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Invasive Crayfish: Drivers or Passengers of Degradation in Freshwater Ecosystems?

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ABSTRACT

Invasive species, such as the freshwater crayfish *Procambarus clarkii*, reportedly negatively influence the abundance of various aquatic species. Moreover, these invaders are increasingly linked to ecological degradation of aquatic ecosystems, as invaded habitats show increased levels of turbidity, nitrogen, and organic matter concentration. *P. clarkii* has, among other impacts, been associated with eutrophication in invaded habitats. However, observations suggest that the presence of *P. clarkii* is often not accompanied by ecosystem degradation, raising the question of whether they are drivers of degradation or function as passive passengers, with the degradation being caused by other stressors. To investigate these contrasting hypotheses, we conducted a full factorial experiment in 24 mesocosms with *P. clarkii* and nutrient pollution (specifically N, P, and K), a ubiquitous stressor in aquatic ecosystems. Here, we assessed the effects on community compositions of morphologically identified macrophytes and chironomids, as well as the compositions of bacteria, phytoplankton, and diatoms identified using environmental DNA (eDNA) metabarcoding. Nutrient pollution induced significant shifts in macrophyte biomass and in the composition of the bacterial, diatom, and phytoplankton communities. All microbial communities exposed to nutrient pollution initially diverged from the control, after which the bacterial and phytoplankton communities converged back to the control in the final weeks. In contrast, we found only marginal effects of *P. clarkii*, rendering it unlikely as a significant short- to medium-term driver of the tested biodiversity. As microbial communities respond quickly to changes in the environmental conditions, these results signify that the mesocosms used in the study were relatively stable in spite of the presence of *P. clarkii*. The crayfish density and timeframe studied may be leveraged as threshold values in the design and execution of freshwater management strategies that aim to avert potential negative impacts of *P. clarkii* on ecosystem structure. Ultimately, the importance of nutrient pollution is reinforced as a driver of environmental change in aquatic ecosystems.

1 | Introduction

Freshwater ecosystems are globally threatened (Collen et al. 2014; Dudgeon et al. 2006; Reid et al. 2019) by drivers such as climate change, pollution, and invasive species (Gallardo et al. 2016; Strayer 2010; Tickner et al. 2020). *Procambarus clarkii*, a crayfish species native to northeastern Mexico and south-central USA, serves as a textbook example of a successful invasive species in freshwater ecosystems (due to its early maturity, large number of offspring, rapid growth rates, and short

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life spans) as it has spread across all continents except Australia and Antarctica (Ficetola et al. 2012; Gherardi 2006; Oficialdegui et al. 2019; Oficialdegui, Sánchez, and Clavero 2020). *P. clarkii* was first introduced in Spain for aquaculture in 1973 (Gherardi 2006; Souty-Grosset et al. 2016). However, improper management led to the escape of *P. clarkii* into natural freshwater bodies in the early 1980s, facilitating its spread through the rest of Europe, which was further accelerated by its popularity in the aquarium trade (Gherardi 2006; Souty-Grosset et al. 2016). Concurrent with the spread of *P. clarkii* in Europe, freshwater ecosystems have faced increasing impacts from other anthropogenic pressures, such as agricultural runoff (pesticides and nitrogen/phosphorus), climate change, and the local extinction of vertebrate predators (e.g., otter and eels). The co-occurrence of multiple pressures makes it difficult to disentangle their relative contributions to the observed degradation of these systems, that is, the human-induced changes in ecosystem structure, dynamics, and functions (Gurevitch and Padilla 2004; Thompson et al. 2013).

The presence of *P. clarkii* has frequently been observed in degraded freshwater ecosystems and has therefore been linked to a number of significant changes in these systems. *P. clarkii* is hypothesized to disturb invaded ecosystems through three key pathways. First, similar to other invasive crayfish, *P. clarkii* is a generalist and opportunistic omnivore (Gherardi 2006; Joaquín Gutiérrez-Yurrita et al. 1998), feeding on detritus, algae, submerged macrophytes, and invertebrates (Angeler et al. 2001; Gherardi 2007). Through this feeding behavior, *P. clarkii* occupies a flexible position in the food web and affects multiple trophic levels of invaded freshwater ecosystems (Dorn and Wojdak 2004; Loureiro et al. 2015). Second, in addition to its feeding behavior, *P. clarkii* has been well-documented to trigger significant declines in macrophyte abundance due to excessive nonconsumptive cutting and uprooting (Gherardi 2007; Matsuzaki et al. 2009; Rodríguez, Bécares, and Fernández-Aláez 2003; Rodríguez et al. 2005), destroying more aquatic vegetation than they are able to consume (Lodge 1991; Nyström and Strand 1996). Third, crayfish-associated bioturbation caused by walking, burrowing, and foraging habits may lead to the resuspension of sediment and the recycling of sediment-bound nutrients into the water column (Angeler et al. 2001; Yamamoto 2010). Light availability in the water column subsequently decreases due to the increased levels of suspended solids (Angeler et al. 2001). It is due to a combination of these behaviors that *P. clarkii* may indirectly shift an ecosystem to a eutrophic state under nutrient-rich conditions. In this case, the removal of macrophytes and the subsequent domination of surface microalgae are accompanied by a transfer from a clear to a turbid state (Rodríguez, Bécares, and Fernández-Aláez 2003). Previous studies have observed the presence of *P. clarkii* at high densities in combination with shifts in both the abundance and composition of microalgae (Rodríguez, Bécares, and Fernández-Aláez 2003; Gherardi and Lazzara 2006). Because of these ecosystem-modifying behaviors, a number of studies have proposed that *P. clarkii* has a significant impact on local freshwater communities and, as such, is regarded as a prime driver of ecological degradation of these systems (Dorn and Wojdak 2004; Rodríguez, Bécares, and Fernández-Aláez 2003; Yamamoto 2010; Gherardi and Acquistapace 2007).

However, the correlated presence of nonindigenous species in altered habitats is often presumed to be the cause of disturbances in those environments (Gurevitch and Padilla 2004). As an alternative explanation for the proliferation of nonindigenous species in invaded habitats, MacDougall and Turkington (MacDougall and Turkington 2005) have proposed the "passenger" model as opposed to the "driver" model. According to the "passenger" model, *P. clarkii* would proliferate because it is less susceptible than the local community to alternative environmental stressors. If the latter of these hypotheses is valid, it might be better suited to spend the limited budget for ecosystem restoration on the root cause of the degradation, rather than on the suppression of nonindigenous species. The effects of *P. clarkii* on freshwater macrophyte abundance have been well-documented (Matsuzaki et al. 2009; Carreira, Dias, and Rebelo 2014; Geiger et al. 2005; Twardochleb, Olden, and Larson 2013), and various studies have also assessed *P. clarkii*'s impact on macroinvertebrates, noting significant negative effects on both their abundance and diversity (Souty-Grosset et al. 2016; Correia and Anastácio 2008; Chucholl 2013; Correia, Bandeira, and Anastácio 2005). However, findings on *P. clarkii*'s role in overall ecosystem degradation remain mixed. For instance, observations have been made where the presence of *P. clarkii* did not coincide with any noticeable level of environmental degradation (Tangerman et al. 2021). Additionally, the interactive effects of *P. clarkii* with other concurrent environmental stressors, such as nutrient pollution, are often overlooked. Therefore, it remains unclear whether *P. clarkii* is an influential driver of the observed environmental degradation, or a passenger within already degraded ecosystems.

As stated above, *P. clarkii* may indirectly induce a shift to a turbid ecosystem state, characterized by surface microalgal domination, through the removal of macrophytes and the recycling of nutrients. Recent research has demonstrated environmental DNA (eDNA) metabarcoding to be an effective tool to register the impact of nutrient pollution within microbial communities (Beentjes et al. 2021; Clark et al. 2020; Li et al. 2018). In fact, microbial communities surveyed with eDNA have appeared more responsive to nutrient pollution (N, P, and K) than morphologically identified macrofauna (Beentjes et al. 2021; Clark et al. 2020), perhaps in part due to the rapid turnover of microbial generations (Luna, Manini, and Danovaro 2002). As such, eDNA metabarcoding of microbial communities appears to be a suitable method for identifying the broader indirect effects associated with *P. clarkii*. The objective of this study is to determine whether *P. clarkii* is a likely driver or a passenger of freshwater ecosystem degradation. For this purpose, a large-scale experiment was performed in the "Living Lab" in Leiden, the Netherlands, using 24 mesocosms with a full factorial design. To examine environmental change, this study assessed the impact of environmentally relevant concentrations of a ubiquitous stressor in freshwater ecosystems, nutrient pollution, and the crayfish *P. clarkii* at environmentally relevant densities, on ecosystem structure, including macrophytes, chironomids, phytoplankton, diatoms, and bacteria. The impacts of both stressors were tested individually and in combination to assess any interactive effects. These community responses were recorded using a combination of traditional morphological and novel eDNA survey methods.

2 | Materials and Methods

2.1 | Experimental Design

Measurements and samples were collected at the Living Lab facility of the University of Leiden (located in Oegstgeest, 52°10′16.2″ N; 4°26′58.0″ E), which harbors several manually excavated earthen experimental ditches, that is, mesocosms, representing Dutch freshwater ecosystems (Barmentlo et al. 2019) with a length of approximately 8.5 m, a width of 0.6 m, and a depth of 0.35 m (Figure S1).

To ensure ecological similarity of the different mesocosms, they were ecologically reset between the 8th and 15th of March 2019 by pumping them dry and removing the sediment layer. Adjacent to the mesocosms lies a small freshwater reservoir, which supplied the mesocosms with water and local fauna during a colonization phase over the course of 6 weeks. In addition, each mesocosm received approximately 11.2 L of filtered and homogenized sediment to commence macrophyte colonization on March 19, 2019. After colonization, the mesocosms were isolated by installing an acrylic sheet (1000 × 500 × 2 mm) that blocked all aquatic movement between the mesocosms and the reservoir. All treatments and biodiversity samples were, therefore, applied and collected in a 5-m-long closed-off subsection of the mesocosms. Approximately 50 g of *Elodea nuttallii* was manually introduced into each replicate mesocosm to accelerate colonization.

The experiment commenced in Week 18 (May 1, 2019) and concluded in Week 30 (July 23, 2019) (Figure S1). The treatments were spatially allocated in blocks. This scheme allowed for an even distribution of treatments across space, such that any bias—for example, through gradients in substrate composition and other physico-chemical parameters—could be accounted for. The treatments consisted of two components: (A) the simultaneous introduction of two same-sized (sexually immature juvenile or young adult) female *P. clarkii* in Week 22 (May 27, 2019), and (B) the addition of a common source of nutrient pollution: fertilizer ("Osmocote"; N:P:K = 15:9:11). Exclusively female crayfish were selected to control for sex-related differences in size and behavior (Bovbjerg 1956). The nutrient pollution treatment was administered in Week 18 (May 1, 2019) through slow-releasing granulates in three sachets, each containing 60 g of fertilizer pellets. In Week 23 (June 7, 2019), the granulates were depleted and replaced in equal proportions. The concentrations of nutrient additions were based on representative values commonly found in Dutch water bodies near agricultural practices (Barmentlo et al. 2019; Vijver et al. 2008). Each treatment had six replicates, and they were assigned in the following order to maximize distance between similar components: (1) crayfish and nutrient pollution, (2) crayfish, (3) nutrient pollution, and (4) control. To prevent migration of the crayfish between the mesocosms, submersible cages were built in the approximate shape of trapezoidal prisms to span the width, depth, and most of the mesocosm length (length: 400 cm; top width: 45 cm; bottom width: 25 cm; height: 40 cm). The cages consisted of coated chicken mesh (mesh size of 6.3 × 6.3 mm). The movement of the crayfish was restricted to these 4-m-long cages within the 5-m-long experimental mesocosms. As the cages were positioned closely along the sides and bottom of the mesocosms, and because macrophytes were allowed to grow through the chicken mesh, the crayfish were able to interact with biota, including the macrophyte community, from within the cages. It proved impossible to allow for natural burrowing behavior, as uncaged crayfish could leave the mesocosms. To provide hiding space for the crayfish in case of aggressive behavior by cohabitants, artificial crayfish burrows were added using small PVC pipes (20 cm in length) with a fine mesh to close off one end. The resulting density of 2 individuals m^{-2} is comparable to that of a small local Dutch field survey (Rip et al. 2021): 1.53 individuals m^{-2}, exceeds the recently established critical threshold (for *P. clarkii* to have an impact on macrophytes, macrofauna, or water quality) of 0.9 individuals m^{-2} (Lemmers, Crommbaghs, and Leuven 2018), and falls within an internationally observed density range of 0.2–15 individuals m^{-2} (Gherardi and Acquistapace 2007; Maezono et al. 2005; Rhodes and Avault 1986).

2.2 | Macrophyte Biomass and Chironomid Survey

Community response to *P. clarkii* was monitored for macrophytes, chironomids, bacteria, phytoplankton, and diatoms. Macrophyte community structure is directly affected by *P. clarkii* (Carreira, Dias, and Rebelo 2014) and was therefore monitored by collecting singular end-point biomass measurements of the most abundantly observed macrophyte taxa in Week 30 (July 23, 2019). The morphological survey of macrophytes yielded four different macrophyte taxa: *Potamogeton berchtoldii*, *Chara globularis*, *E. nuttallii*, and FLABs (filamentous algae beds). Macrophytes present on and in the cages were collected from the mesocosm replicates and sorted by taxa. Dry weights of *E. nuttallii*, *C. globularis*, and *P. berchtoldii* were obtained by extrapolation from wet weight measurements using pretested calibration curves (Figure S2). A calibration curve was also created for FLABs, but this curve was not adequate for extrapolation. All FLABs were, therefore, dried in an oven before being weighed separately.

Chironomidae (Diptera) were selected as one of the measures of community response since chironomids represent a portion of *P. clarkii*'s diet (Correia and Anastácio 2008), are frequently used as indicators of water quality (Molineri et al. 2020), and function as a proxy for the macrofaunal community. For these reasons, emerging adult chironomids were sampled in Weeks 21 (May 21, 2019) and 30 (July 22, 2019) of the experiment from emergence traps that were placed over 12 experimental mesocosms (three for each of the treatments). This sampling method has previously been used to show the impact of agrochemicals on emerging macrofauna (Barmentlo et al. 2021). All chironomids were stored in 70% ethanol prior to identification. From the 12 mesocosms, 646 emerged chironomids were captured and separated from non-chironomid taxa using a stereo microscope. Chironomids were then identified to the species level following the protocol described in Barmentlo et al. (2021), yielding a total of 40 species.

2.3 | eDNA Sampling and Extraction

Surface water of the 24 mesocosms was sampled at 11 selected evenly spaced locations in each mesocosm using 50 mL BD

Plastipak sterile syringes (VWR International, Radnor, PA, USA). Nitrile gloves were worn during sampling and were replaced for each mesocosm. The 11 subsamples per mesocosm were combined, of which 300 mL was filtered. The 300 mL filter volume was chosen to maximize the filtered volume while preventing clogging, and it has proven adequate in past research for registering community response (Beentjes et al. 2019a). Sample filtration was performed with Millipore Express PLUS polyethersulfone membrane filters (Sartorius, Göttingen, Germany) (diameter 45 mm, pore size 0.45 μm), which were placed in sterilized Nalgene filter units (Thermo Fisher, Waltham, MA, USA) attached to a vacuum pump (Datura Molecular Solutions BV, Wageningen, the Netherlands). All materials that made contact with the environmental samples (i.e., the filtering setup) and the designated workplace were bleached, rinsed and dried prior to filtration rounds to prevent contamination between samples. After filtration, the polyethersulfone filters were stored in 700 μL cetyltrimethylammonium bromide (CTAB) (PanReac AppliChem, Darmstadt, Germany) at −20°C. A CTAB extraction protocol was adapted from Beentjes et al. (2021) for DNA extraction. DNA extracts were subsequently stored at −20°C until analysis.

The first set of eDNA samples was taken prior to the implementation of any treatment, in Week 18 (May 1, 2019), thus serving as a baseline measurement. The other eDNA samples were taken in Week 22 (May 27, 2019), right before introducing the crayfish, and then in Week 24 (June 11, 2019), Week 27 (July 2, 2019), and Week 30 (July 22, 2019), resulting in a total of five sampling rounds and 120 samples.

2.4 | DNA Amplification and MiSeq Sequencing

The impact of the treatments was studied by monitoring the community composition of three taxonomic groups: phytoplankton, diatoms, and bacteria. For each of these different taxonomic groups, a different marker was targeted: a 390–410 bp fragment within the V4 subregion of the 18S rRNA gene for phytoplankton, a 273 bp fragment within the V4 region of the 16S rRNA gene for bacteria, and a 312 bp fragment of the rbcL plastid gene for diatoms (for primers, see Table S1). These microbial biodiversity groups were selected because of their high turnover on small timescales (Beentjes et al. 2021, 2019a), which allows the composition of these groups to quickly respond to the applied treatments (Glasl, Webster, and Bourne 2017).

A fourth primer pair was used to analyze the family *Chironomidae*, but this dataset presented a high level (74% of molecular operational taxonomic units; MOTUs) of non-target amplification. After bioinformatic processing, the remaining chironomid data did not allow for robust statistical analyses. Indications of contamination were obtained through the inclusion of positive and negative control samples during amplification of these chironomid replicates. Estimates of cross-contamination in the chironomid replicates were assumed to be representative of the other taxonomic groups, as the same methods, protocols, and plate layouts were used regardless of the targeted taxonomic group, and were subsequently used to correct for this contamination. For the chironomid replicates, six empty wells per plate were filled with four negative and two positive controls. Negative control wells contained milli-Q water (mQ) instead of template DNA. Positive controls instead consisted of DNA selected from the following specimens: (1) *Hylephila phyleus*, (2) *Microvelia* sp., (3) *Allograpta* cf. *fuscisquama*, and (4) *Lucillia* sp., all of which do not occur in the Netherlands, but are successfully amplified with the chironomid primers. As a result of their absence in the Dutch environment, genetic material of the positive controls should not appear in the samples if no inter-sample contamination took place. For the remaining three species groups, no positive controls were implemented, as the rate of cross-contamination was assumed to be the same for all markers. Therefore, the approximated contamination rate of the chironomid replicates was used for all markers.

Dual-indexed MiSeq amplicon libraries were prepared using a two-step PCR protocol. During the first-round PCR, taxon-specific primers tailed with 5′ Illumina adapters were used (Table S1). The PCR mix consisted of 14.4 μL mQ, 5.0 μL PCR buffer (Thermo Fisher, Waltham, MA, USA), 1.3 μL of both forward and reverse primers, 0.5 μL of dNTPs (concentration of 2.5 mM), 0.5 μL of Phire Hot Start II Polymerase (Thermo Fisher, Waltham, MA, USA), and 1.5 μL template DNA. The thermal-cycling regime was 98°C for 30 s, followed by 35 cycles of 98°C for 5 s, 50°C for 5 s, 72°C for 15 s, and a final extension of 72°C for 5 min. DNA was amplified in triplicate to address the imperfect detection, which is regularly observed in population surveys (Deiner et al. 2016; Schmidt et al. 2013).

The PCR products of the first round were checked on E-Gel 96 precast agarose gel (Thermo Fisher, Waltham, MA, USA). PCR replicates were then combined and cleaned with a one-sided size selection using NucleoMag NGS beads (Macherey-Nagel, Düren, Germany), in a 1:0.9 ratio. Amplification in the second-round PCR occurred in 20 μL reaction mixes, each consisting of 5 μL mQ (Ultrapure), 10 μL 1× TaqMan Environmental Master Mix 2.0 (Thermo Fisher, Waltham, MA, USA), 3 μL DNA, and 1 μL of both forward and reverse primers (each at 10 pmol μL^{-1}). The thermal-cycling regime was 95°C for 10 min, followed by 8 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 30 s, and a final extension of 72°C for 7 min. Concentrations were measured on a 5200 Fragment Analyzer (Advanced Analytical Technologies Inc., Orangeburg, NY, USA), and samples were pooled equimolarly by the QIAgility pooling robot. The pools were then cleaned with a one-sided size selection using NucleoMag NGS Beads (Macherey-Nagel, Düren, Germany), in a 1:0.9 ratio. Validation of the end pool was performed through electrophoresis using the TapeStation (Agilent Technologies Inc., Santa Clara, CA, USA). The samples were then sequenced on separate runs of an Illumina MiSeq (v3 Kit, 2°×°300 paired end) at BaseClear BV (Leiden, the Netherlands).

2.5 | Bioinformatics

Quality filtering and clustering occurred in a custom pipeline on the OpenStack environment of Naturalis Biodiversity Center through a Galaxy instance (Jalili et al. 2020). With the use of this pipeline, spurious sequences were removed (e.g., chimeric sequences, and other nonsensical by-products of the PCR workflow) and MOTUs were generated using pre-defined parameters for separation. The raw sequential data were merged using

FLASH v1.2.11 (minimum overlap 10 bp and mismatch ratio 0.25), after which primers were trimmed away using Cutadapt v.2.8 (maximum allowed error rate 0.2 and minimum match 5 bp; primer sequences in Table S1). Trimming windows were established to filter based on read length (248–254 bp for bacteria, 390–420 bp for phytoplankton, and 253–273 bp for diatoms), using corresponding literature (Chonova et al. 2019; Klindworth et al. 2013; Zimmermann, Jahn, and Gemeinholzer 2011) and visual analysis of read size distribution. The visualization of size distribution and subsequent trimming of sequences were both done using PRINSEQ v1.0. Through the application of these trimming windows, sequences of the targeted taxonomic groups were retained, as well as target sequences that displayed minute length variations as a result of, for example, primer slippage (Elbrecht, Hebert, and Steinke 2018). Dereplication and clustering of sequences into MOTUs (with a cluster identity of 98% and minimal accepted abundance of 2 reads (Beentjes et al. 2022)) was done using VSEARCH v2.14.2. The cluster identity percentage was kept consistent across all markers, as there was no indication that any of the markers needed a specific cutoff value. To compensate for potential inter-sample contamination in all taxonomic groups, MOTU tables were corrected using an observed spread rate of 0.054% in the chironomid eDNA dataset. For this spread correction, a tool based on work by Larsson et al. (2018) was used (https://github.com/sandberg-lab/Spreading-Correction), which excluded MOTUs from a sample with a MOTU occurrence lower than 0.054%. Absolute read abundance was then converted to relative read abundance by normalizing read counts per sample using Microsoft Excel 2013. Taxonomic assignment was established using an extended BLAST+ script (Beentjes et al. 2019b) in accordance with different databases for each biodiversity group. The R-Syst::diatom v7 database (Rimet et al. 2016) functioned as a reference for the diatom dataset, whereas for the phytoplanktonic and bacterial datasets, respectively, 18s of Genbank (Release 236; sequences downloaded March 5, 2020) (Benson et al. 2018) and the Silva SSU Parc (Release 138) (Quast et al. 2013) reference databases were consulted. Because of a low amount of species-level identifications, and as the consulted databases are incomplete, a custom lowest common ancestor (LCA) approach was followed for identification up to the genus level for the three taxonomic groups. A description of the custom LCA approach and its parameters can be found in Beentjes et al. (2019b). The output from the LCA was used to remove unidentified and nontarget MOTUs, as well as to visualize the taxonomic distribution. Further statistical analyses were performed using MOTU-level data.

2.6 | Statistical Analyses

Using the macrophyte biomass measurements, correlational models were built to test for possible spatial competition by assessing whether biomass could be affected by the other sampled macrophyte species. To capture broad differences in community composition within the macrophyte and chironomid datasets, nonparametric multivariate tests (PERMANOVA) with the Bray–Curtis dissimilarity index and 999 permutations were performed to evaluate dissimilarity in distance matrices, using the *adonis* function from the *vegan* package (Version 2.4-7) (Oksanen et al. 2020). The chironomid PERMANOVA captured the response to the different treatments, time, and their interactions. The macrophyte PERMANOVA included only the treatments and their interactions. Subsequently, a Bonferonni-corrected pairwise analysis was performed on the macrophyte PERMANOVA, using the *pairwise.adonis2* function from the *pairwiseAdonis* package (Adonis 2020).

Previous research has shown compositional dissimilarity to effectively reflect community response (Beentjes et al. 2021; Barmentlo et al. 2019). As such, to capture the broad effects of the treatments in community dissimilarity of phytoplankton, diatoms, and bacteria, a PERMANOVA was performed using 999 permutations and the Bray–Curtis dissimilarity index with the adonis function. The model captured the response of the remaining MOTUs due to the separate treatment components (crayfish and nutrient pollution), time (the sampling week numerically), and all possible interactions, while mesocosm location was added as strata to compensate for localized biases. Additionally, the sampling week of each replicate was added, as temporal species turnover accounted for a significant portion of the explained variance. At the end of the experimental campaign (Week 30), small juvenile crayfish were found in mesocosms that were not treated with crayfish. This observation specifically concerns one control replicate (Mesocosm Number 7) and four nutrient pollution replicates (Mesocosm Number 5, 11, 17, and 29). To account for the contamination of mesocosms with small juvenile crayfish into the experimental mesocosms, separate PERMANOVAs were performed per sampling week, wherein compromised mesocosms were removed prior to analysis. Dissimilarity matrices were created using the *vegdist* function from the *vegan* package, with the Bray–Curtis dissimilarity index, and were subsequently used as input for the creation of principal coordinates analysis (PcoA) plots (Figure S3) via the *pcoa* function from the *ape* package (Version 5.4-1) (Paradis and Schliep 2019). The degree of variation between the differently treated communities (i.e., beta dispersion) was investigated—at two time points after the treatments were applied—by testing the distances to their relative centroids (potential convergence/divergence after treatment) using the *betadisper* function (package *vegan*). This function analyzed multivariate homogeneity of group dispersion for each of the applied treatment components using the distance matrices created by *vegdist*. To test for significant differences in a multidimensional space between the treated replicates and the control replicates, distances to the control centroid were fitted using a linear model, after which a Dunnett's multiple comparison test was performed to test every replicate against the control centroid, using the *emmeans* and *contrast* functions from the *emmeans* package (Version 1.5.5-1) (Lenth 2021). All eDNA and chironomid statistical analyses were performed in R Version 4.0.3. All macrophyte analyses were separately performed in R Version 4.0.2.

3 | Results

3.1 | Sequence Run Statistics and Contaminants

Overall, the total eDNA–based assessment yielded 14,575 MOTUs for bacteria, 7389 MOTUs for diatoms, and 8959 MOTUs for phytoplankton. After performing the LCA, filtering, and a final check for read depletion, 2647 bacterial, 1660 diatom, and 1737 phytoplanktonic MOTUs remained for analysis. The most prominent

phyla within the bacterial data were Proteobacteria (989 MOTUs, 37.4% of total MOTUs) and Cyanobacteria (483 MOTUs, 18.2% of total MOTUs). The vast majority of Proteobacteria (773 MOTUs, 78.2% of Proteobacteria MOTUs) originated from the class Gammaproteobacteria. The most abundant phytoplankton taxa were Chrysophyceae (544 MOTUs, 31.3% of total MOTUs), Bacillariophyta (342 MOTUs, 19.7% of total MOTUs), and Chlorophyta (240 MOTUs, 13.8% of total MOTUs). The largest groups within the diatom data were the orders Bacillariales (513 MOTUs, 30.9% of total MOTUs), Fragilariales (156 MOTUs, 9.4% of total MOTUs), and Naviculales (125 MOTUs, 7.5% of total MOTUs). Another 38.6% of the dataset (640 MOTUs) could not be identified beyond Bacillariophyceae, the taxonomic class synonymous with the diatom clade.

Of the eDNA data, three samples showed only a small amount of product for the phytoplankton amplicons. The impact of these three phytoplankton samples was tested by repeating the PERMANOVAs while omitting them; however, this did not alter the results. Implemented negative controls presented negligible read counts relative to the sample contents after filtering. In the diatom and phytoplankton datasets, respectively, negative control samples contained an average of 27 reads (with 23,531–21,652 average reads per mesocosm sample). The bacterial negative controls presented high read counts of genus *Ralstonia*, which has been reported as a known contaminant of reagents used for DNA analysis (Salter et al. 2014). These MOTUs were discarded from the dataset prior to analysis. In a similar fashion, MOTUs that were predominantly present only in the negative controls were removed from the dataset, as these were assumed to represent similar contaminants.

3.2 | Macrophyte Results

Generally, the results indicate pronounced effects of nutrient pollution on all macrophyte taxa, and species-specific yet significant effects of *P. clarkii*. The results demonstrate a significant negative effect of FLABs on the other macrophyte species tested ($p = 0.0074$). Biomass samples (dry weight measurements of every species) were analyzed for every treatment, for each species individually as well as in combination, to study compositional change among the four aquatic plant species.

FLABs were positively affected by the nutrient pollution component when compared to the control, as the biomass of the FLABs (Figure 1A) was significantly higher than the control in both the nutrient pollution ($F = 6.307$, $p < 0.001$) and crayfish:nutrient pollution ($F = 4.437$, $p < 0.01$) treatments. With regards to *E. nuttallii* (Figure 1B), in the individual species analysis, a significantly lower biomass in the replicates treated with crayfish:nutrient pollution was detected compared to the control ($F = -2.444$, $p < 0.05$). *C. globularis* (Figure 1C) biomass was significantly lower in the control than in both the nutrient pollution ($F = -3.570$, $p < 0.05$) and crayfish:nutrient pollution replicates ($F = -3.317$, $p < 0.05$). Biomass of *P. berchtoldii* (Figure 1D) was significantly lower in both the crayfish mesocosms ($F = -2.818$, $p < 0.05$) and the combined crayfish:nutrient pollution mesocosms ($F = -3.024$, $p < 0.05$) when compared to the control. All of the significant results for the crayfish:nutrient pollution treatment were in line with the correlation models, which checked for spatial competition. The mesocosms treated with nutrient pollution and with crayfish:nutrient

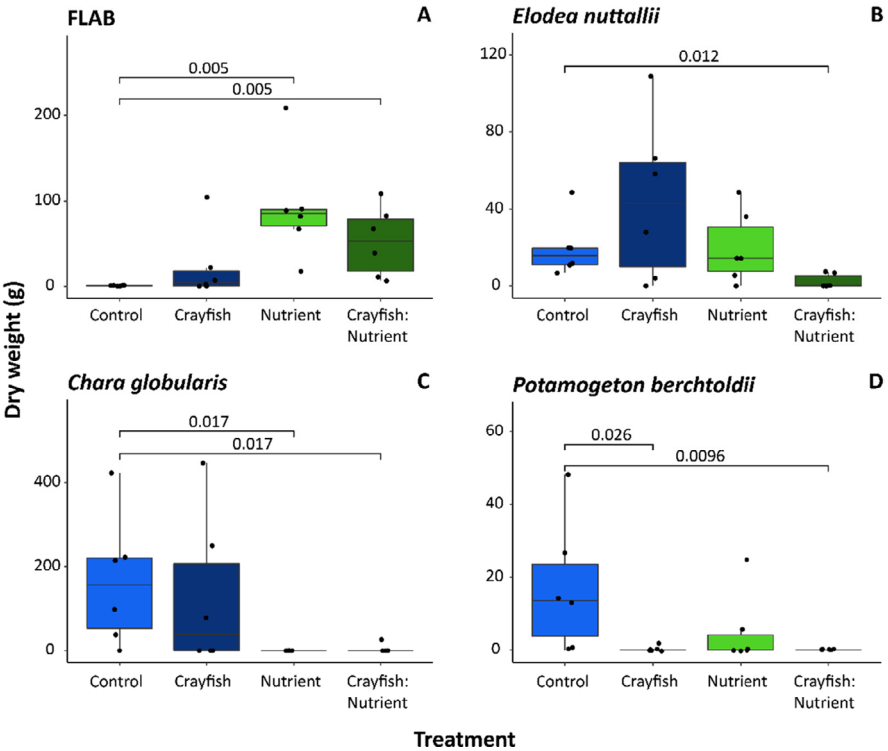

FIGURE 1 | Biomass dry weight measurements (12 weeks after the initiation of the experiment) in grams separated per treatment for four aquatic plant species: (A) Filamentous algae beds, (B) *Elodea nuttallii*, (C) *Chara globularis*, and (D) *Potamogeton berchtoldii*. Statistically significant output of Wilcoxon's signed-rank tests are shown via brackets above the boxplots.

pollution were found to be significantly dissimilar compared to the control ($F = 9.5825$ and $F = 8.1072$, respectively).

3.3 | Chironomid Results

From the PERMANOVA, only the sampling time was found to have a significant effect on community dissimilarity of the morphologically identified chironomids ($p = 0.004$; Table S2), which suggests general species turnover over the course of the experiment. No treatment interactions were found to have impacted the structure of the chironomid community.

3.4 | eDNA Results

A marginal effect of *P. clarkii* on the community composition was detected for all three eDNA–targeted taxonomic groups in the general PERMANOVA ($p < 0.001$; Table S2), with an R (Dudgeon et al. 2006) equal to or less than 0.01 for all taxonomic groups. In a separate PERMANOVA, which was implemented for every time point (Table 1), the crayfish component did not significantly explain any variation in the composition of bacteria, diatoms, or phytoplankton. No significant effects were recorded in the beta dispersion of the community for the treatments containing crayfish (Figure 2). In addition, community composition in the crayfish treatment did not diverge significantly from the community composition of the control according to Dunnett's multiple comparison test (Figure 3) for any of the taxonomic groups at any time point.

The nutrient pollution treatment, on the other hand, strongly affected dissimilarity within the community compositions (Table S2). Beta dispersion in treatments containing nutrient pollution was significantly lower for the phytoplankton communities ($p < 0.05$; indicating convergence of the replicates) and significantly higher for the bacteria communities ($p < 0.05$; indicating divergence of the replicates) (Figure 2). Furthermore, as the community compositions resulting from the nutrient pollution treatments diverged from the control centroid (Figure 3), Dunnett's multiple comparison test also yielded significant results for both the nutrient pollution and crayfish:nutrient pollution treatments in Weeks 22, 24, and 27 for the phytoplankton and diatom communities, along with Week 30 for the diatoms. In the bacterial communities, distance to the control centroid was significantly distinguishable from the control replicates in Weeks 22 ($p < 0.01$) and 24 ($p < 0.05$) for the nutrient pollution treatment. Additionally, in Weeks 24 ($p < 0.001$) and 27 ($p < 0.05$), the crayfish:nutrient pollution treatment replicates were significantly distanced from the control centroid. Distance to the control centroid peaked in Week 24 for the single and combined nutrient pollution treatments in all taxonomic groups. In the diatom group, both the nutrient pollution and crayfish:nutrient pollution treatments maintained a significant distance to the control centroid in subsequent weeks (Dunnett's multiple comparison test: $p < 0.001$ for both nutrient pollution treatments), whereas the significant distance to the control centroid gradually dissipated in the final weeks (27 and 30) for the bacteria and phytoplankton groups.

A slight interactive effect of *P. clarkii* in combination with the nutrient pollution treatment was found in the general

TABLE 1 | PERMANOVA results (F-statistic, R^2, and p values) analyzed separately for all time points for the different treatments. *Procambarus clarkii* was introduced in Week 22, and nutrients were added in Weeks 18 and 23. Data of Weeks 18 and 22 was gathered prior to treatment introduction. The significant values are highlighted in bold.

		Phytoplankton			Diatoms			Bacteria		
		F	***R²***	***p***	***F***	***R²***	***p***	***F***	***R²***	***p***
Week 18	Crayfish	0.707	0.032	0.747	0.069	0.003	0.998	0.414	0.020	0.924
	Nutrients	0.717	0.033	0.655	0.496	0.024	0.695	0.386	0.018	0.950
	Crayfish:nutrients	0.628	0.028	0.806	0.464	0.022	0.739	0.364	0.017	0.957
Week 22	Crayfish	0.876	0.032	0.575	1.848	0.061	0.094	0.993	0.035	0.378
	Nutrients	5.531	0.203	**<0.001**	7.330	0.242	**<0.001**	6.478	0.226	**<0.001**
	Crayfish:nutrients	0.840	0.031	0.635	1.065	0.035	0.334	1.173	0.041	0.251
Week 24	Crayfish	0.965	0.031	0.378	1.568	0.050	0.141	1.407	0.049	0.157
	Nutrients	9.244	0.296	**<0.001**	8.944	0.285	**<0.001**	6.653	0.230	**<0.001**
	Crayfish:nutrients	1.007	0.032	0.337	0.848	0.027	0.481	0.803	0.028	0.590
Week 27	Crayfish	0.665	0.027	0.885	1.146	0.040	0.290	1.389	0.055	0.125
	Nutrients	2.822	0.117	**<0.001**	5.761	0.203	**<0.001**	3.285	0.129	**0.002**
	Crayfish:nutrients	0.733	0.030	0.800	1.444	0.051	0.168[a]	0.754	0.030	0.747
Week 30	Crayfish	0.802	0.032	0.714	0.515	0.018	0.898	0.944	0.039	0.490
	Nutrients	2.752	0.111	**0.002**	7.150	0.251	**<0.001**	1.350	0.055	0.135
	Crayfish:nutrients	1.342	0.054	0.171	0.855	0.030	0.558	2.093	0.086	**0.009**

[a]This value changed to $p = 0.047$ after the removal of the compromised mesocosm replicates from the analysis (see Section 4).

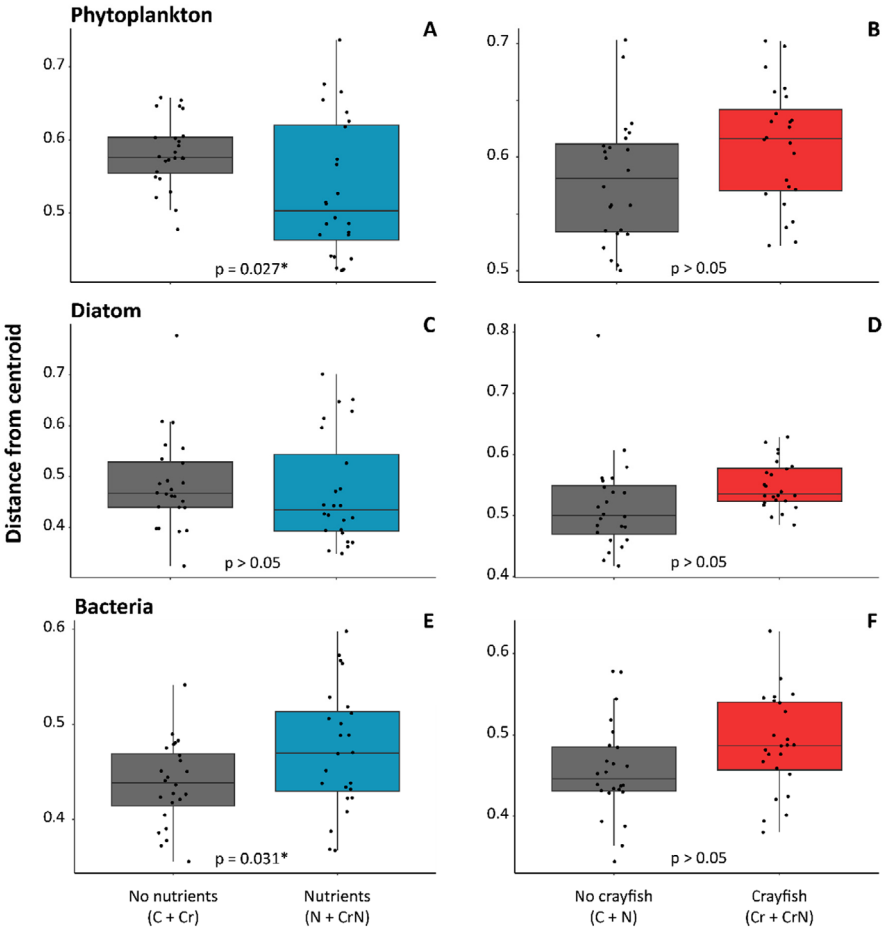

FIGURE 2 | Beta dispersion in Weeks 24 and 27 after being exposed to the different treatments. The phytoplankton community after nutrient pollution treatment (A) and crayfish treatment (B). Diatoms under the nutrient pollution treatment (C) and the crayfish treatment (D). Bacteria under the nutrient pollution treatment (E) and the crayfish treatment (F). Significant *p* values were obtained using an ANOVA, and are provided in the corresponding panels. C = control, Cr = crayfish, CrN = crayfish and nutrient pollution, *N* = nutrient pollution. **p* < 0.05.

PERMANOVA ($p < 0.001$ for all taxonomic groups, phytoplankton: $F = 1.021$, $R^2 = 0.007$; diatoms: $F = 1.563$, $R^2 = 0.010$; bacteria: $F = 0.928$, $R^2 = 0.006$; Table S2). In the secondary PERMANOVA per distinct time point, besides in Week 30 for the bacterial community ($p = 0.009$), no interactions could explain additional dissimilarity as they presented insignificant *p* values (Table 1). After removal of the compromised ditches, a single additional interaction between the nutrient pollution and crayfish treatments was found to be significant in Week 27 for the diatom community ($p = 0.047$). The distance to the control centroid for the nutrient pollution and crayfish:nutrient pollution treatments proceeded similarly through the time points in all taxonomic groups (Figure 3A,B,E), without any synergistic, antagonistic, or additive interactions. Comparatively, the community composition within crayfish:nutrient pollution replicates generally diverged more from the control centroids than the crayfish replicates for the three microbial groups in the weeks subsequent to treatment application, with the exception of Week 30 (for bacteria and phytoplankton).

The effect of time on community dissimilarity was observed in all taxonomic groups (phytoplankton: $F = 13.245$, $R^2 = 0.096$, $p < 0.001$; diatoms: $F = 19.774$, $R^2 = 0.126$, $p < 0.001$; bacteria: $F = 31.093$, $R^2 = 0.202$, $p < 0.001$). An interaction between the nutrient pollution treatment and time resulted in significant dissimilarity in community structure of the phytoplankton and diatoms (phytoplankton: $F = 2.008$, $R^2 = 0.014$, $p = 0.007$; diatoms: $F = 3.940$, $R^2 = 0.025$, $p = 0.002$). In contrast, no interaction between crayfish and time was found to significantly affect community composition in any of the three groups. A potential three-way interaction between crayfish, nutrient pollution, and time yielded insignificant *p* values for all three taxonomic groups.

4 | Discussion

This study aimed at determining the potential impact of a globally relevant invasive crayfish species, *P. clarkii*, relative to that of another ubiquitous stressor, nutrient pollution, which is strongly associated with eutrophication in aquatic ecosystems. The impact of both stressors on ecosystem structure was tested by monitoring the chironomid, macrophyte, and microbial communities. In line with previous studies, our results revealed small, yet significant effects of *P. clarkii* on the macrophyte composition (Matsuzaki et al. 2009; Gherardi and Acquistapace 2007; Ilhéu, Guilherme, and Bernardo 2002; van der Wal et al. 2013). Conversely, our results demonstrated small to nonexistent effects of *P. clarkii* on bacteria, phytoplankton, or diatom communities in the 8-week time

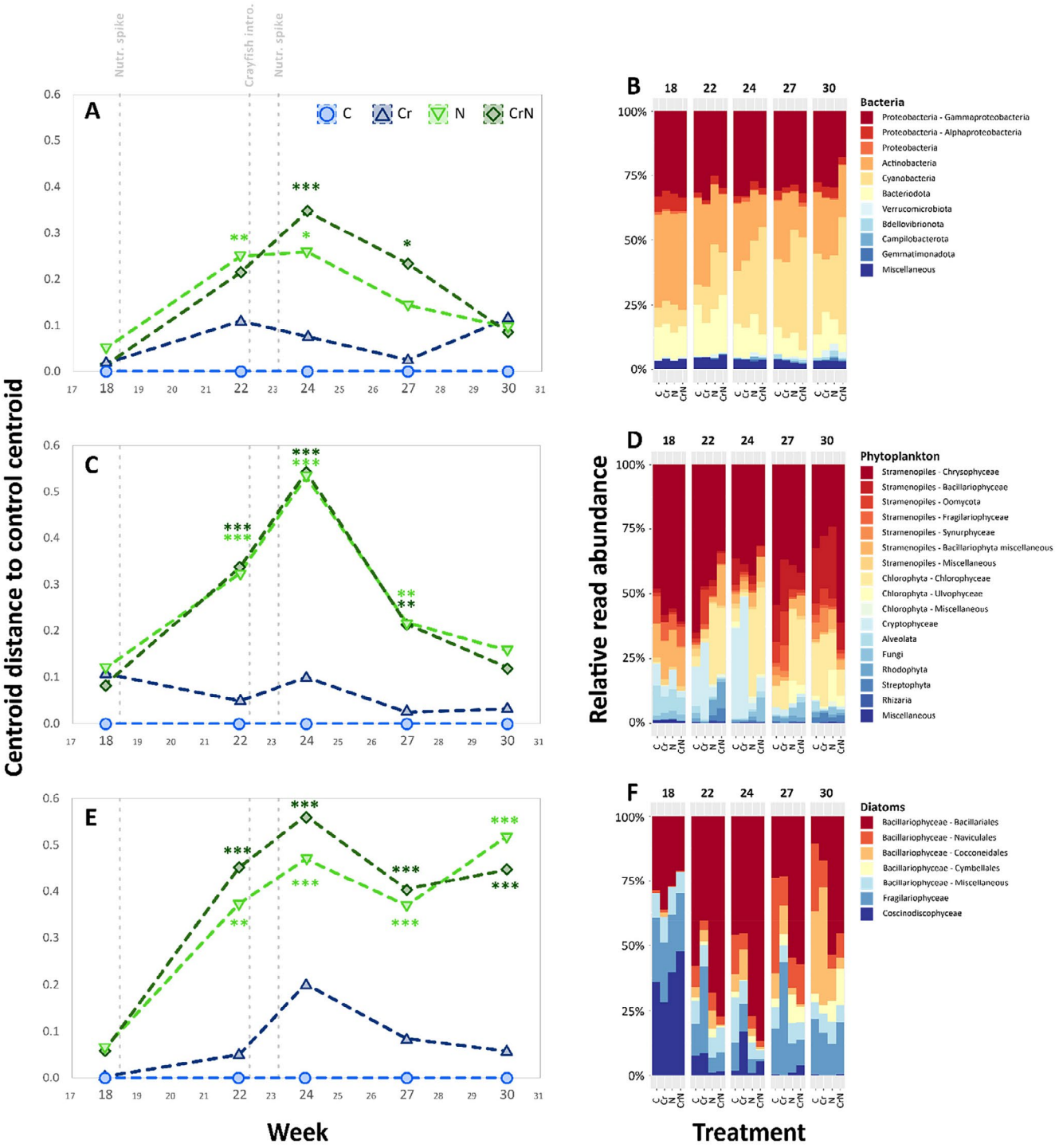

FIGURE 3 | Centroid distance to the control centroid (Principal Coordinates Axes 1 and 2) and relative read abundance taxonomic bar plots per week for each of the taxonomic groups: (A, B) Bacteria, (C, D) phytoplankton, and (E, F) diatoms. Treatments were applied in Weeks 18 (nutrient pollution), 22 (crayfish), and 23 (nutrient pollution). *p* values were from Dunnett's multiple comparison test. C = control, Cr = crayfish, CrN = crayfish and nutrient pollution, *N* = nutrient pollution. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

span after introduction, whereas the effect of nutrient pollution appeared to be a pivotal driver of community composition of each of the aforementioned microbial taxonomic groups. These results indicate that in these experimental mesocosms, *P. clarkii* was a less important driver of community change than previously proposed, and further suggest that it could be a mere passenger, at least in the short term.

4.1 | Unexpected Guests

While the cages in the experimental enclosures were successful in enclosing the introduced crayfish, some cages were colonized by extra crayfish, which potentially impacted our results. In a study by Alcorlo, Geiger, and Otero (2008), it was noted that small *P. clarkii* juveniles passed through their traps (mesh

size of 6 mm) and would unintentionally remain uncaught. Comparably, early-juvenile *P. clarkii* individuals were likely able to enter the cages used in this study (mesh size of 6.3 mm), as several extra individuals were found in a few untreated mesocosm replicates at the end of the experiment. Most of the extra individuals were relatively small sized when compared to the manually introduced full-grown female crayfish, and their impact on the outcome of the study was therefore likely marginal. To test for the possible effect of the trespassing crayfish, separate PERMANOVAs were run after removal of all compromised mesocosms. The results from this secondary dataset closely resembled the ones that were reported from the primary dataset. The only observable difference was an additional statistically significant interaction between the crayfish and nutrient pollution treatments in Week 27 of the diatom group.

4.2 | Macrophyte Composition

Our results show that *P. clarkii* had a negative effect on the biomass of rooted aquatic plant species *P. berchtoldii*, but no significant effect was found on the other macrophyte taxa. Macrophyte consumption by crayfish has been reported to be selective (Cirujano, Camargo, and Gómez-Cordovés 2004). Carreira, Dias, and Rebelo (2014) further proposed the hypothesis that *P. clarkii* may remove macrophytes sequentially, from the most to the least preferred species, thereby differentially affecting systems based on their respective macrophyte compositions. Unlike *E. nuttallii, P. berchtoldii*, and *C. globularis* are macrophytes that strongly depend on their root system and die after the stem has been separated from the root. This might make them vulnerable targets for the snipping behavior of *P. clarkii*, regardless of consumption. In contrast, a decrease in biomass of *E. nuttallii* was only observed in the combined crayfish:nutrient pollution treatment, thus suggesting that this macrophyte species may readily recover at lower grazing pressures. It appears that, as *P. berchtoldii* and *C. globularis* became unavailable to *P. clarkii* in the crayfish:nutrient pollution replicates, the flexible focus of *P. clarkii* shifted to *E. nuttallii*. More than the presence of *P. clarkii*, nutrient pollution treatments significantly drove macrophyte composition, inducing higher amounts of FLAB biomass, which in turn negatively influenced the remaining macrophyte species. The observed response, where submerged macrophytes are replaced by FLAB, is typical for shifts toward a eutrophic or hypertrophic state (Folke et al. 2004; Rader and Richardson 1992; Scheffer et al. 1993, 2001; Smith, Tilman, and Nekola 1999), which has been attributed to severe light competition from floating algae with submerged vegetation (Scheffer et al. 1993; Bornette and Puijalon 2009; Strand and Weisner 1996). Overall, the macrophyte-related results suggest that *P. clarkii* likely had a subordinate role compared to nutrient pollution in shaping the freshwater macrophyte communities under the imposed treatment levels and experimental conditions.

4.3 | The Impact of Nutrient Pollution on Microbial Taxa

The nutrient pollution treatment induced significant shifts in community structure of the monitored microbial communities compared to the control mesocosms. Communities exposed to nutrient pollution peaked in dissimilarity in Week 24 when compared to the control replicates, which coincided with—and was likely caused by—the additional nutrient pollution spike in Week 23. The impact of nutrient pollution demonstrated here is in line with previous work by Beentjes et al. (2021). In their study, similar quantities of nutrient additions triggered significant dissimilarity in bacteria and phytoplankton communities in a comparable fashion. As in the study by Beentjes et al. (2021), bacterial communities diverged in the replicates treated with nutrient pollution as they exhibited significantly higher beta dispersion compared to the replicates untreated with nutrient pollution. Conversely, in our study, phytoplankton communities converged in replicate mesocosms treated with nutrient pollution compared to the control mesocosms. The latter is surprising, as phytoplankton communities are expected to diverge due to higher nutrient availability and increased productivity. The ubiquity of eutrophication and its clear effect on aquatic microbial community compositions highlights the importance of addressing this issue. Even more so because it remains unclear how these shifts in microbial compositions might interact with other stressors (e.g., invasive species) to impact the ecosystem.

4.4 | The Impact of *P. clarkii*

Time was shown to be the most influential explanatory variable for the observed dissimilarity in both the microbial and chironomid community compositions, which suggests high temporal species turnover. This was to be expected, as freshwater systems are known to exhibit strong seasonal variation (Barmentlo et al. 2019; Kratina et al. 2012; Michiels and Traunspurger 2005). Simultaneous sampling of the replicate mesocosms in this seminatural setup allowed for adequate separation between the various effects and regular species turnover. As a result, our study revealed a marginal impact of *P. clarkii* on the composition of the microbial taxa. The removal of time as a major variable, by analyzing time points separately, removed the observed marginal effects by *P. clarkii*. There was no evidence suggesting divergence or convergence of the tested microbial community composition after exposure to *P. clarkii*. In comparison, a study by Matsuzaki et al. (2009) also reported a small significant effect of *P. clarkii* on phytoplankton. It should be noted that our study used a substantially lower crayfish density of 2 individuals m^{-2}, which is 1.5–4.5 times lower than the density used in Matsuzaki et al. (2009). It is plausible that the weak effect of *P. clarkii* on the phytoplankton community, reported in Matsuzaki et al. (2009), did not manifest in some of the analyses in the current study due to these differences in density. Even though the density of crayfish used here was relatively low, it fits into a density range of 0.2–15 individuals m^{-2} recorded in natural settings (Gherardi and Acquistapace 2007; Maezono et al. 2005; Rhodes and Avault 1986). Similarly, the data collected from the chironomid community did not suggest an impact of crayfish on community composition, and, in accordance with Beentjes et al. (2021), no impact of nutrient pollution on the chironomid community was found either. As this experiment mainly addressed the impact of *P. clarkii* on microbial taxa, no definitive conclusions should be drawn for the potential effects on more diverse macrofauna

communities. However, the absence of shifts in the microbial communities indicates that conditions in the experimental mesocosms likely did not change to an extent that would affect other macrofauna, as microbial taxa are particularly sensitive to environmental change (Beentjes et al. 2021; Muylaert, Sabbe, and Vyverman 2009; Rath et al. 2019; Waldrop and Firestone 2006; Lee, Rollwagen-Bollens, and Bollens 2015). It should be noted that this does not rule out the possibility of impacts by *P. clarkii* on the macrofaunal community through other means, for example, through direct predation.

P. clarkii has frequently been reported to detrimentally shift communities in invaded habitats by (in)directly affecting multiple trophic levels within a food web (Souty-Grosset et al. 2016; Dorn and Wojdak 2004). Moreover, considering the reported ecosystem engineering capacity of *P. clarkii*, it is expected that this engineering behavior is reflected in the composition of taxa that are highly sensitive to environmental change. Yet, our findings suggest that the impact of *P. clarkii*, under these experimental conditions, was not significant enough at the short term to affect the tested chironomid and microbial communities at a density of 2 individuals m^{-2}. It should be noted that the experimental conditions might have restricted some aspects of *P. clarkii*'s natural behavior and, consequently, its potential impact. Besides the pathways of impact tested here, others remained beyond the scope of this study. For instance, *P. clarkii* is a known vector of various parasites, such as the crayfish plague (*Aphanomyces astaci*), which is a leading cause of native crayfish decline in Europe, and *Batrachochytrium dendrobatidis*, which causes lethal skin infections among amphibians (Souty-Grosset et al. 2016). Additionally, the burrowing behavior of *P. clarkii* may lead to channel and river bank erosion, and therefore may require sustained consideration depending on the specific environmental context. Other relevant pathways of impact include the predation of *P. clarkii* on local biodiversity and its competition with native species. Although the experimental design did not address *P. clarkii*'s burrowing behavior, the competition with native species, and its role as a vector for disease, these factors remain relevant for the design of management strategies. Therefore, management decisions should not rely solely on the results presented here but should be informed by the broader ecological context, including factors that were beyond the scope of this study. Additionally, these results do not eliminate the possibility of long-term proliferation of *P. clarkii* and a concurrent increased influence on microbial communities. In the long term, the alteration of the macrophyte community by *P. clarkii* may indirectly shape the communities of non-macrophyte biodiversity groups. Further experimental work is required to test any long-term effects, ideally by increasing the length of a similar experiment to at least a year. Assuming that an extension of the studied timescale or an increase of *P. clarkii* density were to lead to structural changes in the composition of indicative taxa, our study provides threshold values of timescale and density at which the impact of *P. clarkii* appears to remain marginal. Keeping in mind the experimental conditions and constraints, these threshold values may be leveraged in the planning and implementation of freshwater management actions, such as rapid response programs and population control measures, to prevent potential adverse effects of *P. clarkii* invasions on ecosystem structure.

5 | Conclusion

To conclude, besides the impact of *P. clarkii* on the macrophyte composition, there was no evidence of major short-term shifts in the chironomid and microbial community composition caused by the presence of *P. clarkii* in a local experimental freshwater ecosystem. In contrast, our results suggested a major impact of commonly found levels of nutrient pollution on every tested taxonomic group in the same system. These findings re-emphasize the impact of nutrient pollution on freshwater communities, placing it firmly on the agenda of freshwater ecosystem management. Under the assumption that higher crayfish densities would effectively shape community compositions, this study provides a minimum density of 2 individuals m^{-2}, at which *P. clarkii* was inadequate at significantly shaping the tested chironomid and microbial community compositions within the investigated timescale. While carefully considering the experimental conditions and constraints, the investigated timescale and crayfish density may inform the planning and implementation of freshwater management actions to prevent potential changes in ecosystem structure caused by *P. clarkii*. Given our findings, we encourage future research to address the timescales and densities at which *P. clarkii* could drive ecosystem change. Simultaneously, long-term experimental work could further elucidate whether *P. clarkii* suits the driver or the passenger model.

Author Contributions

Jelle A. Dercksen: writing – original draft, formal analysis, investigation, visualization. **Maarten J. J. Schrama:** writing – original draft, conceptualization, supervision. **Kevin K. Beentjes:** writing – review and editing, visualization, supervision. **Bob N. Bastiaans:** writing – review and editing, formal analysis, investigation, visualization. **Rody Blom:** writing – review and editing, investigation, supervision. **André van Roon:** writing – review and editing. **Peter W. Lindenburg:** writing – review and editing, funding acquisition. **Krijn B. Trimbos:** writing – original draft, conceptualization, supervision.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw NGS output is available at Dryad: doi: 10.5061/dryad.kd51c5bh9.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.