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Zooplankton community turnover in response to a novel TiO₂-coated nano-formulation of carbendazim and its constituents☆

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ABSTRACT

Novel nanomaterial-based pesticide formulations are increasingly perceived as promising aids in the transition to more efficient agricultural production systems. The current understanding of potential unintended (eco)toxicological impacts of nano-formulated pesticides is scarce, in particular with regard to (non-target) aquatic organisms and ecosystems. The present study reports the results of a long-term freshwater mesocosm experiment which assessed responses of individual zooplankton taxa and communities to a novel TiO₂-coated nano-formulation of the fungicide carbendazim. Population- and community trends were assessed and compared in response to the nano-formulation and its constituents applied individually (i.e. nano-sized TiO₂, carbendazim) and in combination (i.e. nano-sized TiO₂ & carbendazim). Minimal differences were observed between effects induced by the nano-formulation and its active ingredient (i.e. carbendazim) when applied at equivalent nominal test concentrations (4 µg L^{-1}). Nano-sized TiO₂ was found to affect zooplankton community trends when applied separately at environmentally realistic concentrations (20 µg L^{-1} nominal test concentration). However, when nano-sized TiO₂ was applied in combination with carbendazim, nano-sized TiO₂ was found not to alter effects on community trends induced by carbendazim. The findings of the current study provide an extensive and timely addition to the current body of work available on non-target impacts of nano-formulated pesticides.

1. Introduction

Various international policy agendas have recently deemed the rates at which pesticides are applied in conventional agriculture as environmentally and economically unsustainable (EC, 2022; EC, 2020; FAO, 2019; FAO, 2018; United Nations, 2015). A prominent reason for this stems from the inefficiency of current application methods, during which according to various estimates generally less than 25% of applied pesticides reach their target crop, and less than 1% reach their target pest (Zhang, 2018; Pimentel & Burgess, 2012). This necessitates that application volumes and frequencies are increased in order to achieve effective doses at target sites and simultaneously results in large amounts of pesticides being translocated to non-target sites and organisms. Studies and reports over the past years have compiled extensive evidence of links between pesticide use and local- (Brühl et al., 2021; Beketov et al., 2013) and global (Dudley et al., 2017; Bijleveld van

Lexmond et al., 2015) biodiversity decline, as well as adverse impacts on human health (Jepson et al., 2020; Whitmee et al., 2015; Tilman et al., 2002).

Innovations and strategies aiming to sustainably enhance agricultural yields are currently developing at a rapid pace. In this regard, nanomaterial-based pesticides (i.e. nano-pesticides) have received widespread interest due to their perceived potential as an effective and more environmental-friendly means towards crop protection (Zhang and Goss, 2022; Wang et al., 2022; Kah et al., 2021; Hofmann et al., 2020; Kah et al., 2019). Nano-pesticides are generally distinguished into two major types, in which the nanomaterial either acts as the active ingredient (i.e. pesticide) or in which an (existing) active ingredient is embedded in a nanomaterial-based matrix (i.e. a nano-formulation) (Wang et al., 2022). In case of the latter, the nano-formulation generally functions as a means to reduce premature loss of the active ingredient (i.e. via improving its adsorption to the target site and reducing its

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rates of evapotranspiration and degradation), and to control its release and bioavailability over space and time (Fraceto et al., 2020). As a result of these functionalities, nano-formulations could potentially lower required application volumes and frequencies of pesticides and simultaneously mitigate runoff rates to adjacent soils and surface waters.

Evidently, nano-formulated pesticides exhibit pesticidal activity, and as such, their potential widescale employment will require a cost-benefit analysis which takes into account their environmental risks (Su et al., 2022). Of particular interest to this end is the extent to which nano-formulations may enhance or decrease non-target toxicity relative to their (conventionally formulated) active ingredients, e.g. via direct effects of the nanomaterial matrix or via indirect effects resulting from altered release kinetics and bioavailability. Recently, several reviews have indicated that in line with intended functionalities, the majority of experimental studies on nano-formulated pesticides reported a trend of increased pesticidal activity, reduced non-target toxicity, and reduced runoff to soils and surface waters (Su et al., 2022; Wang et al., 2022; Zhang & Goss, 2022). The currently available literature in this regard is however highly skewed towards efficacy to target organisms, and when available, non-target toxicity data furthermore has been found to lack representativity for aquatic organisms (Wang et al., 2022; Zhang & Goss, 2022). Although nano-formulations show potential to reduce runoff rates of pesticides to surface waters, they are unlikely to facilitate a comprehensive nullification to this end (Wang et al., 2022). As such, the extent to which nano-formulations differ from conventional formulations in terms of non-target toxicity to aquatic organisms remains of relevance in order to weigh their potential environmental and economic benefits.

Higher-tier effect assessments via the use of simulated ecosystems (i. e. micro- and mesocosms) are well established methods in ecotoxicological studies of pesticides, and their application in evaluations of environmental effects of nanomaterial emissions has steadily increased over the past years (Nassar et al., 2021). In contrast to lower-tier ecotoxicological tests, micro- and mesocosm experiments allow for studying endpoints which emerge from processes beyond the level of individuals of single species (i.e. direct- and indirect effects on community structure and functioning), and furthermore constitute more environmentally realistic representations of exposure scenarios (e.g. due to fluctuating food availability and abiotic conditions) (Surette et al., 2021). To date, few studies have evaluated non-target impacts of nano-formulated pesticides in micro- or mesocosm setups, and as stated by Zhang & Goss (2022), this has resulted in a lack of understanding of their potential adverse environmental impacts at higher levels of ecological organization (Zhang & Goss, 2022).

The current study aimed to address the gap in non-target- and higher-tier aquatic toxicity data of nano-formulated pesticides by means of a long-term mesocosm experiment. Natural zooplankton communities were exposed to a novel TiO2-coated nano-formulation of the fungicide carbendazim, as well as to its individual- (i.e. nano-sized TiO2, carbendazim) and combined constituents (i.e. nano-sized TiO2 & carbendazim). Zooplankton community turnover was subsequently assessed after 7- and 28 days of exposure according to a series of complementary analyses, and patterns of community turnover were compared between treatments. In doing so, the current study ultimately addressed the question to what extent the TiO2-coated nano-formulation mitigated or altered effects of carbendazim on individual zooplankton taxa and zooplankton communities.

2. Methods

2.1. Experimental setup

The experiment took place at the outdoor ecological research facility the 'Living Lab', located in Oegstgeest-Leiden, the Netherlands (see http://mesocosm.org/ for an extensive description of the experimental site). The Living Lab consists of a series of replicated freshwater mesocosm test systems which are designed to mimic agricultural ditches (used length-width-depth: 5–0.8-0.3 m; volume: 1200 L). Prior to initiation of the experiment, ecological communities consisting of microbes, plants, phyto- & zooplankton, and macroinvertebrates were naturally established in each of the 35 ditches over a period of 3 months (January 2019–March 2020) via connections to land, air, and a pond adjacent to the experimental site. Each ditch was subsequently isolated from the adjacent pond using acrylic barriers in order to prevent exchange of organisms, treatments and water during the experimental period, and communities were allowed to settle for an additional month. Physicochemical water quality parameters (i.e. temperature, pH, dissolved oxygen conductivity, NH_4^+ and NO_3^-, chlorophyll A & turbidity) were measured on a weekly basis, starting one week prior to treatment application, using a Hach HQ40d multimeter (Hach Ltd., Colorado, USA), a Vernier LabQuest 3 multimeter (Vernier Software & Technology, Oregon, USA) and a AquaFluor® handheld fluorometer (Turner Designs, Inc., San Jose, USA).

2.2. Treatments

Treatments consisted of a custom synthesized nTiO2-coated controlled-release product of carbendazim (nTiO2-coated carbendazim) and its constituents (i.e. nTiO2 & non-coated carbendazim), which were applied separately and in combination in the experimental design. nTiO2-coated carbendazim was produced using pulsed chemical vapor deposition (pCVD), a technique which has shown promising applications in the development of controlled-release formulations of pharmaceuticals, where similar functionalities are of interest (Zhang et al., 2019; Guo et al., 2017). pCVD was performed in a fluidized bed reactor (Valdesueiro et al., 2015), during which a nano-scale TiO2 layer of amorphous crystalline structure was deposited on the surface of dry carbendazim powder (CAS no. 10605-21-7, 97% purity, Sigma Aldrich, Missouri, USA), effectively resulting in a diffusion layer via which release of the active ingredient (i.e. carbendazim) to the surrounding environment was retarded. The obtained nTiO2-coated carbendazim product showed polydispersity with particle sizes ranging between 100 and 500 μm, as reported in Nederstigt et al. (2022a). Dry carbendazim powder from the same batch as was used in the preparation of the nTiO2-coated carbendazim product was used to establish treatments of non-coated carbendazim. nTiO2 (JRCNM01005a, European Commission – DG JRC, also provided by Degussa/Evonik as AEROXIDE P25®) was obtained from the European Commission's Joint Research Centre (JRC) and consisted of a mixture of ~85% anatase: 15% rutile crystalline structures with a reported particle size of 15–24 nm (JRC, 2014). Water column concentrations of carbendazim and nTiO2 were quantified over the course of the experiment from depth-integrated samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and inductively coupled plasma mass spectrometry (ICP-MS) respectively. Comprehensive descriptions of the applied methodology to this end, as well as to the synthesis of nTiO2-coated carbendazim and the characterization of all applied stressors (including transmission electron microscopy imaging and dynamic light scattering analyses) have previously been reported and discussed in Nederstigt et al. (2022a).

Treatment concentrations for all applied stressors resembled concentrations representative of those currently measured in freshwater ecosystems in various parts of the world. For nTiO2, this constituted a nominal treatment concentration of 20 μg L^{-1} (measured concentrations of anthropogenic TiO2 in European and North-American surface waters range from 0.2 to 450 μg L^{-1}; Peters et al., 2018 & Nabi et al., 2022, respectively). For carbendazim, a nominal treatment concentration of 4 μg L^{-1} was applied, with the aim of achieving an actual time-weighted-average (TWA) concentration of 0.6 μg L^{-1} over the duration of the experiment. The latter concentration resembles current maximum permissible annual average surface water concentrations as set for the Netherlands (BKMW, 2009) and measured surface water concentrations globally (Alejandro et al., 2022; Wan et al., 2021; Merel

et al., 2018). nTiO$_2$-coated carbendazim was applied at a nominal treatment concentration of 4 µg L^{-1} (measured as the concentration of carbendazim), equal to the nominal treatment concentrations of non-coated carbendazim.

Treatments were applied in a single application in parallel to controls over 35 ditches (i.e. n = 7) according to a systematic block design in early May 2020. nTiO$_2$ stock suspensions were prepared in 1 L of Milli-Q water (Milipore Milli-Q reference A+ system, Waters-Millipore Corporation, Milford, MA, USA) and bath-sonicated for 10 min at a calculated energy output of 27 ± 0.2 Watt s^{-1} (Sonicor SC-50-22, Sonicor INC. NY, USA). To ensure a homogenous distribution of stressors throughout the water column, stock suspensions of nTiO$_2$ were diluted in 10 L demineralized water and applied slowly and evenly across the water surface of each ditch via perforated bottle caps. Carbendazim and nTiO$_2$-coated carbendazim stock solutions and suspensions were immediately prepared in 10 L of demineralized water and applied as described for nTiO$_2$.

2.3. Zooplankton community sampling

Zooplankton was sampled one week prior-, and one- and four weeks post treatment application. Depth integrated samples were collected from 5 locations distributed over the length of each ditch using a PVC tube (100 mm diameter) with a rubber stopper, adding up to a total sample volume of 10 L. Samples were concentrated over a zooplankton collection net fitted with a filter with a mesh size of 53 µm (EFE & GB Nets, Cornwall UK). Concentrated samples were preserved and stained in a 1% Lugol (Sigma Aldrich, Missouri USA) solution and stored at 4 °C until processing. Identification and counting of collected specimens was carried out by distributing a total volume of 2 mL per homogenized sample on microscopy slides, which were analyzed using regular light microscopy. Zooplankton specimens were identified to the lowest possible clade (using Floßner, 2000; Notenboom-Ram, 1989 and in in-house identification guide) and counted, and abundances were recalculated to be representative of 1 L of water in the experimental setup.

2.4. Data analysis

Homogeneity between established zooplankton communities was analyzed based on samples collected preparatory to treatment applications (see section 2.5.1). Taxon accumulation curves (function 'specaccum', R package 'vegan') were generated for each sampling moment to determine whether collected zooplankton samples were adequately representative of the community present in the experimental setup (Oksanen et al., 2020). Differences in physicochemical water quality parameters between treatments and controls over the course of the experiment were assessed via linear mixed effect models (including ditch number as a random variable to account for repeated measures) and pairwise comparisons per timepoint moment were conducted based on estimated marginal means. Data was transformed accordingly when model assumptions (i.e. normality of residual distributions and homogeneity of variance) were not met, and *p*-values were Benjamini-Hochberg-corrected to account for multiple testing.

Zooplankton community turnover was assessed according to a series of complementary analyses focusing on (1) community (dis)similarity at individual sampling moments (i.e. non-temporal β-diversity indices) (2) rates of community turnover (i.e. temporal β-diversity indices), and (3) the relative contributions of more rare- and common taxa to community turnover (i.e. ζ-diversity profiles). With exception of analyses of ζ-diversity profiles (as substantiated in section 2.5.3), all analyses were performed on datasets which were filtered to reduce the contribution of potentially under-sampled taxa by excluding taxa with fewer than 3 occurrences amongst contrasts of interest. Data analysis was performed using R version 4.2.2 (R Core Team, 2022) and outcomes of statistical tests were considered statistically significant at P < 0.05.

2.4.1. β-diversity indices (non-temporal)

β-diversity indices allow to calculate a case-specific coefficient of (dis)similarity between two assemblages of taxa (i.e. communities) on the basis of their matching and mismatching components (i.e. taxa). Coefficients obtained from calculations of β-diversity indices are commonly inversed to denote dissimilarity scaled between 0 and 1, constituting minimum- and maximum dissimilarity respectively. A multitude of β-diversity indices have been proposed and developed over the years (see e.g. Legendre & De Cáceres, 2013 for a review on the properties of commonly used β-diversity indices in ecology), all of which can primarily be distinguished into being based either on incidence (i.e. presence-absence) or on integrated measures of abundance and incidence (e.g. counts, biomass, etc., hereafter referred to as abundance-based indices). Calculation and comparison of both abundance- and incidence-based β-diversity indices provides insight into the extent to which dissimilarity between communities of interest can be attributed to differences in taxonomic composition (i.e. via incidence-based indices) and/or differences in abundances of taxa (i.e. via abundance-based indices).

In the current study, we calculated incidence- and abundance-based dissimilarity between zooplankton communities using the Sørensen- and Bray-Curtis index respectively. Statistical analyses of dissimilarity between treatment and control communities in terms of β-diversity indices consisted of permutational analyses of variance (PERMANOVAs, function 'adonis2', R package 'vegan'), and differences in homogeneity of multivariate dispersions (i.e. β-dispersion) were determined through permutation-based tests as well (function 'permutest', R package 'vegan'). Permutations (max. 999) were restricted within the applied systematic block design to control for the potential presence of a natural gradient across the spatial distribution of the experimental setup and *p*-values were Benjamini-Hochberg-corrected to account for multiple testing. The relative contribution of individual taxa to overall observed dissimilarity between communities was determined by extracting and ranking PERMANOVA model coefficients for each sampling moment. Population trends of the 5 taxa which were found to contribute most to observed dissimilarity between treatments and controls were compared using generalized linear models fitted with negative binomial distributions (function 'manyglm', R package 'mvabund') followed by likelihood ratio tests (corrected for multiple testing via a step-down resampling procedure with 999 iterations).

Count data are often transformed prior to calculation of abundance-based β-diversity indices to balance the contributions of more- and less abundant taxa to calculated dissimilarity coefficients (Tebby et al., 2017). All analyses of abundance-based β-diversity indices in the current study were performed on raw, $\log_{10}(x+1)$ and square-root transformed count data. As differences between analyses of raw and transformed data were found to be minimal, the analyses presented throughout the current study are based on raw count data (results based on transformed data are provided in the appendix).

2.4.2. Temporal β-diversity indices

Temporal β-diversity indices (TBIs) were calculated to better understand how treatments affected rates of community turnover over the experimental timeframe. In contrast to β-diversity indices calculated between treatments at individual sampling moments, TBIs are calculated on the basis of rates of change in community composition in each experimental (or more generally, observational-) unit over time (Legendre, 2019). An important advantage that follows from this is that TBIs allow for the partitioning of temporal community change (i.e. community turnover) into its fundamental components, i.e. the losses and gains of (individuals of) taxa over time. To this end, Legendre (2019) proposed a TBI in which losses per taxon (B_j) are calculated as the fraction of the abundance (or incidence) of a taxon which is higher at the first-than at the second timepoint under consideration, and gains per taxon (C_j) are calculated vice versa. The total unscaled sums of losses (B) and gains (C) across the community are subsequently obtained by summing the values
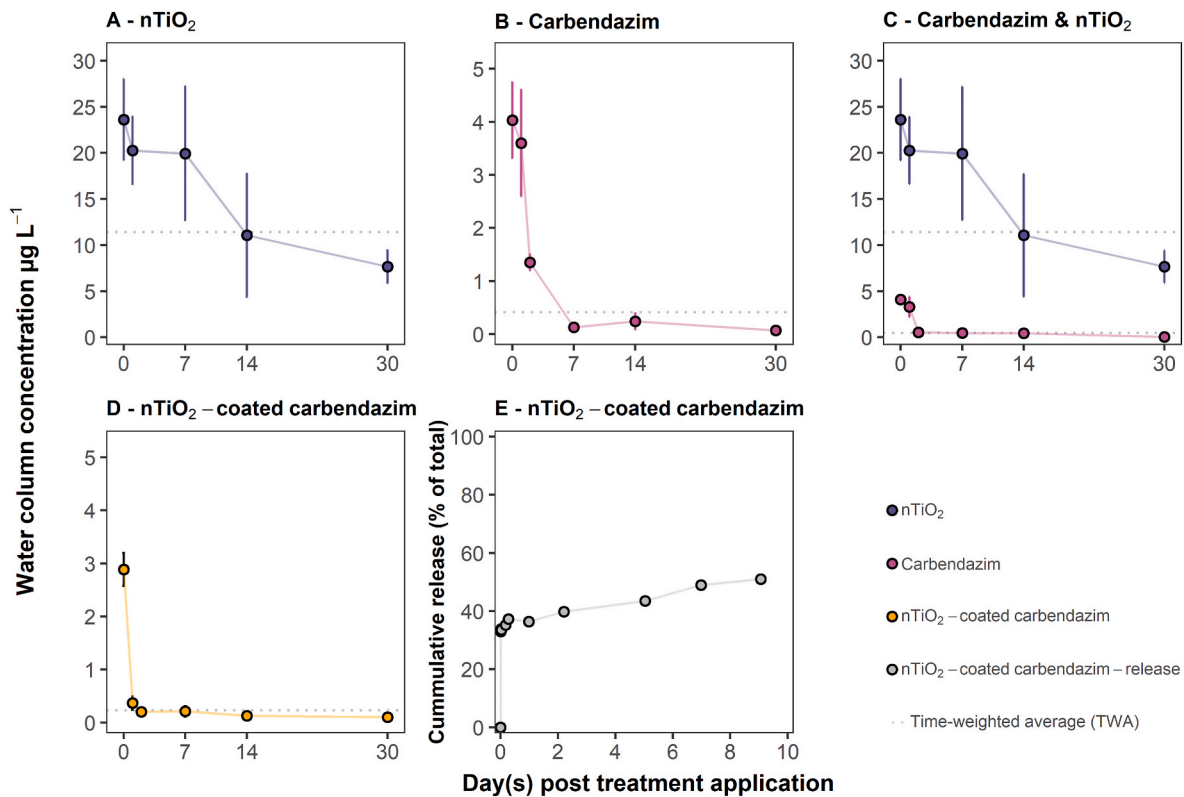
Fig. 1. Water column concentrations (mean ± standard error) of applied treatments across the timeframe of the experiment (**A–D**) & *in vitro* cumulative release of carbendazim from the nTiO$_2$-coated product in demineralized water (**E**). nTiO$_2$-coated carbendazim concentrations are expressed as total carbendazim concentrations (i.e. released & retained carbendazim). Dotted grey lines represent time-weighted average concentrations. Part of this data has previously been reported in Nederstigt et al. (2022a).

of B_j and C_j obtained for each taxon, and the net change can be denoted as (C–B). Similar to non-temporal β-diversity indices, rates of gains and losses may be scaled as a measure of dissimilarity to range between 0 and 1, and can be calculated on the basis of abundance or incidence data (see Legendre, 2019 for an extensive discussion on temporal β-diversity indices).

Within the current study, incidence- and abundance-based rates of losses, gains and total change across communities were calculated using the 'TBI' function in R package 'adespatial', according to the Sørensen- and Bray-Curtis (i.e. percentage difference-) index respectively (Dray et al., 2018). Calculated rates of change were compared between treatment and control communities using linear mixed effect models including ditch number as a random effect to account for repeated measures. Pairwise comparisons per sampling moment were conducted using estimated marginal means and *p*-values were Benjamini-Hochberg-corrected to account for multiple testing. Model assumptions (i.e. normality of residual distributions and homogeneity of variance) were checked prior to analyses and data where transformed accordingly when required. Taxon-specific contributions to rates of losses, gains and total change were manually calculated according to the equations implemented in the 'adespatial' package.

2.4.3. ζ-diversity profiles

ζ-diversity indices are used to describe patterns of compositional turnover across time or space based on the number of taxa shared (i.e. the value of ζ-diversity) by a given number (i) of assemblages (Hui & McGeoch, 2014). Crucially, in contrast to β-diversity indices, the calculation of ζ-diversity indices thereby does not require pairwise measures when >2 assemblages are compared and values of ζ-diversity can instead be calculated for each number of assemblages available. Doing so allows for partitioning of the contributions of more rare (as these are predominantly represented at lower ζ-orders) and common

(which are predominantly represented at higher ζ-orders) taxa to community turnover (Latombe et al., 2017). Values of ζ-diversity can be used to calculate a variety of metrices (see e.g. McGeoch et al., 2019) and may be normalized by dividing over the total, mean or minimum number of taxa present in the comparisons of interest.
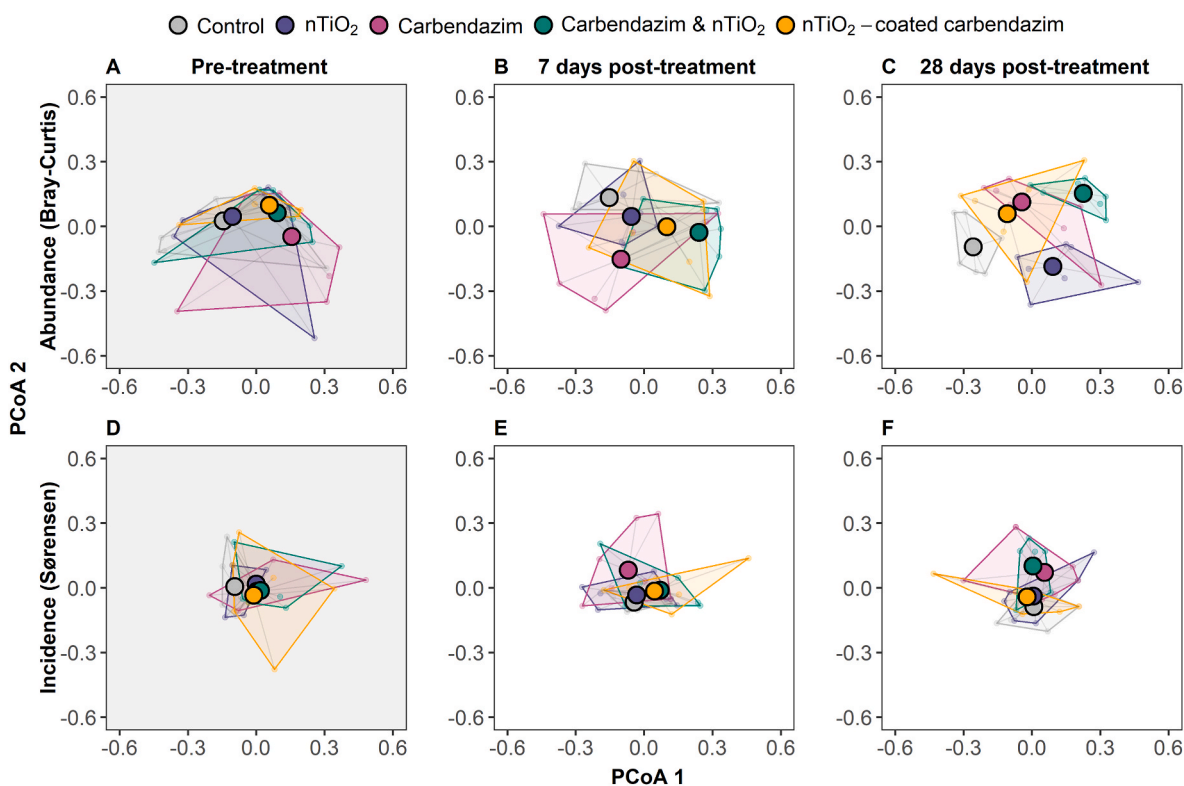
Within the current study, we calculated ζ-decline (i.e. the decline in the fraction of shared taxa with increasing ζ-order) and the taxon retention rate (i.e. the likelihood of a taxon being retained with increasing ζ-order) over time as additional measures of incidence-based turnover to the more commonly used Sørensen index. As stated previously, data of taxa with low occurrences was retained for all calculations of ζ-diversity indices, as the contribution of rare taxa to ζ-diversity values is inherently partitioned over ζ-orders. ζ-diversity indices were calculated over all possible sample combinations for each ζ-order using the 'zeta.decline.mc' and 'zeta.order.mc.multi' functions (R package 'zetadiv') (Latombe et al., 2020) and normalized by dividing over the average number of taxa per sample (i.e. Sørensen normalization). The form of ζ-decline was assessed by fitting exponential- and power-law models to ζ-diversity values over ζ-orders. A superior fit of ζ-decline values to exponential models indicates a comparatively equal probability for each taxon to be present across compared samples, whilst a superior fit to power-law models indicates a comparatively unequal probability in this regard, thus indicating the presence of forces driving community structure (Hui and McGeoch, 2014). The goodness of fit of obtained models was compared using the Akaike information criterion. As the number of comparisons performed to obtain average ζ-diversity values is large (i.e. over 1000 for ζ-orders 1–10 in the current example), statistical evaluations are likely to result in low *p*-values, even when differences may be far from ecologically relevant. As such, interpretation of slopes of ζ-decline curves and differences in values of ζ-diversity per ζ-order was performed on the basis of visually comparisons.

Table 1

Summarized output of statistical tests evaluating differences in abundance- (Bray-Curtis) and incidence- (Sørensen) based community composition (permutational ANOVA) and dispersion (β-dispersion, ANOVA) between treatments and controls at respective timepoints. Displayed *p*-values of composition tests are Benjamini-Hochberg-corrected and displayed *p*-values of dispersion tests are Tukey-corrected to account for multiple testing. Test statistics for comparisons between treatments are provided in Appendix Table 2.

| | Full model | Control vs. | | | | | | | |
| | | nTiO$_2$ | | Carbendazim | | Carbendazim & nTiO2 | | nTiO$_2$.coated carbendazim | |
		p	R^2	p	R^2	p	R^2	p	R^2
	Composition				*Pre-treatment*				
Bray-Curtis	$F_{(4,34)} = 1.37$, R^2 = 0.15, p = 0.14	0.58	0.06	0.21	0.18	0.21	0.15	0.21	0.17
Sørensen	$F_{(4,34)} = 0.50$, R^2 = 0.06, p = 0.92	0.19	0.09	0.58	0.08	0.06	0.21	0.46	0.09
	Dispersion								
Bray-Curtis	$F_{(4,34)} = 0.39$, p = 0.81	0.99		0.99		0.95		0.89	
Sørensen	$F_{(4,34)} = 1.62$, p = 0.19	0.79		0.71		0.99		0.95	
	Composition				*7 days post-treatment*				
Bray-Curtis	$F_{(4,34)} = 1.75$, R^2 = 0.19, ***p*=0.01***	0.72	0.07	0.25	0.13	0.06	0.25	0.06	0.19
Sørensen	$F_{(4,34)} = 1.87$, R^2 = 0.20, p = 0.06	0.67	0.05	0.34	0.19	0.34	0.14	0.34	0.18
	Dispersion								
Bray-Curtis	$F_{(4,34)} = 0.91$, p = 0.47	0.99		0.97		0.95		0.88	
Sørensen	$F_{(4,34)} = 0.87$, p = 0.44	0.69		0.36		0.69		0.63	
	Composition				*28 days post-treatment*				
Bray-Curtis	$F_{(4,34)} = 4.01$, R^2 = 0.35, ***p*=1e^{-3}*****	**0.02***	0.38	**0.02***	0.28	**0.02***	0.45	**0.02***	0.23
Sørensen	$F_{(4,34)} = 1.77$, R^2 = 0.19, p = 0.05.	0.38	0.07	0.09	0.26	0.06	0.24	0.33	0.17
	Dispersion								
Bray-Curtis	$F_{(4,34)} = 0.37$, p = 0.83	0.93		0.89		0.87		0.83	
Sørensen	$F_{(4,34)} = 0.38$, p = 0.82	0.83		0.95		0.93		0.82	

Asterisks indicate statistical significance (. p = 0.05, *p < 0.05, **p < 0.01, ***p < 0.001). *p*-values <0.05 are indicated in bold.

Control ⬤ nTiO$_2$ ⬤ Carbendazim ⬤ Carbendazim & nTiO$_2$ ⬤ nTiO$_2$ − coated carbendazim

Fig. 2. Principle coordinate analysis plots (PCoAs) displaying β-diversity-based dissimilarity in community composition per time point based on abundance (**A-C**, Bray-Curtis) and incidence (**D-F**, Sørensen) data. Centroids represent mean PCoA scores per treatment and polygons are drawn around PCoA scores of individual replicates (i.e. ditches).
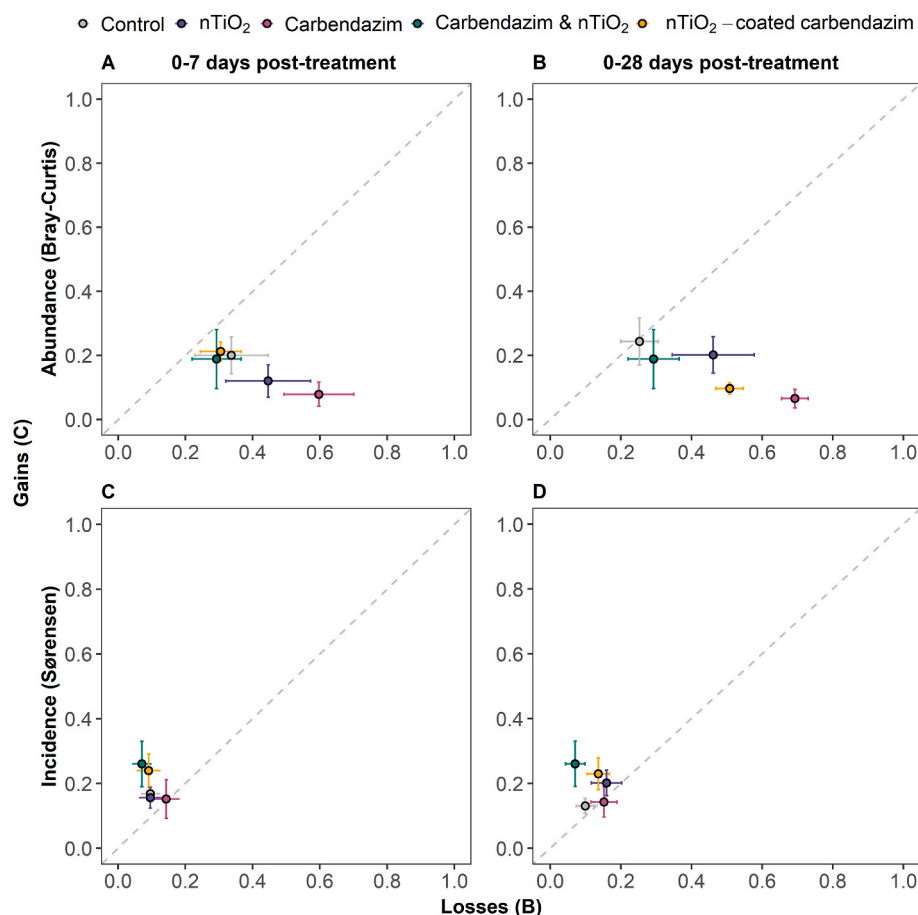
Fig. 3. B–C plots showing rates (mean ± standard error) of losses (*B*) and gains (*C*) in abundance (**A** & **B**, Bray-Curtis index) and incidence (**C** & **D**, Sørensen index) in zooplankton communities at respective timepoints relative to pre-treatment conditions. Rates are scaled between 0 and 1, where 0 denotes no, and 1 denotes maximum losses or gains of (individuals of) taxa within a ditch. Gains are expressed as the sum of abundances or incidences of taxa that were higher at each respective timepoint post-than prior to treatment application. Losses are expressed as the sum of abundances or incidences of taxa that were higher prior to treatment application than at each respective timepoint. Dotted grey lines represent the value at which losses equal gains.

3. Results & discussion

3.1. Experimental conditions

Initial measured water column concentrations of $nTiO_2$ were 23.6 ± 4.4 µg L^{-1} (mean ± standard deviation) and subsequently declined to reach a time-weighted average (TWA) concentration of 11.4 ± 1.1 µg L^{-1} (Fig. 1 A). *In vitro* analysis in water from the test systems showed that $nTiO_2$ aggregated to particle sizes of approximately 500 nm within 24 h of incubation, after which aggregation rates stabilized (Nederstigt et al., 2022a). In the carbendazim- and carbendazim & $nTiO_2$ treatments, water column concentrations of carbendazim initially were 4.0 ± 0.7 µg L^{-1} and 4.1 ± 0.4 µg L^{-1}, respectively and subsequently showed a sharp decline, resulting in TWA concentrations of 0.4 ± 0.04 µg L^{-1} (i.e. 66 ± 6% of intended TWA) and 0.5 ± 0.03 µg L^{-1} (i.e. 83 ± 5% of intended TWA) respectively (Fig. 1 B & D). Total carbendazim (i.e. the sum of coated and released) concentrations in the $nTiO_2$-coated carbendazim treatments were initially approximately 25% lower than nominal treatment concentrations, and subsequently declined resulting in a TWA concentration of 0.23 ± 0.02 µg L^{-1} (Fig. 1 D). An extensive discussion on exposure conditions and release kinetics of carbendazim from the $nTiO_2$-coated product is available in Nederstigt et al. (2022a).

Natural variation in zooplankton community composition was present across the experimental setup prior to treatment applications, but this did not manifest in statistically significant measures of dissimilarity (Table 1, Fig. 2 A & D). Overall, 35 zooplankton taxa were identified over the course of the experiment of which 2 to subclass-, 3 to order-, 7 to genus-, and 23 to species level. Total taxon richness across treatments and controls declined slightly over time from 31 prior-to 29 at 7- and 25 at 28 days post-application of treatments, and the most abundant taxa identified across all samples belonged to Copepoda and Daphniidae.

Sampling efforts were sufficient to characterize the taxon richness present across the experimental systems as indicated by taxon accumulation curves, which showed that on average < 1 previously unidentified taxon was collected after sampling 8, 6 & 5 ditches for successive time points (Appendix Fig. 2).

Physicochemical water quality parameters (temperature, pH, dissolved oxygen conductivity, NH_4^+ and NO_3^-, chlorophyll A & turbidity) fluctuated over the timeframe over which zooplankton community dynamics were assessed (Appendix Fig. 1). With the exception of a small (~0.3) relative increase in pH in treatment communities at 3 weeks after treatments application, no differences between treatments were observed for any of the measured parameters (Appendix Table 1).

3.1.1. Zooplankton community turnover in response to stressors – insights from β-diversity-based analyses

Seven days post treatment application, abundance-based dissimilarity between treatment- and control communities increased relative to pre-treatment conditions but remained statistically insignificant (Table 1, Fig. 2 B & E). Temporal β-diversity indices (TBIs) indicated that within this timeframe, all communities experienced a net loss of cumulative abundances across taxa, but no differences in rates of losses, gains or total change were observed between treatments and controls (Fig. 3 A, Appendix Table 4). Incidence-based indices of dissimilarity, both temporal- and non-temporal, also showed minimal differences between controls and treatments, and all communities on average exhibited a slight net gain in taxon richness relative to pre-treatment conditions (Fig. 3 C).

Over time, differences between treatment- and control communities became more pronounced. This resulted in statistically significant abundance-based measures of dissimilarity at 28 days after treatment application for all treatments relative to controls (Table 1, Fig. 2 C).
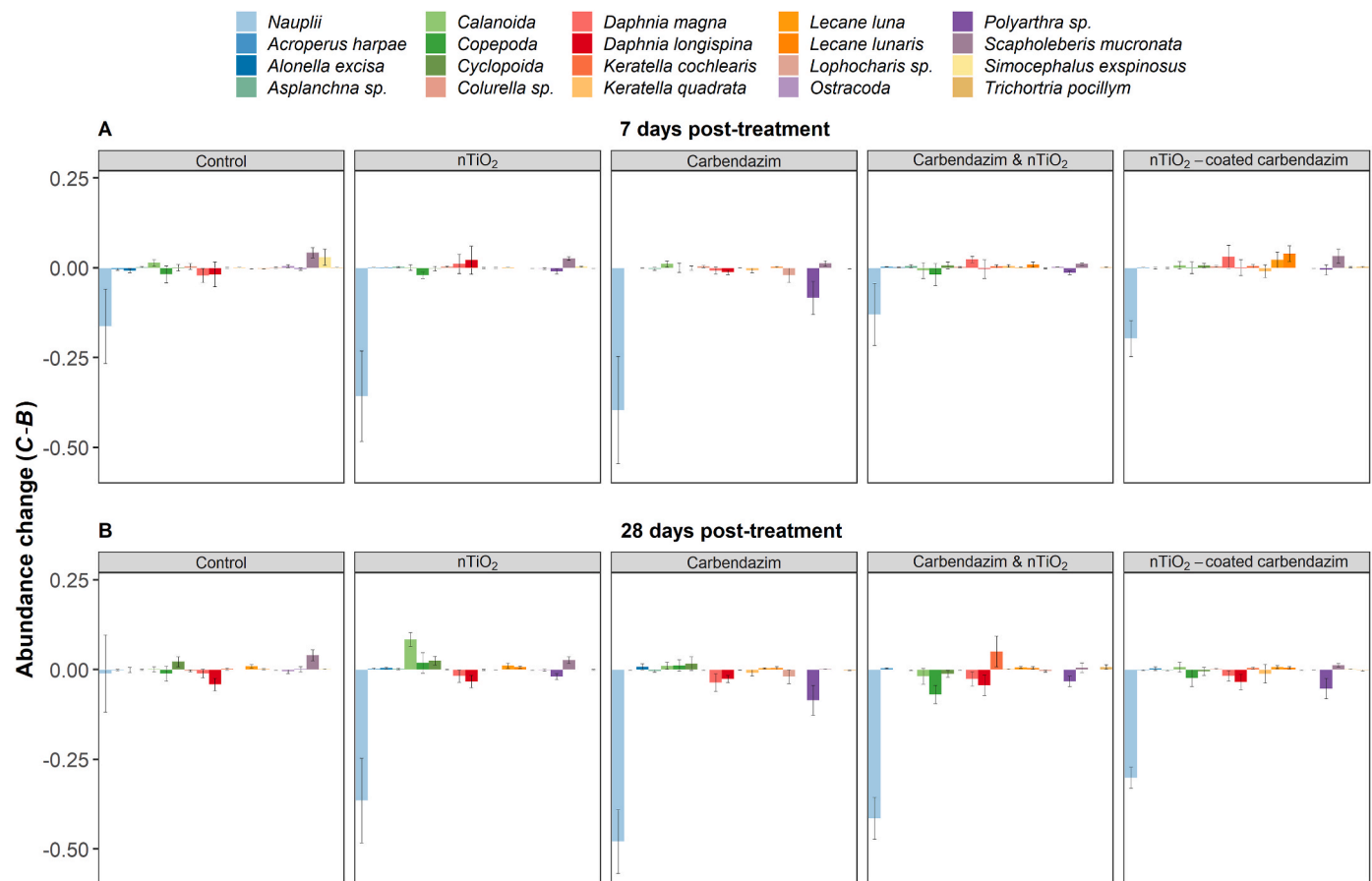
Fig. 4. Plots showing the net abundance change (*B–C*, mean ± standard error) per taxon at respective timepoints relative to pre-treatment conditions. Nauplii belong to Copepoda, including Calanoida and Cyclopoida.
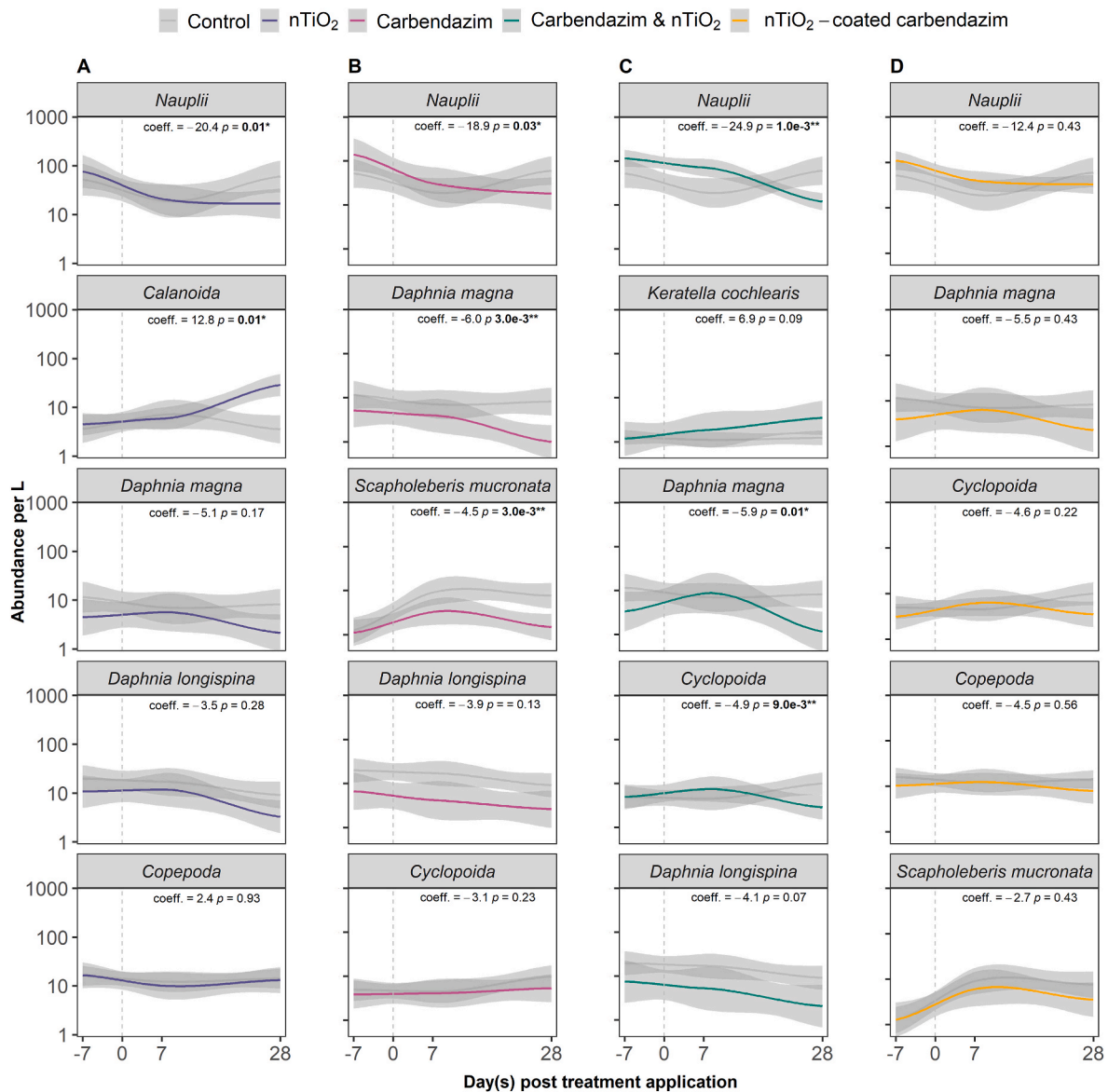
Similar to 7 days post treatment application, incidence-based measures showed no significant dissimilarities between treatments and controls, although an increase in variance attributed to carbendazim and carbendazim & $nTiO_2$ treatments was observed in PERMANOVA models (Table 1). Overall, this suggests that dissimilarity between treatment and control communities could predominantly be ascribed to varying abundances of taxa rather than to differences in taxonomic community composition (Fig. 2 E). Analysis of multivariate dispersions (i.e. within-treatment variation in community composition) showed no differences between controls and treatments at any of the assessed timepoints. This indicates that treatments acted predominantly as a deterministic (i.e. non-random) driver of community turnover (Anderson and Walsh, 2013). Losses in abundances in treatment communities were more pronounced at 28- than at 7 days post treatment application, and net rates of change in control communities were approximately zero (Fig. 3). For carbendazim and carbendazim & $nTiO_2$ treatments, this resulted in significant differences from controls in both rates of abundance loss (carbendazim: $t_{60} = -3.7$, $p = 3.5e^{-3}$ & carbendazim & $nTiO_2$: $t_{60} = -3.4$, $p = 0.01$) and net rates of change ($t_{60} = -3.5$, $p = 7.1e^{-3}$ & $t_{60} = -3.0$, $p = 0.02$). In concurrence with observations of non-temporal measures of dissimilarity, incidence-based TBIs showed smaller differences between controls and treatments than abundance-based TBIs, and all communities on average remained to display a net gain in taxa (Fig. 3 D).

In comparisons between treatments, rates of losses, gains and total change indicated no significant differences at any of the assessed timepoints (Appendix Table 4). Pairwise comparisons of abundance-based measures of non-temporal β-diversity indices after 28 days however

showed significant dissimilarity for all possible treatment combinations (Appendix Table 2). This suggests that although all treatments over time induced alterations in community structure from controls, the specific responses in terms of affected taxa varied between treatments.

3.1.2. Taxon-specific contributions to abundance-based zooplankton community turnover

In all treatments, observed dissimilarity from controls over time could predominantly be ascribed to differing abundances of taxa belonging to Copepoda (including nauplii, i.e. their juvenile stages) and Daphniidae (Figs. 4 and 5). Twenty eight days after treatment application, abundances of nauplii, *Daphnia magna* and *Scapholeberis mucronata* were significantly reduced in carbendazim treatments in comparison to controls (LR-test = 9.1, $p = 0.03$; LR-test = 17.6, $p = 3.0e^{-3}$ & LR-test = 15.6, $p = 3.0e^{-3}$, respectively; Fig. 4). Similarly, nauplii and *D. magna* were found to be significantly reduced in carbendazim & $nTiO_2$ treatments at the same timepoint (LR-test = 21.0, $p = 1.0e^{-3}$ & LR-test = 12.0, $p = 0.01$, respectively), and a significant reduction in abundances of Cyclopoida (LR-test = 11.8, $p = 9.0e^{-3}$) was observed as well. In $nTiO_2$ treatments, only nauplii showed a significant reduction in abundances relative to controls (LR-test = 11.4, $p = 0.01$), whereas a significant increase in abundances of Calanoida (LR-test = 11.9, $p = 0.01$) was observed. This suggests that in $nTiO_2$ treatments, Calanoida experienced a competitive advantage as a result of reductions in abundances of other taxa. Interestingly, whereas abundance trends of multiple taxa showed significant differences from controls in carbendazim and carbendazim & $nTiO_2$ treatments, trends were less pronounced in $nTiO_2$-coated carbendazim treatments, showing no differences from controls

Fig. 5. Abundance trends of the 5 taxa contributing most to observed abundance- (i.e. Bray-Curtis) based dissimilarity from controls per treatment at 28 days post-treatment application. Nauplii belong to Copepoda, including Calanoida and Cyclopoida. Taxa were selected according to ranked PERMANOVA model coefficients (denoted in figures by coeff.) and are displayed accordingly (top to bottom). Lines represent means and ribbons represent 95% confidence intervals. Displayed p-values (corrected for multiple testing via a step-down resampling procedure with 999 iterations) are derived from likelihood ratio tests of generalized linear models fitted with negative binomial distributions and refer to differences between treatments and controls at the 28 days timepoint specifically. Full test statistics including comparisons between treatments for all assessed taxa are provided in Appendix Table 5. Asterisks indicate statistical significance (. $p = 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). p-values <0.05 are indicated in bold.

for any of the individually assessed taxa (Fig. 5).

3.1.3. Zooplankton community turnover in response to stressors – additional insights from ζ-diversity-based analyses

ζ-decline curves showed superior fit to power law-over negative exponential distributions (the two most common parametric forms of ζ-decline) in all analyzed communities of treatments and controls over time, indicating that communities were predominantly shaped by deterministic assembly processes (Fig. 6 C & D, Appendix Table 6, Latombe et al., 2018). For treatment communities, this is in concurrence with observations of multivariate dispersions from non-temporal β-diversity indices, which also indicated directional (i.e. deterministically driven-) treatment-induced effects on community composition. Over time, the fraction of core taxa (i.e. the taxa present across all compared samples from the timepoint of interest and prior to treatment application), as defined by ζ-diversity values at the highest ζ-order (i.e. ζ_{14}) in ζ-decline curves, were lower in all treatments than in controls (Fig. 6 A & B). Similarly, taxon retention rates in treatments generally showed a more pronounced difference from those in controls at lower ζ-orders (Fig. 6 E & F). For carbendazim, carbendazim & nTiO$_2$ treatments and nTiO$_2$-coated carbendazim treatments, the fraction of shared taxa between communities sampled prior to- and after application of treatments decreased most steeply at the lower ζ-orders (i.e. ζ_{1-5}), both when considering communities at 7- and 28 days post treatment application. For nTiO$_2$ treatments, a more gradual decline in ζ-diversity values across ζ-orders was observed, which persisted at orders where control communities approached a plateau. As such, whereas communities in carbendazim, carbendazim & nTiO$_2$ treatments and nTiO$_2$-coated
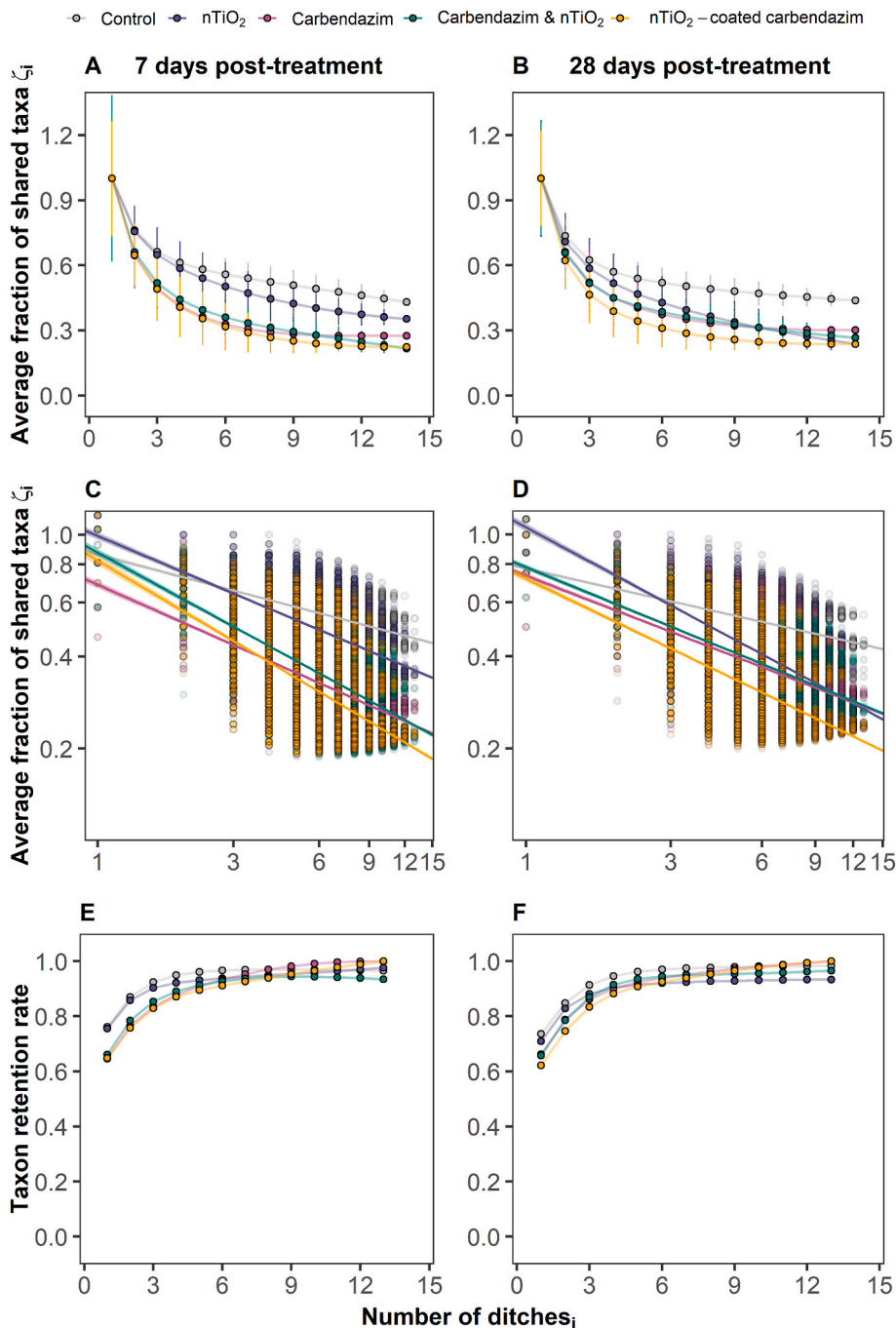
Fig. 6. A & B: ζ-decline curves showing the change (mean ± standard error) in the average ratio of shared taxa (i.e. Sørensen-normalized ζ-diversity) with increasing ζ-order (i.e. number of compared ditches). **C & D:** Power-law regression fits of ζ-decline curves (mean ± 95% confidence intervals). **E & F:** Taxon retention rates based on Sørensen-normalized ζ-diversity with increasing ζ-order. ζ-diversity values were derived from aggregated community data from samples from the timepoint of interest and samples from the same treatments prior to treatment application.

carbendazim treatments primarily exhibited a reduction in relative occurrences of more common taxa, $nTiO_2$ treatments displayed a reduction across taxa of different orders or commonness. Overall, ζ-decline curves and power-law model slopes of carbendazim & $nTiO_2$ treatments and $nTiO_2$-coated carbendazim treatments showed a stronger resemblance to carbendazim-than to $nTiO_2$ treatments, indicating that carbendazim acted as a more powerful driver of community turnover than $nTiO_2$.

Interestingly, ζ-diversity indices demonstrated more pronounced responses to treatments than incidence-based measures of β-diversity. This is best demonstrated by trends of ζ-diversity values calculated across ζ-orders. At $ζ_2$, ζ-diversity values provide a measure of similarity based on pairwise comparisons between individual sites which is equivalent to incidence-based measures of β-diversity (i.e. Sørensen

diversity). In concurrence with findings presented in section 3.1.1, these measures show only minor differences between treatment- and control communities, and differences to this end only become apparent when higher ζ-orders are considered (Fig. 6). As such, the partitioning of diversity measures across the spectrum of rarity and commonness as facilitated by ζ-diversity indices appears hold promise as a basis for sensitive assessments of incidence-based community turnover in ecotoxicological studies.

3.2. Zooplankton community turnover in response to stressors – synopsis of observed effects

Zooplankton communities in all treatments developed distinctly

from those in controls over the course of the current experiment, and chronic- (and possibly indirect-) rather than acute effects were found to act as the predominant drivers in this regard. Cumulative differences in abundances across taxa (i.e. community-level effects) provided a more responsive measure of treatment-induced disturbances than analyses of trends of individual taxa. As such, the findings of the present study highlight the more often emphasized importance of community-level approaches as a sensitive method of effect assessment in ecotoxicological studies (Iwasaki et al., 2018; Rohr et al., 2016; Mikó et al., 2015).

Treatment concentrations of $nTiO_2$ applied in the current experiment were within the upper range of measured concentrations of anthropogenically derived TiO_2 in freshwater ecosystems as reported by Peters et al. (2018) & Nabi et al. (2022). To the best of our knowledge, the current study comprises the first experimental assessment of community-level effects of $nTiO_2$ on zooplankton. To date, available data on $nTiO_2$ toxicity towards zooplankton species has predominantly been derived from standardized single-species laboratory studies which have often focused on *D. magna* or other Cladocerans (i.e. according to OECD TG 202, 2004 & OECD TG 211, 2012). In many cases, such studies reported minimal or no adverse effects resulting from test concentrations which were several orders of magnitude higher than those applied in the current study (Book & Backhaus, 2022). Notably however, several studies in which effects of $nTiO_2$ on *D. magna* were assessed under more environmentally realistic test scenarios (e.g. under exposure across multiple generations, limited food availability or in the presence of UV light) have previously demonstrated that standardized single-species laboratory assays may underestimate the extent to which $nTiO_2$ is able to induce adverse effects in freshwater ecosystems (Nederstigt et al., 2022b; Ellis et al., 2021; Jacobasch et al., 2014).

$nTiO_2$-coated carbendazim treatments induced slightly less pronounced effects on zooplankton community turnover than treatments of carbendazim in its non-coated form, both when the latter was applied individually and when it was applied in combination with $nTiO_2$. Although less pronounced, patterns and forms of community turnover in $nTiO_2$-coated carbendazim treatments nevertheless showed a strong resemblance to those in treatments of non-coated carbendazim. This resemblance was less striking in comparisons with treatments where $nTiO_2$ was applied separately, which suggests that effects of $nTiO_2$-coated carbendazim treatments in the current experiment could predominantly be ascribed to carbendazim rather than the applied coating material.

The current commercial availability and application of nano-formulated pesticides encompasses only a few cases world-wide, although it is expected that numbers will rise in the near future as many products are now moving to market-ready stage (Wang et al., 2022). At present however, the lack of commercially available products limits the harmonized collection of non-target toxicity data on comparable products, which in turn restricts inter-study validation of findings to general trends observed across tested nano-formulated pesticides. To this end, the findings of the current experiment indicate that the applied nano-formulation would pose a similar- or slightly reduced hazard to freshwater zooplankton communities in comparison to its active ingredient, which is largely in concurrence with observations of non-target toxicity of nano-formulated pesticides in general as reviewed by Wang et al. (2022) and Zhang & Goss (2022).

4. Conclusions & outlook

Nano-formulated pesticides are perceived as promising applications of nanotechnology which could minimize collateral environmental damage from agricultural production. To date, evaluations of non-target toxicity of nano-enabled pesticides have remained sparse, and where available, comparisons with their conventional counterpart are often lacking (Zhang & Goss, 2022). The results of the current study provide a first indication that the evaluated $nTiO_2$-coated nano-formulation of carbendazim at best exhibited a slightly reduced hazard to freshwater

zooplankton communities in comparison to non-coated carbendazim. A comprehensive hazard assessment would benefit from future studies in which dose-related responses are derived, e.g. at the level of individual taxa which have been identified as main contributors to observed community-level impacts of components of the $nTiO_2$ formulation in the current study. Ultimately, the extent to which hazards associated with nano-formulated pesticides would culminate into alterations of environmental risks is dependent on factors such as efficacy of the respective formulations in crop protection and on resulting application- and runoff rates. We therefore argue that in order to facilitate comprehensive cost-benefit analyses, data on the risk and usage profile of nano-formulated pesticides should be holistically collected and evaluated in future studies.

Author contributions

TN: Conceptualization; Investigation; Methodology; Data curation; Formal analysis; Visualization; Writing - original draft; BB: contributed to the presented work as a trainee at ULEI: Methodology; Data Collection; Writing-review & editing; WP: Conceptualization; Supervision; Resources; Writing-review & editing; RO: Resources; Interpretation; Writing-review & editing; MV: Conceptualization, Supervision, Resources, Funding acquisition, Project administration, Writing-review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121894.

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