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Aquatic macroinvertebrate community responses to pollution of perfluoroalkyl substances (PFAS): Can we define threshold body burdens?

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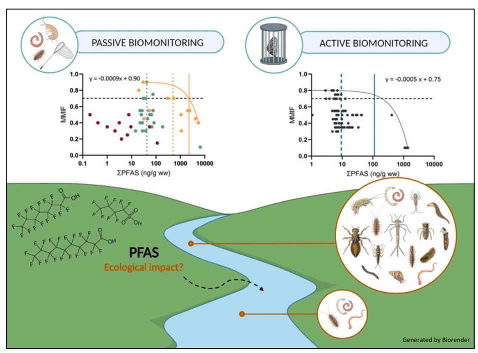
HIGHLIGHTS

- Field-based effect studies of PFAS on aquatic communities are generally lacking.
- PFAS in macroinvertebrates and mussels associate with a reduced ecological quality.
- First study reporting threshold body burdens for a set of PFAS in invertebrates

GRAPHICAL ABSTRACT



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ABSTRACT

The pollution of *per-* and polyfluorinated alkyl substances (PFAS) in aquatic environments is a worldwide concern of which the ecological impact is still not well understood. Especially field-based effect studies in aquatic ecosystems are generally lacking, creating a knowledge gap that goes along with monitoring and regulatory challenges. Therefore, this study examined if bioaccumulated PFAS concentrations could be related to ecological responses assessed by changes in the macroinvertebrate community structure. In addition, threshold body burdens that are protective of ecological damage were estimated. Aquatic macroinvertebrates were sampled in 30 streams across Flanders (Belgium) and 28 PFAS target analytes were measured in three resident taxa (*Gammarus* sp., *Asellus* sp. and *Chironomus* sp.) and translocated zebra mussels (*Dreissena polymorpha*). The macroinvertebrate community structure was assessed by calculating the Multimetric Macroinvertebrate Index Flanders (MMIF). Primarily long-chain perfluorinated carboxylic acids (PFCAs) were detected in both resident taxa (passive biomonitoring) and zebra mussels (active biomonitoring). Based on a 90th quantile regression model, safe threshold body burdens could be calculated for PFTeDA (7.1 ng/g ww) and ΣPFAS (2264 ng/g ww) in *Gammarus* sp. and for PFOA (5.5 ng/g ww), PFDoDA (1.7 ng/g ww), PFTrDA (0.51 ng/g ww), PFTeDA (2.4 ng/g ww), PFOS (644 ng/g ww) and ΣPFAS (133 ng/g ww) in zebra mussel. An additional threshold value was calculated for most compounds and species using the 95th percentile method. However, although these estimated thresholds are pertinent and indicative, regulatory applicability requires further lines of evidence and validation. Nevertheless, this study offers first-time evidence of associations between accumulated PFAS concentrations in

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invertebrates and a reduced ecological water quality in terms of macroinvertebrate community structure and highlights the potential of *Gammarus* sp. and zebra mussels to serve as reliable PFAS biomonitoring species.

1. Introduction

The pollution of *per*- and polyfluorinated alkyl substances (PFAS) in aquatic environments is a significant global environmental concern with complex regulatory challenges (Ankley et al., 2021). PFAS, characterized by strong carbon-fluorine bonds and an amphiphilic nature, are water and oil repellent and exhibit resistance to heat making them highly useful in various industrial and commercial applications since the 1940s (Buck et al., 2011). However, the widespread application of PFAS has resulted in both direct and indirect releases into the environment. Once in the environment, PFAS are persistent and tend to bioaccumulate in aquatic organisms (Houde et al., 2011). While most scientific studies have primarily focused on the distribution and bioaccumulation of PFAS, the ecological impact of PFAS contamination on aquatic communities has received comparatively less attention (Ankley et al., 2021).

This limited understanding is coupled with a lack of regulatory measures, particularly the derivation and implementation of appropriate quality standards (QS). So far, in Europe, biota and environmental quality standards have only been established for a very limited subset of PFAS compounds, despite the production of over 7 million compounds (Schymanski et al., 2023). Additionally, the Biota Quality Standard (BQS) for perfluorooctane sulfonic acid (PFOS; 9.1 ng/g ww) primarily aims to protect human health and to prevent secondary poisoning of top predators but does not consider the protection of aquatic communities (European Union, 2013). In October 2022, the European Commission proposed new thresholds for PFAS in biota ($QS_{biota,sec\ pois}$), in particular for fish (22.3 μg/kg ww) and bivalves (6.2 μg/kg ww) expressed as PFOA equivalents (SCHEER, 2022). However, these BQSs were also derived based on a secondary poisoning assessment. For the protection of human health, a more stringent BQS of 0.077 μg/kg ww has been derived, also expressed in PFOA equivalents (SCHEER, 2022). So far, no BQS has been derived for the protection of aquatic organisms at community or ecosystem level.

Furthermore, the monitoring of PFAS contamination predominantly relies on measurements taken from the abiotic environment, such as water and sediment (Kördel et al., 2013). However, these measurements only provide a momentary snapshot of the pollution status and fail to account for variations in bioavailability caused by fluctuating abiotic (e.g. water hardness, temperature, …) and biotic factors (e.g. feeding mode, physiological status, …) (Van Ael et al., 2017). As a result, derived water and sediment quality criteria may not always be adequate for the protection of aquatic communities. The monitoring of PFAS in biota can address the limitations of monitoring solely in the abiotic environment by considering bioaccumulated levels that integrate over time and reflect the bioavailability of PFAS. Two different biomonitoring approaches can be distinguished, namely passive and active biomonitoring. Passive biomonitoring (PBM) includes the collection of indigenous organisms as representatives of the in-situ conditions (Lacroix et al., 2015). Benthic macroinvertebrates have been considered to be a good indicator group to conduct passive biomonitoring as they are sedentary, easy to collect and identify, they can integrate fluctuations of environmental conditions, and have a wide geographical abundance (Resh et al., 1995; Basset et al., 2004; Li et al., 2010; Lin et al., 2020). They comprise a diverse group of organisms that play a crucial role in freshwater ecosystems by facilitating essential ecological processes, including nutrient cycling through organic matter decomposition, sediment mixing, and serving as a pivotal link in the aquatic food web (Nieto et al., 2017). Given that certain invertebrate taxa are more sensitive to pollution than others, the composition and structure of macroinvertebrate communities can serve as an indicator of the cumulative impact of both short-term and long-term pollution events (López-López and Sedeño-Díaz, 2015). Several studies on metal pollution demonstrated that bioaccumulated metal concentrations in aquatic macroinvertebrates can be associated with the macroinvertebrate community structure, offering potential for deriving biota quality standards that provide community-level protection (De Jonge et al., 2015; Rainbow et al., 2012; Bervoets et al., 2016; Méndez-Fernández et al., 2017). Active biomonitoring (ABM), on the other hand, refers to translocating caged organisms collected from a reference population to a specific site of interest (Wepener, 2013). After a fixed period of exposure, the biota will accumulate a contaminant profile that reflects the state of the local environment. Several animal groups can be used for ABM in aquatic environment including bivalves, crustaceans and fish (Moschino et al., 2016; Bertrand et al., 2018; Santana et al., 2018; Teunen et al., 2021). One of the most commonly used species in Europe is the zebra mussel (*Dreissena polymorpha*), which has been shown to have a high uptake efficiency for metals and Persistent Organic Pollutants (POPs) and is tolerant to a wide range of contaminants (Smolders et al., 2002; Riva et al., 2008; Besse et al., 2012; Evariste et al., 2018). Both PBM and ABM have their advantages and disadvantages. Therefore, it is suggested to integrate both methods in order to achieve a comprehensive picture.

To date, most knowledge on the ecological effects of PFAS is based on laboratory toxicity data, while field-based effect studies are generally lacking (Ankley et al., 2021). In order to understand the potential effects of PFAS on wildlife, it is necessary to integrate field studies that examine the effects of PFAS at a community and population level. Therefore, the objective of the present study is first to study the relationship between bioaccumulated PFAS concentrations (in macroinvertebrates and mussels) and the ecological responses assessed as the macroinvertebrate community structure. Secondly, we aim to evaluate the applicability of zebra mussels as ABM for PFAS pollution. Thirdly, we aim to determine wether this approach is suitable to derive threshold body burdens that are protective of ecological damage. To the best of our knowledge this is the third field-based effect study that has ever been conducted on the macroinvertebrate community responses to PFAS pollution (Bakke et al., 2010; Rusconi et al., 2015).

2. Material and methods

2.1. Passive and active biomonitoring

Between September 2020 and December 2020, as well as from September 2021 to November 2021, resident macroinvertebrates were collected from 30 sites located in small rivers and streams across Flanders, Belgium (Fig. 1; Table A.1). The recommended sampling period for macroinvertebrates by Gabriels et al. (2010) is spring, summer and autumn to avoid extreme regimes and temperatures. However, weather in Belgium is shifting towards milder winters and warmer summers, culminating in unprecedented temperature records during the summer months (De ridder et al., 2020). Therefore, the sampling period of the present study was selected to avoid extreme weather conditions during summer. Sampling was conducted by collecting a 10-meter stretch at each site using a standard hand net with a mesh size of 0.5 μm. The kick sampling technique, as outlined by De Pauw and Vanhooren (1983) was employed to ensure the capture of a diverse range of macroinvertebrates. In the laboratory, all samples were carefully sorted, and the three most abundant invertebrate species, namely larvae of the non-biting midge *Chironomus* sp., the isopod *Asellus* sp., and the amphipod *Gammarus* sp., were selected and stored in a freezer at −20 °C for subsequent PFAS analysis. All other invertebrate taxa were preserved in 70 % ethanol to facilitate further identification.

To ensure an appropriate representation of individuals from the same species across all locations, active biomonitoring was also employed. Zebra mussels (*Dreissena polymorpha*) were exposed at 23 sites, which coincided with the locations where resident taxa were sampled (Fig. 1). In order to minimise potential differences in bioavailability and to allow for comparison, mussels were exposed during the same macroinvertebrate sampling period. The mussels were sourced from a reference lake, the Blaarmeersen in Gent, known for its low background concentrations of pollutants (Teunen et al., 2021). Upon collection, the mussels underwent a two-week acclimatization period in artificial ponds containing dechlorinated tap water at the mesocosm facility of the University of Antwerp. Subsequently, the mussels were exposed for a duration of six weeks in cages constructed from polyethylene pond baskets (11 × 11 × 22 cm) with a mesh size of 2 × 4 mm, facilitating water circulation within the cages (Smolders et al., 2002). In total, 15 individual mussels were exposed per site. The cages were positioned approximately 20–30 cm above the bottom to prevent sediment accumulation. As previously described by Bervoets et al. (2004), a six-week exposure period is found optimal for zebra mussels, allowing for the accumulation of comparable pollutant levels to those found in indigenous mussels. Unfortunately, recollection of the mussels was only possible for 15 out of 23 sites, because many cages got destroyed or lost during the exposure time. Following collection, the mussels underwent a depuration phase in particle-free water obtained from the corresponding sampling sites, with a duration of 24 h at a temperature of 15 °C, before subsequent dissection. Given that zebra mussels are non-native species, and on top of the existing evidence of widespread distribution of *Dreissena* species in Flanders (Bervoets et al., 2004), the exposure was intentionally carried out during autumn and winter when water temperatures below 12 °C act as a threshold to mussel reproduction (Mackie and Schloesser, 1996).

2.2. Ecological quality assessment

In order to evaluate the macroinvertebrate community responses to PFAS pollution, the Multimetric Macroinvertebrate Index of Flanders (MMIF) was calculated, following the methodology outlined by Gabriels et al. (2010). Specifically developed for surface waters in Flanders, this index incorporates five equally weighted metrics along with considerations for the specific water body type. The metrics used in the MMIF calculation include taxonomic richness (TAX), the number of EPT-taxa (Epheremeroptera, Plecoptera, Trichoptera), the number of other sensitive taxa (NST), the Shannon-Wiener Diversity index (SWD), and the mean tolerance score (MTS). These metrics are recognized as indicators sensitive to pollution in general, with particular emphasis on the diversity of EPT-taxa as markers for micropollutants (Berger et al., 2018). The resulting MMIF scores range from 0 to 1, where a score of 0 indicates poor ecological quality and a score of 1 indicates high ecological quality (Table A.2).

2.3. PFAS analyses

Before the extraction process, the mussels were dissected, and the soft tissue was homogenized with a bead homogeniser (TissueLyser LT Qiagen GmbH, Germany) with stainless steel beads (5 mm; Qiagen GmbH, Germany). Approximately 0.3 g ww of each individual mussel was weighed and placed in a 50 ml polypropylene (PP) tube. To ensure an adequate amount of tissue for the extraction of the other, resident, macroinvertebrates, five to ten individuals per species and per location were pooled together, homogenized with a bead homogeniser, and weighed in separate 50 ml PP tubes. The extraction procedure for biotic samples, as described by Powley et al. (2005), was carried out with minor modifications (supplementary information in appendix A). In total, 28 target analytes were analysed using Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS) (Table A.3). Further information regarding the target analytes and specific instrumental settings can be found in Groffen et al. (2021). As a quality control measure, a procedural blank containing 10 ml of acetonitrile (ACN) per every 10 samples was included during the extraction process. The limit of quantification (LOQ) for each target analyte was determined in matrix, based the integrated peak area of a low concentration peak with a corresponding a signal-to-noise ratio of 10 (Table A.4). To prevent carry-over effects to the next sample, ACN was regularly injected during PFAS analysis as an instrumental blank.

2.4. Statistical analyses

In addition to PFAS bioaccumulation, the macroinvertebrate community structure can also be influenced by various factors, including other pollutants, water characteristics, habitat quality, and food availability. To specifically examine the relationship between accumulated PFAS levels and the response of the invertebrate community, a 90th quantile regression analyses was employed. Hereby, only the maximum ecological response is considered, in order to correct for these unmodelled factors. All quantile regression models were performed using the software package R (version 4.1.3) and a significance level of p-value < 0.05 was considered. To define threshold body burdens above which a good ecological water quality is not achieved, two methods can be used.

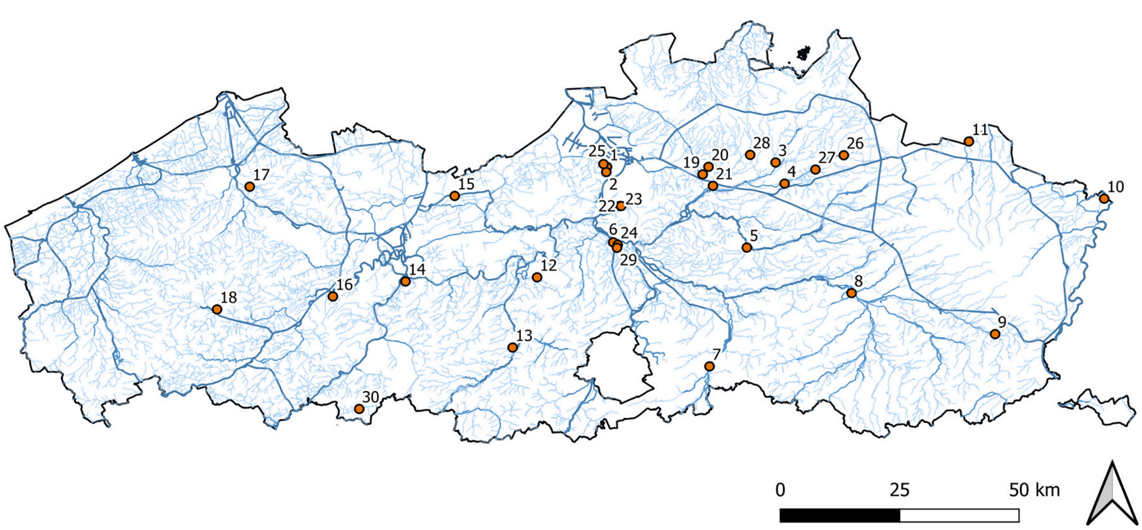

Fig. 1. Overview of sampling locations in Flanders, Belgium (Map made with QGIS version 3.4.15).

The first and more robust approach is based on the 90th quantile regression line. In cases where no significant relationship was identified, the 95th percentile of accumulated PFAS concentrations measured at locations exhibiting good ecological quality (MMIF $\geq$ 0.7) was calculated (Gabriels et al., 2010). Two-way ANOVA analyses were performed to compare PFAS concentrations between species. Post-hoc comparisons were performed using Tukey's honestly significant difference (HSD) adjusted post-hoc test or the non-parametric equivalent Dunn's post-hoc test when model assumptions (homogeneity of variances and normality of residuals) were not met.

3. Results

3.1. PFAS profiles

From the resident taxa, *Asellus* sp. could be sampled at 22 out of 35 sampling sites and *Gammarus* sp. and *Chironomus* sp. were each collected at 12 sites. To have enough datapoints, regression analyses were only done for compounds with a detection frequency $\geq$ 50 % as shown in Fig. 2. Based on the detection frequency profiles, similar profiles were found for *Asellus* sp., *Gammarus* sp. and zebra mussels with mainly long-chain perfluoroalkyl carboxylic acids (PFCAs) such as perfluorooctanoic acid (PFOA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic

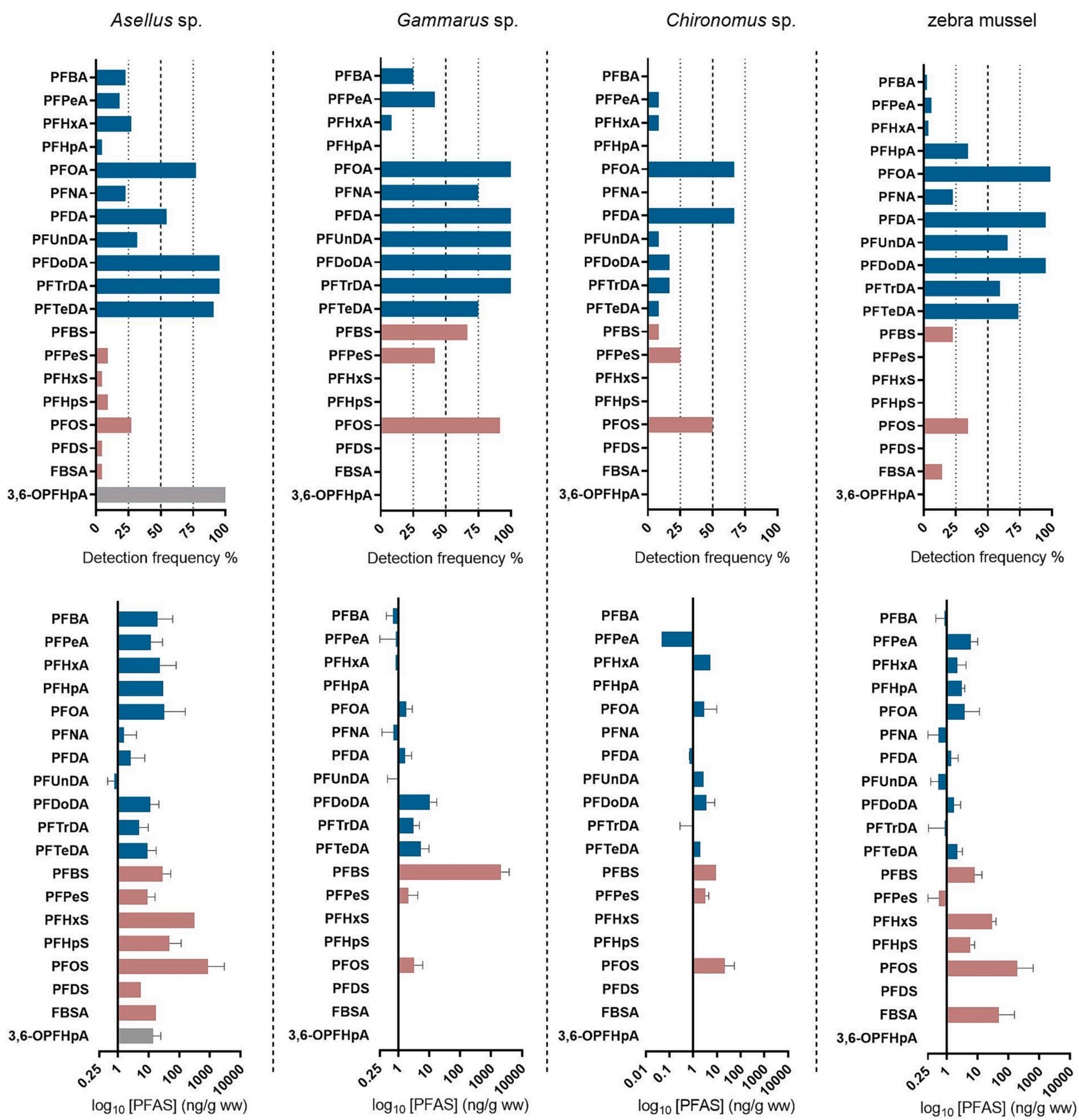

Fig. 2. Detection frequencies (%) and mean PFAS concentrations with Standard Deviation (SD) (ng/g ww) detected over all sites per species: *Asellus* sp., *Gammarus* sp., *Chironomus* sp. and zebra mussel. Perfluoroalkyl carboxylic acids (PFCAs) are indicated in green, perfluoroalkyl sulfonic acids (PFSAs) and sulfonamides in pink and perfluoropolyether carboxylic acids (PFECAs) in grey. Note that the x-axis showing the PFAS concentrations is on a log scale.

acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA) with a frequency $\geq$ 50 %. Remarkable is the detection of perfluoro-3,6-dioxaheptanoic acid (3,6-OPFHpA) in only *Asellus* sp. and not in any other species, even not at the same locations. Despite the detection of 3,6-OPFHpA throughout the whole sampling area, elevated concentrations were mainly detected in rivers restricted to the Nete catchment area and the lower Scheldt catchment area. For Chironomids, the detection frequency is less pronounced with only PFOA, PFDA and PFOS being $\geq$50 %. This is also reflected in the concentration profiles, where PFAS concentrations are generally lower in *Chironomus* sp. compared to the other species. The lowest average concentration of ΣPFAS was found in *Chironomus* sp. (17 ng/g ww, min. 0.15 – max. 105 ng/g ww), followed by *D. polymorpha* (89 ng/g ww, min. 1.1 – max. 1388 ng/g ww), *Asellus* sp. (360 ng/g ww, min. 17–6324 ng/g ww) and *Gammarus* sp. (1409 ng/g ww, min. 18 – max. 5405 ng/g ww). However, the comparison of these concentration profiles must be made with some caution, as they are highly dependent on the different sites and therefore different conditions to which a given species is exposed. For example, *Asellus* sp. and zebra mussels were collected and exposed at a specific hotspot location, which explains the accumulation of compounds such as perfluorobutanesulfonamide (FBSA), perfluoroheptanoic acid (PFHpA), perfluorohexanesulfonic acid (PFHxS) and perfluoroheptanesulfonic acid (PFHpS), which were not detected at other locations and in other species. Comparisons between all four species within the same site could not be made because they were never sampled or exposed all together. For three species, *Gammarus* sp., *Asellus* sp. and zebra mussel comparisons could be made based on five sites (Fig. 3). Significant higher concentrations were found for PFDoDA in *Asellus* sp. (p < 0.01) and *Gammarus* sp. (p < 0.001) compared to zebra mussel with mean concentrations being $7.4_{Asellus\ sp.}$, $13_{Gammarus\ sp.}$ and $1.4_{\mathrm{zebra\ mussel}}$ ng/g ww. For all other compounds no significant difference was found for this comparison. However, generally higher PFTrDA and PFTeDA concentrations were detected in *Asellus* sp. and *Gammarus* sp. compared to zebra mussels with mean concentrations being $3.1_{Asellus\ sp.}$, $3.1_{Gammarus\ sp.}$ and $0.38_{\mathrm{zebra\ mussel}}$ ng/g ww for PFTrDA and $5.6_{Asellus\ sp.}$, $5.7_{Gammarus\ sp.}$ and $1.7_{\mathrm{zebra\ mussel}}$ ng/g ww for PFTeDA. Significantly higher PFDoDA concentrations were also found when comparing only *Gammarus* sp. to zebra mussels based on 11 sites (p = 0.017). For the other comparisons such as *Gammarus* sp. vs. *Asellus* sp. (based on eight sites) and *Gammarus* sp. vs zebra mussel (based on five sites), no significant difference was found for any of the compounds. For *Chironomus* sp. it was only possible to statistically compare with *Asellus* sp. for PFOA (based on five sites) and no significant difference was found. All other compounds could not be compared as they were often not detected in *Chironomus* sp. at these specific sites, resulting in insufficient data for statistical comparison. The same was true for *Chironomus* sp. vs. zebra mussels, which overlapped at 4 sites. *Gammarus* sp. and *Chironomus* sp. were never sampled at the same sites. An overview of all concentrations detected per species and per location is shown in Table A.5–8.

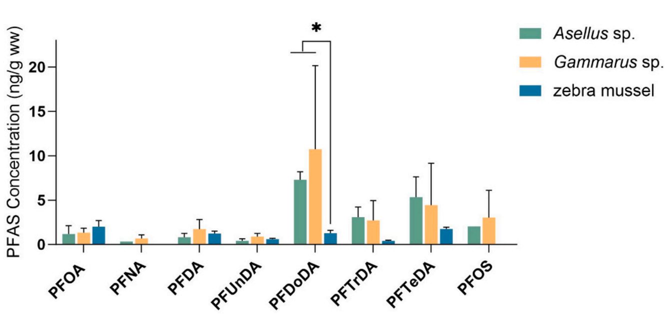

Fig. 3. Comparison of PFAS concentrations and standard deviation (ng/g ww) among *Asellus* sp. *Gammarus* sp. and zebra mussels for five sampling sites where all three species were sampled or exposed.

3.2. Passive biomonitoring: [PFAS] $_{resident\ taxa}$ vs. ecological water quality

Fig. 4 displays scatterplots illustrating the relationship between the MMIF and the accumulated PFAS concentrations in resident taxa, specifically *Asellus* sp., *Gammarus* sp., and *Chironomus* sp. However, based on the 90th quantile regression analyses, no significant relationship was observed between the ecological water quality and accumulated PFAS concentrations, except for PFTeDA (p = 0.03) and ΣPFAS (p < 0.01) in *Gammarus* sp. Consequently, threshold body burdens for PFTeDA and ΣPFAS in *Gammarus* sp. were calculated using the quantile regression model, resulting in a value of 7.1 ng/g ww and 2264 ng/g ww respectively. Despite the absence of significant relationships, threshold body burdens could still be derived for PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFOS, and ΣPFAS in both *Asellus* sp. and *Gammarus* sp. using the 95th percentile method (Table 1). Additionally, a threshold value of 35 ng/g ww was calculated in *Asellus* sp. for 3,6-OPFHpA. For *Chironomus* sp., no threshold value could be derived due to the absence of a significant relationship between accumulated PFAS concentrations and the ecological water quality. Additionally, it is important to note that the sites where *Chironomus* sp. were sampled consistently demonstrated MMIF values below the threshold of 0.7. As a result, the alternative 95th percentile method could also not be applied to derive threshold burdens for *Chironomus* sp. in relation to the accumulated PFAS concentrations.

Hence, besides the MMIF, the relationship between each individual metric and accumulated PFAS concentrations was also examined. This was done to investigate whether specific metrics might have distinct associations that are overshadowed by other metrics within the composite MMIF. Hereby, significant negative relationships were found between PFTeDA concentrations in *Gammarus* sp. and the number of EPT taxa (p = 0.04), number of other sensitive taxa (p = 0.04) and the mean tolerance score (p = 0.03) (Fig. A.1). For *Asellus* sp. a significant negative relationship was found between PFTeDA and taxonomic richness (p < 0.01) (Fig. A.2).

3.3. Active biomonitoring: [PFAS] $_{zebra\ mussel}$ vs. ecological water quality

Fig. 5 depicts the scatterplots illustrating the relationship between the MMIF and the accumulated PFAS concentration in translocated zebra mussels. Significantly observed quantile regression models (p < 0.01) were found for the compounds PFOA, PFDoDA, PFTrDA, PFTeDA, PFOS, and ΣPFAS. Consequently, threshold body burdens for these compounds in zebra mussels were calculated using both methods and are presented in Table 1. Since PFOS was not detected in the mussels at sites exhibiting a good ecological quality (MMIF>0.7), the threshold value could not be calculated using the 95th percentile method. However, this method was applicable for deriving the threshold values of PFDA and PFUnDA only (Table 1).

Based on the quantile regression analysis with the individual metrics significant relationships (p < 0.01) were found for PFOA, PFDoDA, PFTrDA, PFTeDA and ΣPFAS versus all individual metrics (TAX, EPT, SWD and MST) except for NST. For PFOS a significant relationship was found with EPT, TAX, MTS and NST but not with SWD (p < 0.01). For NST, a significant relationship was only found for PFOA, PFOS and ΣPFAS (p < 0.01). As a result, EPT is the only index that showed significant models for all the same compounds as for the MMIF. The scatterplots of these significant relationships can be found in the appendix (Fig. A. 3–7).

4. Discussion

4.1. PFAS profiles

The PFAS profiles observed in the present study exhibit similarity among the different invertebrate species, with long-chain PFCAs being the most dominant compounds. This finding aligns with previous

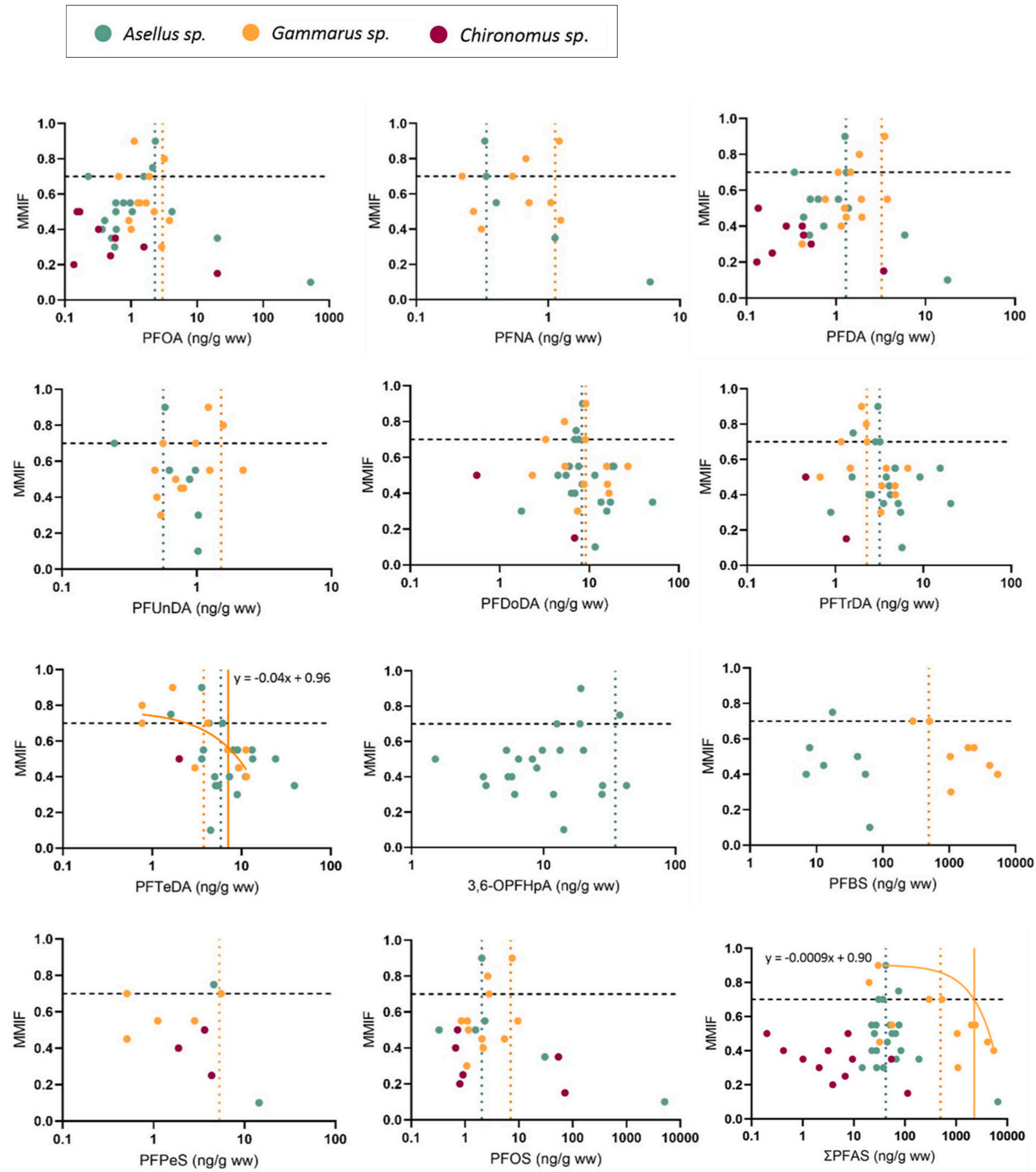

Fig. 4. Scatterplots of the relationship between bioaccumulated PFAS concentrations in resident taxa versus the ecological water quality index (indicated as the MMIF). The horizontal dashed line indicates the threshold for a good ecological water quality (MMIF >0.7). The vertical dashed lines indicate the threshold body burdens per species (green = *Asellus* sp., yellow = *Gammarus* sp. and red = *Chironomus* sp.) calculated based on the 95th percentile method. Vertical solid lines indicate the threshold body burdens calculated based on the 90th quantile regression model.

research conducted by Lasier et al. (2011), who exposed aquatic oligochaete worms (*Lumbriculus variegatus*) to PFAS-contaminated field sediments and observed that PFOS and long-chain PFCAs exhibited the highest bioaccumulation potential. A similar trend was also observed in the study conducted by Bertin et al. (2016), where Biota Sediment Accumulation Factors (BSAFs) for Gammarids increased with increasing chain length of PFAS. A study involving the comparative analysis of PFAS accumulation kinetics in *Chironomus riparius* and *Gammarus* sp.

Table 1
Derived threshold body burdens (ng/g ww) for PFAS compounds in zebra mussels and resident taxa based on the 95th percentile and the 90th quantile regression model. 'ns' means that no significant relationship was found. '-'indicates that the threshold body burden could not be calculated because this compound was not detected at any location with an MMIF ≥7 or that the compound was not detected in this species.

	95th Percentile			90th Quantile regression model		
ng/g ww	*Asellus* sp.	*Gammarus* sp.	*D. polymorpha*	*Asellus* sp.	*Gammarus* sp.	*D. polymorpha*
PFOA	2.3	3.0	3.2	ns	ns	5.5
PFNA	0.34	1.1	–	ns	ns	ns
PFDA	1.3	3.2	1.8	ns	ns	ns
PFUnDA	0.56	1.5	0.92	ns	ns	ns
PFDoDA	8.3	9.1	1.6	ns	ns	1.7
PFTrDA	3.2	2.3	0.51	ns	ns	0.51
PFTeDA	5.9	3.8	2.3	ns	7.1	2.4
PFBS	–	493	–	ns	ns	–
PFPeS	–	5.3	–	–	–	–
PFOS	2.0	7.0	–	ns	ns	644
3,6-OPFHpA	35	–	–	–	–	–
ΣPFAS	42	493	9.4	ns	2264	113

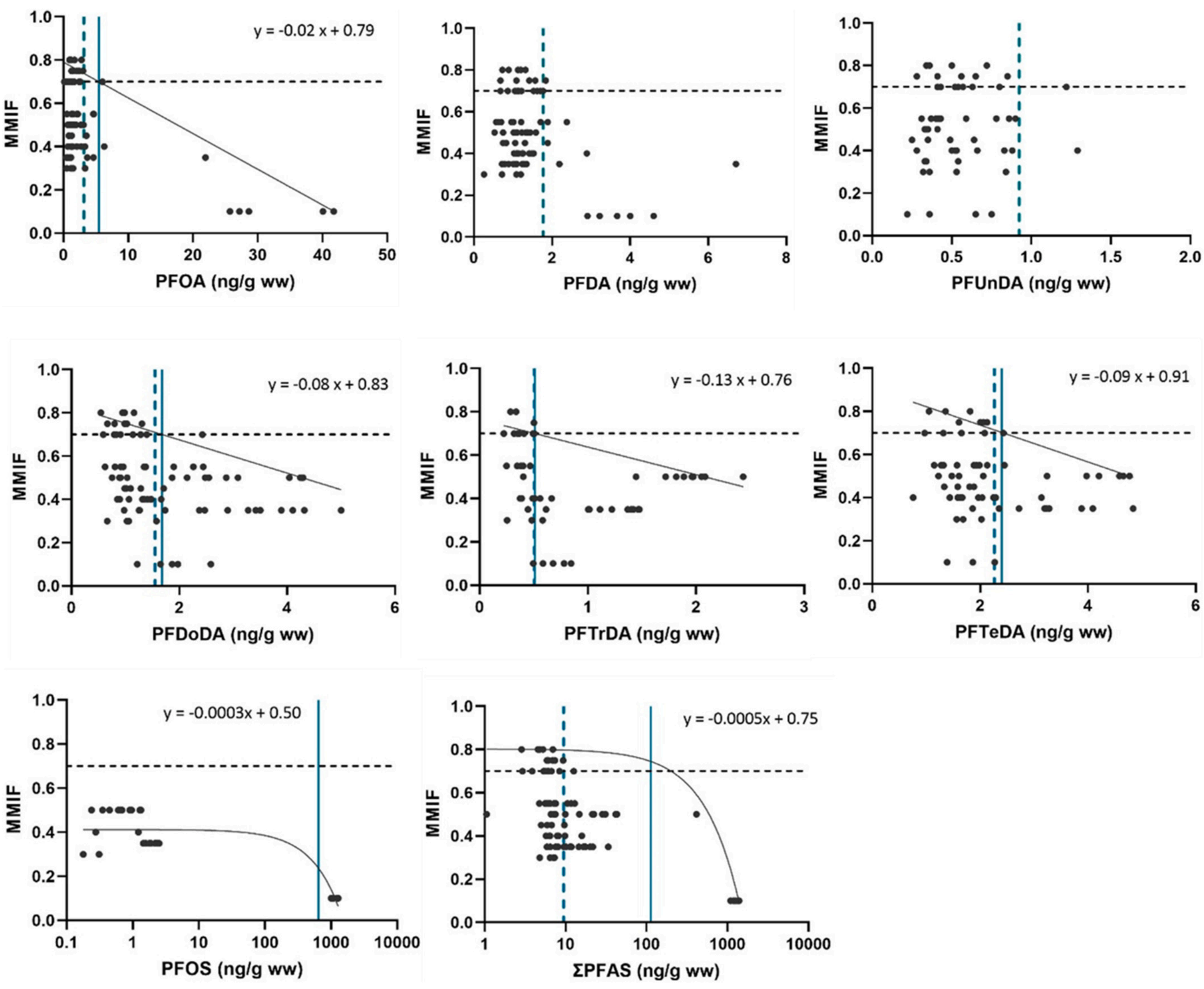

Fig. 5. Significant 90th quantile regressions between the accumulated PFAS concentrations (ng/g ww) in zebra mussels (*D. polymorpha*) and the ecological water quality index (MMIF). The horizontal dashed line indicates the threshold for a good ecological water quality (MMIF >0.7). The vertical dashed lines indicate the threshold body burdens based on the 95th percentile method and the vertical solid lines indicate the threshold body burdens calculated based on the 90th quantile regression model.

revealed notable distinctions (Bertin et al., 2014, 2016). Specifically, the elimination rate of PFAS compounds within chironomids was observed to be higher than within gammarids, and correspondingly, the BSAFs were found to be higher in gammarids compared to chironomids. This is in line with the present study, where the overall patterns show that the detection frequencies and the accumulated concentrations of PFAS are generally lower in chironomids. In a study on PFAS bioaccumulation in the Rhone River, invertebrate samples were pooled and ΣPFAS concentrations ranged from 6.56 ng/g ww in a chironomid sample to 356 ng/g ww in a gammarid sample (Babut et al., 2017). In the present study mean ΣPFAS concentrations range from 17 ng/g ww in *Chironomus* sp. to 1409 ng/g ww in *Gammarus* sp. However, it should be noted that our study covers multiple water bodies, while the study by Babut et al. (2017), focuses on one river system.

Furthermore, it is important to consider that the different foraging behaviour of the studied species may result in different PFAS exposure routes and profiles. Zebra mussels, being filter feeders, are primarily exposed to PFAS through their diet and water, while the studied resident taxa, which dwell in sediments, experience additional exposure through sediment contact (Lewis et al., 2022). In contrast with our findings, it was initially anticipated that the relative contribution of short-chain PFAS would be higher in mussels than in bottom-dwelling resident taxa, given the greater water solubility of short-chain PFAS (Li et al., 2020). The dominance of long-chain PFAS in zebra mussels could be attributed to their higher bioaccumulation potential compared to short-chain PFAS (Goodrow et al., 2020). Additionally, it should be noted that the placement of mussel cages in close proximity to the sediment in relatively shallow streams may also have influenced the bioaccumulation pattern. A similar pattern in mussels was observed in a recent study by Teunen et al. (2022), where quagga (*Dreissena rostriformis bugensis*) and Asiatic clams (*Corbicula fluminea*) were exposed in larger rivers across Flanders. Consistent with our findings, the profile was mainly characterized by PFOA (approximately 40 %), followed by other long-chain PFCAs such as PFUnDA, PFDoDA and PFTrDA. Although accumulated concentrations in zebra mussels were lower than in resident taxa for some compounds such as PFDoDA, PFTrDA and PFTeDA, zebra mussels and resident taxa have similar detection profiles, suggesting that zebra mussels may provide an overview of compounds to which resident taxa are exposed. Additionally, the present study shows some of the highest concentrations of PFOS and PFOA that have ever been reported in mussels with mean PFOS concentrations of 1137 ng/g ww and mean PFOA concentrations of 37 ng/g ww measured at a specific hotspot location. PFOS concentrations in zebra mussels from the Great Lakes ranged from 2.4 to 3.1 ng/g ww (Kannan et al., 2005). However, most data on PFAS accumulation in mussels is on marine species and often restricted to PFOS and PFOA. In brown mussels from Guanabara Bay, Brazil, PFOS and PFOA concentrations ranged from 3.5 to 4.5 and 2.1 to 6.0 ng/g ww (Quinete et al., 2009); PFOS concentrations in blue mussels from Danish coastal waters ranged from 0.11 to 0.58 ng/g ww (Bossi et al., 2008); South China and Japanese coast concentrations of PFOS and PFOSA in mussels ranged from 0.11 to 0.59 and from 0.04 to 2.9 ng/g ww (So et al., 2006). High PFOS concentrations were also reported in Mediterranean mussel (36.8–126 ng/g ww) collected in Portugal (Cunha et al., 2005). The mean ΣPFAS concentration reported for dreissenid mussels in Lake Huron was 4.1 ± 0.53 ng/g ww, while the mean ΣPFAS in the present study for zebra mussels was 89 ng/g ww (range 1.1–1388 ng/g ww) (Ren et al., 2022). Again, it should be noted that the present study does not focus on one specific water body, but covers several streams and small rivers throughout Flanders. In the study by Teunen et al. (2021), which also covers several river systems in Flanders, ΣPFAS in bivalves (quagga mussels and Asian clams) ranged from 8.56 to 157 ng/g ww. In Manila clams (*Venerupis philippinarum)* from the Po River Delta in Italy, concentrations of PFOA and PFOS dominated, ranging from 1.6 to 5.8 ng/g ww (PFOA) and from 0.3 to 1.6 ng/g ww (PFOS) (Mazzoni et al., 2016). Conversely, in blue mussels (*Mytilus galloprovincialis)* from the same region, the highest concentrations were observed for PFOA, PFDoDA and PFUnDA, ranging from 0.2 to 1.1 ng/g ww, 0.2 to 0.7 ng/g ww and 0.1 to 0.2 ng/g ww, respectively (Mazzoni et al., 2016). Notably, the concentrations in blue mussels were significantly lower compared to those in Manila clams. A separate study by Corsolini et al. (2014) also found that the ΣPFAS concentrations in Manila clams were markedly higher than those in blue mussels sampled at the same locations. This underscores the importance of selecting the right organisms and species for a successful active biomonitoring campaign. In general, the above-mentioned PFAS concentrations are relatively low compared to the concentrations measured in the present study, suggesting that zebra mussels have potential to serve as an effective biomonitoring species for PFAS.

4.2. Macroinvertebrate community responses to PFAS pollution

4.2.1. Resident taxa

Studying the relationship between accumulated micropollutant levels in resident taxa and macroinvertebrate based biotic indices has been found to be a good method to predict ecological effects and derive critical threshold values that are protective of ecological damage (Luoma et al., 2010; Rainbow et al., 2012; De Jonge et al., 2013; Bervoets et al., 2016; Méndez-Fernández et al., 2017). However, to our knowledge this type of research has not been done for PFAS up until now. Overall, research on the effects of PFAS on aquatic wildlife is lacking compared to controlled laboratory studies, especially for macroinvertebrates. Based on the review study of Ankley et al. (2021), 39 field-based effect studies were reported with only two studies focussing on aquatic invertebrates. One of these studies investigated the macrobenthic community structure up- and downstream of a PFOA point source in Northern Italy (Rusconi et al., 2015). Although more sensitive taxa were found upstream compared to downstream no significant differences were observed based on a multimetric index. Therefore, the relationship between each individual metric and accumulated PFAS concentrations was also examined in the current study. As such, individual metrics showed inconsistent results, making the MMIF the preferred ecological endpoint for assessing community responses as it also takes into account watercourse type, which mitigates the effect of external factors on the index (Gabriels et al., 2010).

Although *Chironomus* species are well known for their tolerance to pollutants, they were only found in sufficient numbers at 12 out of 30 sites and never at the sites of good ecological quality (Saether, 1979; Lindegaard, 1995; Mousavi et al., 2003). Among aquatic invertebrates, chironomids are one of the most studied taxa regarding PFAS toxicity. However, findings from laboratory studies present conflicting outcomes. For example, Ankley et al. (2021) report that *Chironomus riparius* is one of the most sensitive species to PFOS, with adverse reproductive effects occurring at a concentration of 4 μg PFOS/l. Conversely, the multigenerational study conducted by Marziali et al. (2019), examining the effects of PFOS, PFOA, and PFBS on *Chironomus* sp. for 10 generations to 10 μg/l nominal concentrations, did not identify any reproductive or population-level impacts, suggesting a limited influence under field conditions. Crustaceans have also been identified as one of the more sensitive groups. However, most toxicity studies focus primarily on Cladocerans (particularly *Daphnia* sp.), leaving a gap in toxicity data for other crustaceans such as *Asellus* sp. and *Gammarus* sp. (Ankley et al., 2021).

Based on the scatter plots (Fig. 4) it can be observed that certain locations exhibit a low ecological quality, despite low accumulated PFAS concentrations. This is likely due to the influence of other factors such as environmental stressors (e.g., low oxygen, high nutrient loads, habitat degradation, …), as well as the presence of other contaminants. This observation lends further support to the notion that accumulated pollutants and the ecological water quality do not necessarily reflect a causal relationship. Therefore, the use of 90th quantile regression models appear to be a good strategy to relate internal PFAS concentrations to the maximum ecological response to mitigate the effects of unmodeled factors. For the resident taxa, a significant negative relationship was only found for PFTrDA and ΣPFAS in *Gammarus* sp. The lack of significant relationships for the other compounds may be because the accumulated concentrations in lower trophic levels, such as invertebrates, are too low to cause changes in macroinvertebrate community structure. However, sites exhibiting higher levels of accumulated PFAS concentrations consistently remain below the threshold value for a good ecological quality (MMIF = 0.7). Furthermore, in sites characterized by an MMIF>0.7 the accumulated concentration of 9.0 ng/g ww was never exceeded for any of the compounds except for PFBS in *Gammarus* sp. It is important to note that the nature of this fieldwork introduces challenges to consistently sample the same species across all sites. Consequently, the available dataset per compound and species is often constrained, representing a pivotal limitation in this study.

4.2.2. Zebra mussels

Translocated zebra mussels were exposed to overcome the limitations of passive biomonitoring with resident taxa. This active biomonitoring technique has been used for decades to assess the bioavailability of pollutants in aquatic environments (Mersch et al., 1996; Bervoets et al., 2004; Minier et al., 2006). Although zebra mussels have been found to be a good bioindicator for predicting the impact of contaminants such as metals and pesticides on aquatic ecosystems (Bervoets et al., 2016; Bashnin et al., 2019), literature on the use of this approach for PFAS is limited (Teunen et al., 2021). In the present study it was found that increasing PFOA, PFDoDA, PFTrDA, PFTeDA, PFOS and ΣPFAS concentrations in zebra mussels can be related to a decreasing ecological water quality, as assessed by the MMIF. Overall, as was also found for resident taxa, the congruity between outcomes derived from individual metrics and the multimetric index (MMIF) suggests that the MMIF is a good approach. This suggests that translocated zebra mussels can be a suitable biomonitoring tool to study the bioavailability of PFAS in aquatic environments and to predict the ecological responses of macroinvertebrate communities. However, the experimental study of Sanjuan et al. (2013) indicated that the bioaccumulation potential of PFOS and PFOA in zebra mussels is low compared to other aquatic organisms. It was found that above an accumulated concentration of 9 ng/g ww, the bioaccumulation kinetics of PFOS and PFOA are inversely related to the activity of efflux transporters. It is therefore possible that some of the accumulated compounds in mussel tissues are being actively excreted. However, in the present study, no major differences in accumulated PFAS concentrations were observed compared to resident species, supporting our statement that zebra mussels are suitable biomonitoring species. In addition, as described above, the present study shows some of the highest concentrations of PFOS and PFOA that have ever been reported in mussels, suggesting that they are good bioaccumulators of PFAS. Although ABM offers great potential for biomonitoring PFAS, we also experienced some drawbacks, including losing 8 out of 23 cages during our exposure period. Some cages were simply taken out of the water, while other cages had ropes cut through, probably as a result of riverbank maintenance practices such as mowing. For this reason, we recommend to place the cages on small bridges over the stream, rather than along the banks.

4.3. Derivation of threshold body burdens

This type of field-based effect study allows to provide evidence of pollutant bioavailability and has been used to estimate critical body levels to protect aquatic invertebrate communities for a range of pollutants (Meador et al., 2014; Bervoets et al., 2016; Bashnin et al., 2019). For PFAS, to the best of our knowledge only the study of Teunen et al. (2022) has applied this approach to estimate threshold body burdens for PFOS in two fish species, eel (*Anguilla anguilla*) and perch (*Perca fluviatilis*). In perch the threshold values were 9.5 ng/g ww (95th percentile) and 12 ng/g ww (90th quantile) and in eel a threshold of 49 ng/g ww (95th percentile) was derived. In general, invertebrates have been found to be more sensitive to contaminants than fish (Buckler et al., 2005; Ankley et al., 2021). Therefore, a lower threshold body burden was expected for invertebrates compared to fish. This was the case for *Gammarus* sp. and *Asellus* sp. with PFOS thresholds of 7.0 and 2.0 ng/g ww respectively, but not for zebra mussels (644 ng/g ww). This is likely because the body burden of PFOS (90th quantile) in zebra mussels was only based on four sites including one hotspot. When the hotspot site is excluded from the dataset, the quantile regression is no longer significant. In general, the quantile regression method can be considered as the most robust method for the derivation of threshold values as it is based on a significant quantile regression model. However, compared to the 95th percentile method, this method is more likely to be influenced by hotspots as shown in the present study. The 95th percentile on the other hand is solely based on concentrations measured at sites with a good ecological quality. This method is often applied as no relationship was observed which might indicate that the threshold or turning point is not even reached yet in the dataset. Therefore, it is possible that the effective threshold is higher. The advantages and disadvantages of both methods must be considered for further validation of the threshold body burdens.

Despite that ΣPFAS concentrations are often reported in literature and monitoring studies, the present study indicates that this approach is less relevant for the estimation of thresholds. For example, remarkable high threshold values of 492 ng/g ww (95th percentile) and 2264 ng/g ww (Quantile) were estimated for ΣPFAS in *Gammarus* sp. This is mainly due to the high PFBS concentrations, ranging from 281 to 5367 ng/g ww, detected in *Gammarus* sp. at eight out of 12 sites. When PFBS was excluded from the ΣPFAS, the quantile regression was not significant anymore and the 95th percentile threshold resulted in 29 ng/g ww. Therefore, the threshold for ΣPFAS is likely to be biased, given that all compounds are accorded equal weight, thereby failing to account for variations in the toxicity of distinct compounds.

An additional crucial aspect requiring further investigation to validate these threshold values is the impact of season on critical body levels. The seasonal fluctuation in water temperatures can alter the metabolic activity of aquatic organisms, consequently affecting the bioaccumulation kinetics (Vidal et al., 2019). For example, in mussels, cooler water temperatures often lead to reduced respiration and feeding rates in response to lower metabolic demands, potentially resulting in diminished contaminant bioaccumulation (Rao and Khan, 2000; Wang et al., 2005; Maulvault et al., 2018; Kibria et al., 2021). Conversely, a contrasting trend has been noted for the bioaccumulation of Hg in zebra mussels, where reduced metabolism is assumed to weaken excretion processes, leading to lower bioaccumulated concentrations (Wilman et al., 2023). Therefore, it is imperative to gain a clearer understanding of the impact of seasonal variations in water temperature on the bioaccumulation of PFAS in the considered species. Besides temperature, also other factors can contribute to seasonal variations in PFAS bioavailability. For example, PFAS tend to adsorb onto suspended particles entering the aquatic environment through surface runoff, becoming a significant pathway for sediment contamination through deposition (Ahrens et al., 2010; Borthakur et al., 2021). Hence, external elements such as heavy rainfall and increased river flow velocity can profoundly influence PFAS bioavailability in aquatic environments due to increased particle loading and resuspension of river sediments (Borthakur et al., 2021).

Moreover, it is crucial to recognize that due to variations in bioaccumulation kinetics among different species, estimates of critical body burdens are applicable exclusively to the specific species for which they have been established (Bertin et al., 2014, 2016).

Despite some limitations in the estimation approach and the need for further validation through additional research, the precautionary thresholds identified in the present study remain pertinent and indicative. This is particularly significant given the pivotal role played by macroinvertebrates in aquatic ecosystems, serving as a significant biomagnification pathway for PFAS within the aquatic food web (Wallace and Webster, 2003). Therefore, the relevance of these thresholds extends beyond the protection of macroinvertebrate communities to encompass the broader safeguarding of the entire aquatic ecosystem.

5. Conclusion

First, predominantly long-chain perfluorinated carboxylic acids (PFCAs) were consistently detected in both resident taxa and zebra mussels across rivers and streams in Flanders, Belgium. Consequently, we strongly recommend prioritizing these compounds in future environmental monitoring programs. Given that the present study focused on a limited number of PFAS compounds, further testing of PFAS is advised to encompass the influence of precursor compounds and provide a comprehensive understanding of PFAS present in the aquatic systems of Flanders.

Secondly, given the observed relationship between increasing PFAS concentrations in *Gammarus* sp. and zebra mussels and a declining ecological water quality, it can be inferred that both species have the potential to serve as reliable biomonitoring tools. They can fulfil two primary purposes: (I) monitoring the bioavailability of PFAS and (II) predicting the ecological responses of macroinvertebrate communities to PFAS pollution. In contrast, due to the absence of significant relationships, *Asellus* sp. and *Chironomus* sp. appear to be less suitable for predicting the ecological effects of PFAS on aquatic ecosystems. Nevertheless, our results provide first-time evidence of associations between accumulated PFAS concentrations in benthic macroinvertebrates and a reduced ecological water quality in terms of the macroinvertebrate community structure.

Additionally, this study marks the first reporting of threshold body burdens for a set of PFAS in aquatic invertebrates. However, for regulatory applicability, it is imperative to gather additional lines of evidence to validate the estimated threshold values. Specifically, assessing the consistency of findings between laboratory, mesocosm studies (involving invertebrates, fish, birds, etc.) and observations from field-based effect studies involving similar species would be of significant value.

CRediT authorship contribution statement

Cara Byns: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Thimo Groffen:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Lieven Bervoets:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used deepl.com in order to improve the readability of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the publication.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170611.

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