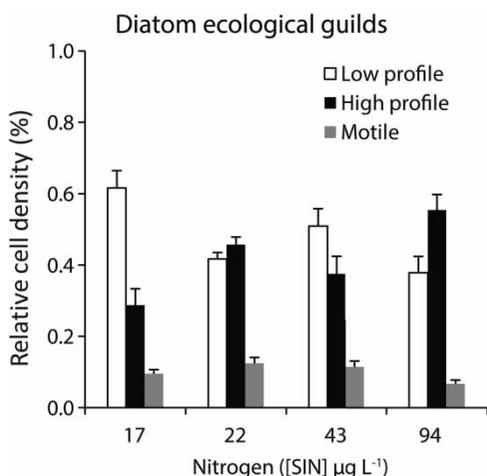


Figure 3 | Relative cell density (%) of diatom ecological guilds (mean \pm 1 SE) in nutrient treatments ($n = 9$ mesocosms samples per nutrient treatment). Algal guilds were motile, low and high profile growth forms.

Benthic invertebrate assemblages

Within-group multivariate dispersion of benthic invertebrate assemblages was consistent across levels of nutrient enrichment and sediment treatments, although dispersion varied significantly among tables (i.e. plots) (Table 3). Since the assumption of homogeneity of dispersion was satisfied for the fixed factors, the same matrix was used for PERMANOVA examining the influence of experimental treatments on benthic assemblages.

The initial PERMANOVA run using the full linear model (Equation (1)) revealed that sediment effects were consistent across tables ($F_{16,60} = 1.02$, $p = 0.43$). Therefore, the Sed \times Table(Nut) interaction term was removed from the model (Equation (2)). The PERMANOVA run with the reduced model (Table 3) revealed that table (i.e. whole-plot error) was the most influential experimental factor ($F_{8, 76} = 2.26$, $p = 0.0002$). Because of this, it is expected that the probability of failure to detect nutrient and sediment effects (β) was very high and so the critical significance level for all subsequent macroinvertebrate analyses was relaxed to $\alpha = 0.10$ (Peterman 1990), and alpha adjustments (e.g. Bonferroni) were not applied.

Because significant nutrient \times sediment interaction was detected ($F_{6, 76} = 1.46$, $p = 0.012$), multiple comparisons of simple main effects were performed (Table 4). Nutrient enrichment directly affected benthic assemblages only at 0% fine sediment, with greatest compositional contrast between 22 and 43 $\mu\text{g L}^{-1}$ (SIN) (BC distance = 18.0, $p = 0.0978$). Significant differentiation also occurred between the lowest and highest nutrient levels (distance = 1.46, $p = 0.0986$).

Overall, compositional differences between sediment levels were more common than with nutrient enrichment. Assemblages in the medium sediment treatment (25% fines) showed greatest distinction from other substrate conditions, with particularly strong contrast between 0 and 25% fines at medium (43 $\mu\text{g L}^{-1}$ (SIN)) nutrient enrichment (distance = 18.3, $p = 0.0006$).

Univariate taxonomic responses

Given the overall significance of the PERMANOVA tests, univariate responses of taxa present in at least 50% of

Table 3 | PERMANOVA and permutational analysis of multivariate dispersions (PERMDISP) on Bray–Curtis dissimilarities of benthic invertebrate assemblages in mesocosms treated with combinations of nutrient enrichment (through manipulation of SIN) and deposited fine sediment

Source	PermDISP (<i>p</i> value)	d.f.	SS	MS	F	P
Nutrient	0.2782	3	0.3663	0.1221	1.1509	0.2588
Sediment	0.6386	2	0.1409	0.0705	1.5029	0.0660
Nutrient × sediment		6	0.4096	0.0683	1.4563	0.0120
Table (nutrient)	0.0008	8	0.8488	0.1061	2.2631	0.0002
Residual		76	3.5629	0.0469		
Total		95	5.3285			

Significant treatment effects indicated by bold font.

samples were tested (Table 5). Of the 12 insect families and two classes of worms examined, five taxa responded to an interaction between nutrient enrichment and fine sediment addition. In each case, the interaction of nutrient and sediment was significant, so multiple comparisons of the simple main effects were examined. Total invertebrate richness and EPT richness did not significantly vary in response to nutrient enrichment ($Chi^2(3) = 1.482$, $p = 0.678$; $Chi^2(3) = 0.611$, $p = 0.892$) or sediment ($Chi^2(2) = 0.094$, $p = 0.962$; $Chi^2(2) = 1.422$, $p = 0.487$).

Table 4 | Pair-wise Bray–Curtis distances between centroids for simple main effects of nutrient (SIN) and sediment (% fines <2 mm) treatments on benthic invertebrate assemblages ($n = 96$)

Nutrient (within sediment)				
Contrasts	0% fines	25% fines	50% fines	
17 versus 94 $\mu\text{g L}^{-1}$	15.9*	13.1	15.0	
17 versus 43 $\mu\text{g L}^{-1}$	15.7	15.4	13.9	
22 versus 94 $\mu\text{g L}^{-1}$	10.2	14.1	18.6	
17 versus 22 $\mu\text{g L}^{-1}$	13.1	12.4	18.6	
22 versus 43 $\mu\text{g L}^{-1}$	18.0*	13.5	16.3	
43 versus 94 $\mu\text{g L}^{-1}$	16.0	7.6	16.2	
Sediment (within nutrient)				
Contrasts	17 $\mu\text{g L}^{-1}$	22 $\mu\text{g L}^{-1}$	43 $\mu\text{g L}^{-1}$	94 $\mu\text{g L}^{-1}$
0 versus 50% fines	12.3	13.7*	13.3	11.9
0 versus 25% fines	13.3*	8.4	18.3***	8.9
25 versus 50% fines	12.7	16.3*	16.6*	9.8

Significant multivariate distances differentiated with non-parametric t-tests represented by bold font and asterisks.

* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$.

In treatments without fine sediment addition, densities of Chironomidae followed an erratic pattern over the nutrient gradient (Figure 4(a)), increasing substantially with minor nutrient enrichment, then declining again at 43 $\mu\text{g L}^{-1}$ [SIN] only to increase once more at 94 $\mu\text{g L}^{-1}$ [SIN]. In contrast, with the addition of fine sediment the range of extremes in the nutrient trends were reduced, resulting in an overall increase in insect density (survivorship) with slight enrichment, but no change in density beyond 22 $\mu\text{g L}^{-1}$ [SIN].

In the treatment with a low amount of fine sediment, the nutrient effect detected for Oligochaete worms was similar to the Chironomidae pattern; the highest densities were observed with slight (22 $\mu\text{g L}^{-1}$ [SIN]) and high (94 $\mu\text{g L}^{-1}$ [SIN]) enrichment (Figure 4(b)). Oligochaeta generally increased with fine sediment addition, and the most significant differentiation of sediment treatments occurred at low (17 $\mu\text{g L}^{-1}$ [SIN]) and at medium nutrient enrichment (43 $\mu\text{g L}^{-1}$ [SIN]). Nematode worms also showed a complex multiple-stressor response, with highest densities observed at 43 $\mu\text{g L}^{-1}$ [SIN] with moderate sedimentation (25% fines; figure not included).

Caddisflies of the family Lepidostomatidae generally responded positively to nutrient enrichment and fine sediment addition; although densities were lower in streams with high concentrations of both stressors (Figure 4(c)). The effects of experimental treatments on total EPT density were largely related to trends in Lepidostomatidae – the most abundant family of EPT in our mesocosms. EPT responded positively to nutrient enrichment and (intermediate) sediments as independent drivers; however these effects were lost or reversed with the addition of a second stressor (Figure 4(d)).

Table 5 | Univariate responses of benthic invertebrates to nutrient (SIN, as N) and sediment (% fines <2 mm) treatments, with significant effects in bold ($\alpha = 0.10$; $n = 96$)

	Transformation	Nutrient <i>P</i>	Sediment <i>P</i>	Interaction <i>P</i>
Invertebrate density				
Chironomidae	⁴ √	0.102	0.595	0.017
Oligochaeta	⁴ √	0.036	0.025	0.017
Ephemerellidae	⁴ √	0.359	0.485	0.881
Lepidostomatidae	⁴ √	0.752	0.399	0.016
Heptageniidae	⁴ √	0.672	0.893	0.555
Elmidae	⁴ √	0.686	0.978	0.251
Hydropsychidae	⁴ √	0.935	0.181	0.611
Simuliidae	⁴ √	0.993	0.107	0.078
Leptophlebiidae	⁴ √	0.046	0.944	0.950
Leptohephidae	⁴ √	0.496	0.400	0.587
Baetidae	⁴ √	0.829	0.161	0.495
Nematoda	⁴ √	0.101	0.737	0.002
Psephenidae	⁴ √	0.802	0.465	0.224
Athericidae	⁴ √	0.154	0.511	0.336
Community metrics				
EPT	⁴ √	0.850	0.388	0.056
EPT richness	none	0.892	0.487	0.927
Total richness	none	0.678	0.962	0.163

P values derived from Wald's Chi-Square significance tests for Type II ANOVA models fit with REML.

Adult insect emergence

Chironomidae represented 87% of all emergents, while six families of Ephemeroptera accounted for 13% of captured adults. Twenty-two Trichoptera (from seven families) and nine Plecoptera (all Perlidae) also emerged. Corresponding to between-table variance in benthic assemblages, emergence of most insects varied substantially across tables, a result that likely masked some effects of nutrient enrichment and sediment (see Methods section). Nonetheless, densities of Chironomidae were found to follow a marginally-significant (Chi^2 (2) = 5.166, $p = 0.082$) unimodal pattern over the sediment gradient; with highest emergence at 25% fines (Table 6). Adult mayflies of the family Leptophlebiidae increased slightly with 25% fine sediment addition (Chi^2 (2) = 5.340, $p = 0.076$) but showed no further increase with higher sediment. Overall, minimal treatment effects on adult insect densities confirmed that negative responses observed in larval organisms were not due to emergence.

Experimental treatments were not found to significantly alter sex ratios of prominent mayfly families. Both nutrient enrichment (Chi^2 (3) = 15.46, $p = 0.001$) and sediment (Chi^2 (2) = 4.914, $p = 0.086$) positively influenced body length of male Baetidae, which ranged from 3.3 to 4.9 mm. The largest Baetidae males were found in high nutrient enrichment (94 $\mu\text{g L}^{-1}$ [SIN]), high fine sediment (50% fines) combinations (Figure 5).

DISCUSSION

This mesocosm experiment showed that nutrient enrichment and sedimentation in combination resulted in a non-additive shift in macroinvertebrate assemblage composition. This supports our first hypothesis that both stressors were influential to final assemblage composition. The significant interaction term also suggests that prediction of multiple stressor impacts on invertebrate assemblages requires the

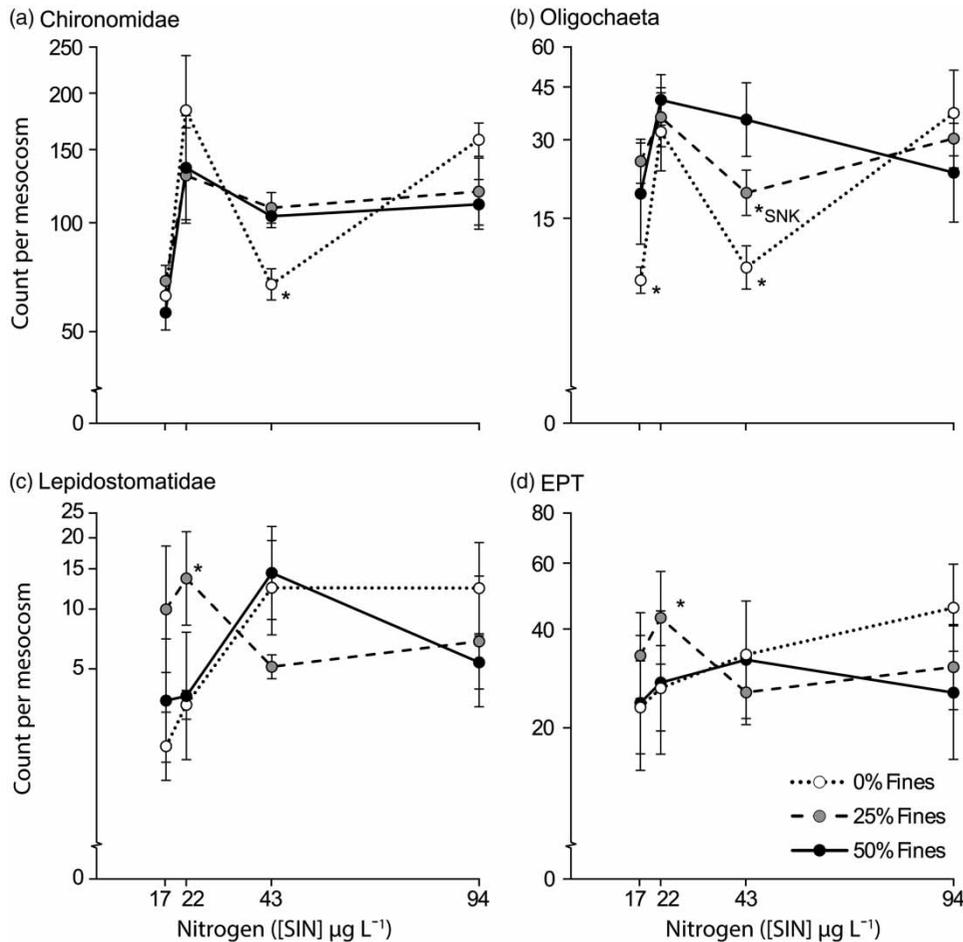


Figure 4 | Mean densities (± 1 SE) of selected macroinvertebrates and total EPT in response to nutrient and sediment treatment combinations. Densities plotted on 4th root transformed scale. Sediment treatments differentiated by Tukey's HSD or SNK indicated by asterisks ($p < 0.10$). Total $n = 96$ mesocosms.

incorporation of complex stressor relationships, meaning that simple additive cumulative effects should not be assumed. For our second hypothesis, increases in survivorship observed for nutrient enrichment and fine sediment addition were essentially nullified when stressors were combined.

Nutrient effects on whole macroinvertebrate assemblages were more prominent when fine sediment content was low. Significant differences were found between the lowest and highest nitrogen concentrations, likely corresponding to the shift in periphyton composition from diatoms to less palatable green algae. The threshold at which green algae abundance surpassed diatoms occurred between 22 and 43 $\mu\text{g L}^{-1}$ [SIN], which was consistent with the greatest change in macroinvertebrate assemblages.

Substrate composition had the greatest effect on macroinvertebrate assemblages within the 43 $\mu\text{g L}^{-1}$ [SIN] nutrient treatment. Sediment effects on algae were not observed because periphyton was collected from the upper surface of cobbles and was therefore not in direct contact with the added fines. Furthermore, turbulence and velocity were not great enough in the mesocosms for resuspension of fine sediment. The observed shift in periphyton with nutrient enrichment, from low-profile diatom dominated communities to thick stands of green algae and high-profile diatoms, is generally consistent with other stream studies (Lowe *et al.* 1986; Passy 2007; Lange *et al.* 2011). The strong overall dominance of chlorophytes relative to diatoms in enriched streams was not found by some other studies that have observed persistent dominance of diatoms (Stelzer &

Table 6 | Adult insect emergence in response to nutrient and sediment treatments, with significant effects in bold and direction of single factor responses in parentheses

	Transformation	Nutrient <i>P</i>		Sediment <i>P</i>	Interaction <i>P</i>
Count					
Chironomidae	$^4\sqrt{\quad}$	0.331		0.082	(+/-)
All Mayflies	$^4\sqrt{\quad}$	0.614		0.258	0.635
Ephemerellidae	none	0.192		0.814	0.622
Baetidae	none	0.483		0.697	0.730
Leptophlebiidae	none	0.657		0.076	(+)
Sex ratio					
Ephemerellidae	$\sqrt{\quad}$	0.107		0.290	0.571
Baetidae	$\sqrt{\quad}$	0.492		0.740	0.110
Leptophlebiidae	$\sqrt{\quad}$	0.633		0.280	0.524
Body length					
Ephemerellidae Female	$\sqrt{\quad}$	0.948		0.676	0.756
Ephemerellidae Male	$\sqrt{\quad}$	0.747		0.857	0.173
Baetidae Female	none	0.691		0.251	0.135
Baetidae Male	none	0.001	(+)	0.086	(+)
Leptophlebiidae Female	$\sqrt{\quad}$	0.804		0.767	0.827
Leptophlebiidae Male	none	0.751		0.167	0.236

P values derived from Wald's Chi-Square significance tests for Type II ANOVA models fit with REML.

Lamberti 2001; Flecker *et al.* 2002), although chlorophytes dominated across enrichment gradients in a survey conducted by Chetelat *et al.* (1999).

Univariate responses of Chironomidae and Oligochaeta also showed the strongest sediment treatment differentiation

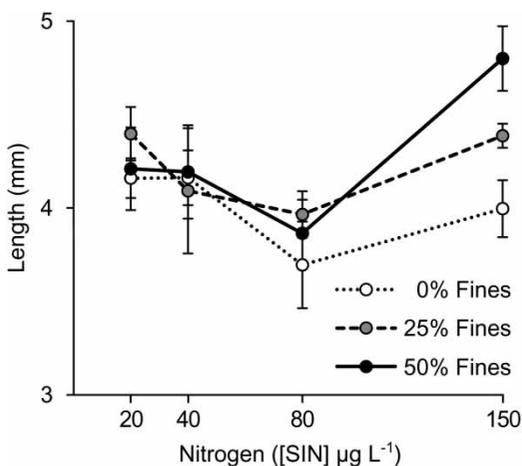


Figure 5 | Body lengths of male Baetidae (mean ± 1 SE) in nutrient and sediment treatments.

within the $43 \mu\text{g L}^{-1}$ [SIN] nutrient treatment. Oligochaete worms appeared to benefit from finer substrata, possibly allowing them to capitalize on increased food availability along the entire enrichment gradient. Chironomidae larvae, which tend to prefer fine substrate in which to burrow, seemed to benefit from sediment addition, though nutrient enrichment was primarily responsible for variation in density. These responses are generally consistent with other studies (e.g. Townsend *et al.* 2008; Jones *et al.* 2011; Wagenhoff *et al.* 2011; Chase *et al.* 2016). Chironomidae, and to a lesser extent Oligochaeta, increased most with only slight nutrient enrichment, as densities generally levelled off or decreased beyond $22 \mu\text{g L}^{-1}$ [SIN]. It is possible that for these two classes of burrowing detritivores, slight enrichment equated to nutritional resource subsidy but habitat alteration occurred at higher nitrogen concentrations, as substrate smothering by periphyton became the overriding mechanism of nutrient influence. Substrate smothering by periphyton in high nutrient conditions is certainly well documented (Biggs 1996), and may largely explain the negative portion of the subsidy-stress response

of Chironomidae to nutrients in the experiment by Wagenhoff *et al.* (2012). Densities of predators, including Athericidae, Chloroperlidae, Dytiscidae, Empididae, Gomphidae, Perlidae, and Perlodidae, were actually lowest in the $43 \mu\text{g L}^{-1}$ [SIN] treatment, though the overall nutrient influence was not significant (one-way nested ANOVA: $F_{3,8} = 2.31$, $p = 0.152$). Thus, we suggest that nutritional subsidy and habitat modification is a stronger explanation for trends observed in Chironomidae and Oligochaetae than the influence of predators.

Motile crawling insect larvae of the EPT orders, especially the family Lepidostomatidae, appeared to take greater advantage of increased nutritional resources, and were only negatively influenced by nutrient enrichment (presumably related to substrate smothering by algae) when habitat was also modified by sedimentation. EPT and Lepidostomatidae in moderate and high sediment mesocosms may have exhibited a subsidy-stress response over the nutrient gradient, as has been shown in other studies (e.g. Wagenhoff *et al.* 2011, 2012). Interestingly, moderate (25%) fine sediment addition alone resulted in higher Lepidostomatidae survivorship, which may reflect their limited ability to construct cases in environments lacking fine sediment (Statzner 2011; Tszedel *et al.* 2006), but also possibly habitat structure in the mesocosms and resource availability. Most Lepidostomatidae were small (<5 mm) and housed in cylindrical cases constructed with particles smaller than 2 mm. Moreover, the fact that similarly high densities were found in the treatment with higher nutrients and 0% fine sediment suggests that Lepidostomatidae may have also been limited by the availability of case materials (Robson & Barmuta 1998). High nutrient enrichment caused a marked increase in benthic algal biomass, which could potentially infill interstitial spaces to produce habitat conditions not unlike those available with moderate sediment addition. Therefore, the comparatively lower Lepidostomatidae densities found in high (50%) fine sediment conditions and high nutrient with sediment added imply that optimum habitat conditions are exceeded beyond a sediment threshold (>25% fines) or when both factors coincide.

In general, sediment generated a mix of positive and negative invertebrate responses even among sensitive taxa. We suspect that the dominantly negative effects observed

in field surveys are principally related to suspended particulate rather than the subsequent habitat modification (Niyogi *et al.* 2007; Jones *et al.* 2011; Wagenhoff *et al.* 2011). Also, the tendency for increased invertebrate drift in response to sedimentation has been well documented (Culp *et al.* 1986; Suren & Jowett 2001), and since drift was impeded in our experiment local sediment-induced depletion of invertebrate populations could not occur. However, this inconsistency between *in situ* and *in vitro* phenomena was partially moderated by the fact that recruitment was also absent; catastrophic drift in reaction to sediment deposition (Culp *et al.* 1986) at actual stream sites would generally be compensated by upstream recruitment (Bournaud *et al.* 1987). Wagenhoff *et al.* (2012) examined macroinvertebrate responses to nutrients and fine sediment in a mesocosm experiment similar to ours, except that their system allowed for some drift and recruitment, and sediment was added following an initial seeding of streams with invertebrates. In contrast to our experiment, invertebrates were directly exposed to suspended material. A strong negative response (in EPT for example) to sediment deposition was observed, which they attributed to drift. Therefore, we propose that local declines in invertebrate populations following sedimentation may be driven primarily by the initial process of particle suspension (turbidity) and deposition, rather than the change in habitat.

CONCLUSIONS

Our research suggests that at higher nutrient concentrations, stressor mechanism overlap occurs, as primary production contributes to the effect of deposited sediment by infilling and smothering hyporheic habitat. Gayraud & Philippe (2001) reported that invertebrate densities were positively related to substrate interstitial space but negatively impacted by increasing organic content in substrates, lending support to the argument that organic material, like fine sediment, can impede the ability of crawling macroinvertebrates to penetrate hyporheic habitat. Pollution-tolerant burrowers are well adapted for homogenous substrates, so unlike larger crawling insects, they respond positively to nutrient-sediment combinations. Wagenhoff *et al.* (2011) also observed such a dichotomy of invertebrate responses to

nutrient and sediment combinations in New Zealand streams, where, for example, %EPT was lowest when stressors coincided, while drivers interacted to bolster *Oligochaete* populations.

In our experiment, nutrient enrichment and fine sediment addition led to increased organism survivorship in several cases. However, positive effects were generally suppressed when both factors were present. In a natural setting these pollutants may interact to cause the loss of organisms and substantial shifts in community structure and function. Multivariate patterns in benthic invertebrates also suggest that assemblages impacted by both stressors are compositionally distinct from those affected by either single factor.

Short-term experiments are not meant to fully characterize the ecological impact of environmental perturbations like sedimentation and nutrient loading, but rather to help in our understanding of how stressors affect natural systems. Through the use of stream mesocosms, this experiment has revealed a novel insight into stressor mechanisms that would be difficult to observe in the field. Finally, this and other studies have shown that the multiple stressor response of biotic communities to additions of nutrients and sediment is highly complex and not easily predicted. It is therefore pertinent that nutrient loading and inorganic sedimentation be considered interconnected ecological drivers and be jointly managed. Agricultural best management practices that effectively reduce both nutrient and sediment runoff should be widely incorporated into watershed management strategies in agricultural regions.

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