Water Research 222 (2022) 118863

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Wastewater treatment plant contaminant profiles affect macroinvertebrate sludge degradation

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ARTICLE INFO

Keywords:
Waste treatment plants
Excess sludge
Macroinvertebrates
Biotic interactions
Stress gradient hypothesis

ABSTRACT

Disposal of the overwhelming amounts of excess wastewater treatment plant (WWTP) sludge is an increasing financial and environmental problem, and new methods to reduce the amount of excess sludge are therefore required. In the natural environment, interactions between multiple macroinvertebrate detritivores mediate the degradation of organic matter. Macroinvertebrates may thus also be able to degrade WWTP sludge, but may meanwhile be impacted by the associated contaminants. Therefore, the aim of the present study was to examine if WWTPs contaminant concentrations and profiles affect the biotic interactions and macroinvertebrate mediated degradation of sludge. Assessing degradation of sludge from three WWTPs differing in contaminant profile by (combinations of) three macroinvertebrate detritovore taxa, revealed that macroinvertebrate enhanced sludge degradation was WWTP and taxa combination specific. Yet, taxa combinations only had an additional positive effect on sludge degradation when compared to single taxa in sludge with a higher contaminant load. This was confirmed by the results of a Cu-spiked sludge degradation experiment, indicating a possible effect of biotic interactions. It was concluded that macroinvertebrates are a potential tool for the reduction of excess WWTP sludge, and that using multispecies assemblages of detritivorous macroinvertebrates may increase the resilience of this additional treatment step.

1. Introduction

The world's wastewater treatment plants (WWTPs) produce unprecedented amounts of excess sludge. The 323 Dutch WWTPs alone already produce over 1.250 million kg of sludge yearly (CBS, 2021). This excess sludge has to be processed and disposed, but options for land-filling and agriculture as a means of disposal have become limited (Collivignarelli et al., 2019). Hence, the costs of sludge processing and disposal can make up to 60% of the total operational costs of a WWTP (Buys et al., 2008). Therefore, there is a high demand for new strategies and technologies to reduce the mass of excess sludge to lower the economic and environmental impact (Christodoulou and Stamatelatou, 2016).

As several macroinvertebrate detritivores play an important role in the degradation of organic matter in the aquatic environment, they may also be capable to degrade WWTP sludge, possibly mitigating the environmental impacts (van der Meer et al., 2021) and costs of excess sludge disposal. To this end aquatic oligochaete worms have been employed, which can feed on WWTP sludge, thereby reducing the amount of organic matter produced (Emamjomeh et al., 2018). While deploying these oligochaetes has produced some promising results on an experimental scale (Li et al., 2019; López-Visoet al., 2021), success rates in larger scale setups have been varying due to complicating factors such as uncontrollable population dynamics of the oligochaetes (Tamis et al., 2011), and low sludge degradation rates (López-Viso et al., 2022). Oligochaete worms are, however, not the only macroinvertebrate detritivores involved in organic matter degradation. In the aquatic environment the degradation of organic matter is a complex process that is mediated by natural assemblages of detritivores. These detritivores exhibit different feeding traits, and can interact during the degradation of organic matter, which can lead to increased organic matter degradation rates (Costantini and Rossi, 2010; Heard, 1994). However,

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https://doi.org/10.1016/j.watres.2022.118863
Received 16 February 2022; Received in revised form 9 June 2022; Accepted 11 July 2022
Available online 13 July 2022

competition between detritivores might reduce organic matter degradation. Therefore, only focusing on oligochaete worms would exclude the numerous traits and feeding habits that macroinvertebrates possess, which may be harnessed for sludge reduction purposes.

Opposing forces are, however, also at play, since WWTPs receive wastewater containing a plethora of contaminants, including heavy metals, pharmaceuticals, polycyclic aromatic hydrocarbons, personal care products and pesticides, which may affect the interactions between the different macroinvertebrate species and reduce sludge degradation rates (Bundschuh et al., 2021). According to the Stress Gradient Hypothesis (SGH) proposed by Bertness and Callaway (1994; see also: Silknetter et al. 2020), when stress levels in the environment increase, competitive interactions will decrease, and positive interactions such as facilitation will increase. Hence, when the environment becomes more stressful due to the presence of toxic contaminants, negative interactions, such as competition for resources, might become less rigorous, while positive interactions such as trophic facilitation might become more prevalent. In a WWTP setup, this could potentially counteract the adverse effects of contaminants on WWTP sludge degradation by detritivorous macroinvertebrates, as the potential decrease in single species degradation rates might be partly compensated by less stringent competitive interactions or even turn into facilitation. However, the effects of WWTP contaminants on sludge degradation by macroinvertebrate assemblages remains currently unknown.

The aim of the present study was therefore to examine if WWTP contaminant concentrations and profiles affect macroinvertebrate mediated degradation of sludge and the biotic interactions. We hypothesized that: 1) higher contaminant loads would reduce macroinvertebrate sludge degradation rates; 2) sludge degradation rates by macroinvertebrate assemblages would be less impacted than sludge reduction by single-taxa at higher contaminant loads, due to altered biotic interactions within the multi-taxa assemblages. To test these hypotheses, we performed a sludge degradation experiment with sludge from three WWTPs differing in contaminant profile and with all possible combinations of three macroinvertebrate detritivores differing in feeding and bioturbation traits. Subsequently, we performed a similar sludge degradation experiment with different concentrations of Cu-spiked sludge.

2. Materials and methods

2.1. Sludge collection

Sludge was collected from the aeration tanks of the WWTP Rhenen ($46{\cdot}10^3$ population equivalents (p.e.), 51°58′26.7″N 5°31′54.9″E), WWTP Mijdrecht ($70{\cdot}10^3$ p.e., 52°12′43.6″N 4°53′06.3″E) and WWTP Alkmaar ($97{\cdot}10^3$ p.e., 52°38′36.8″N 4°44′27.6″E), all located in the Netherlands. Five or six 10 L buckets of sludge were scooped and transported to the laboratory, and further processed within 2 h. Four to five buckets were used for the contaminant profiling, and one was used for the sludge degradation experiment.

2.2. Test organisms and culture conditions

For the experiments, we selected larvae of the non-biting midge *Chironomus riparius*, the snail *Physa acuta*, and *Tubificidae* worms, which all occur in organically rich, contaminated environments, such as WWTP impacted sites (Adler and Courtney, 2019; dos Reis Oliveira et al., 2020; Liang et al., 2006), but differ in their feeding and bioturbation traits. *C. riparius* larvae bioturbate and bioirrigate sediments by constructing and ventilating tubes, and deposit-feed on the upper layer of the sediment, whereas *Tubificidae* worms are gallery diffusors and conveyer-belt feeders, feeding in deeper layers of the sediment, and defecate at the sediment surface (Lagauzère et al., 2009). *P. acuta* snails on the other hand are primarily scraper/grazers that move on top of the sediment (Costantini and Rossi, 2010).

C. riparius and *P. acuta* were cultured in the laboratory at the University of Amsterdam in Dutch Standard Water (DSW: deionized water with an addition of 200 mg•L^{-1} $CaCl_2{\bullet}2H_2O$, 180 mg•L^{-1} $MgSO_4{\bullet}7H_2O$, 100 mg•L^{-1} $NaHCO_3$, and 20 mg•L^{-1} $KHCO_3$) at 20 ± 1 °C with a 12:12 h light-dark photoperiod. The tanks (20 L, 30·20·20 cm (l·w·h)) were gently aerated to maintain adequate (> 7 mg•L^{-1}) oxygen concentrations. DSW was changed 1–2 times a week, and deionized water was added to compensate for evaporation losses. The *C. riparius* tanks contained a sediment layer of 2 cm of quartz sand, whereas *P. acuta* was reared without a substrate. *C. riparius* was fed with a grinded 20:1 Trouvit:Tetraphyll mixture. *P. acuta* was fed grinded Tetraphyll twice a week. *Tubificidae* worms were bought from a fish feed wholesale and acclimatised to the same conditions as *C. riparius* one week prior to the experiment. Additionally, dried and shredded oak leaves were added to create an organic layer for the worms.

2.3. Experimental setup

2.3.1. Degradation of WWTP sludge

A 7-day degradation experiment with sludge from the three WWTPs was run in which eight taxa assemblages were deployed ($n = 5$), consisting of a control without macroinvertebrates to assess initial degradation, and 7 assemblages with equal macroinvertebrate biomass consisting of either one, two or three taxa. Macroinvertebrate survival and sludge degradation were determined at the end of the experiment.

Each experimental replicate consisted of a 50 mL glass beaker (bottom surface area: 11.3 cm^2) to which 25 mL of homogenized sludge and 15 mL of DSW were added. After approximately 30 min the sludge had settled on the bottom of the beaker forming a sediment-like layer. After 1 h, the macroinvertebrates were added using either a plastic 5-mL Pasteur-pipette for the *C. riparius* larvae and *Tubificidae* worms, or plastic tweezers for the *P. acuta* snails. To determine the sludge DW at the start of the experiment, 5 additional beakers were prepared in the same way for each WWTP, after which the overlying water was removed, and the remaining sludge was dried in a stove at 65 °C for 24 h.

To ensure equal biomass in all assemblages (8–9 mg DW), 18, 9, or 6, *C. riparius* larvae; 6, 3 or 2 *P acuta* snails; and 36, 18 or 12 *Tubificidae* worms were used in the single taxa (C, T and P), two taxa (CT, CP and TP), and three taxa (CTP) assemblages, respectively. Based on currently established length-dry weight relationships (Fig. S1), early 4th stage *C. riparius* larvae with a mean length of 9.7 mm (SD: 1.5 mm), and snails with a length of 4.5–5.7 mm were used. The weight of the *Tubificidae* worms was based on the average of 30 individuals. To ensure depuration of the gut content, all macroinvertebrates were starved for 24 h before the start of the 7-day sludge degradation experiment.

During the 7-day experiment, the beakers were kept under controlled conditions, at 20 ± 1 °C with a 16:8-h light-dark photoperiod, and gently aerated to prevent anoxic conditions. At the start, half way and at the end of the experiment, the pH, conductivity, and oxygen concentration were measured using a portable multi-meter (Hach HQ40) with the appropriate probes (PHC101, CDC401, LDO101, respectively). The beakers were checked daily for emerged *C. riparius* adults and *P. acuta* egg packages, and both were subsequently removed. A new *C. riparius* larvae was added for the removed adult, to ensure equal biomass during the 7-day experiment. Out of the 598 initially used larvae, 13 (2%) were replaced due to emergence of the adult. In eleven replicates one adult was replaced, and one replicate 2 adults were replaced.

At the end of the 7-day experiment, the overlying water in the beakers was discarded. To determine the survival of the macroinvertebrates, these were collected using a plastic Pasteur-pipette or plastic tweezers and counted. The remaining sludge was dried at 65 °C for 24 h and subsequently weighed. Sludge degradation was determined as the difference between the average start DW and the individual replicate final DW of the sludge.

2.3.2. Degradation of Cu-spiked sludge

To assess if macroinvertebrate interactions, and macroinvertebrate mediated sludge degradation are affected by increased contaminant concentrations, we performed a 7-day sludge degradation test with Cu-spiked sludge from Rhenen and (combinations of) C. riparius larvae and P. acuta snails. Cu was chosen as a model contaminant, and sludge from Rhenen was selected as it was presumed that it had the lowest initial contaminant loads of the three WWTPs This resulted in 4 species assemblages: *C. riparius* larvae (C), *P. acuta* snails (P), *C. riparius* + *P. acuta* (CP), and a control without macroinvertebrates. In the single species assemblages, either 28 *C. riparius* larvae with a mean length of 7.5 mm (SD: 1.3 mm), or 6 *P acuta* snails with a length of 5.5–6.5 mm were added. In the CP assemblage 14 *C. riparius* larvae and 3 *P acuta* snails were added. All assemblages consisted of 4 replicates. The same methods as in Section 2.3.1 were followed to perform the experiment. At the end of the 7-day experiment, in addition to the methods mentioned in Section 2.3.1, the *C. riparius* larvae were pooled per replicate beaker, dried at 65 °C for 24 h, and weighed to determine the average DW of the larvae. To determine the growth of the larvae during the experiment, the difference between the average initial larval DW (based on the length-DW correlation (Fig. S1)) and average final larval DW was calculated for each beaker. The Cu concentration in the sludge and the overlying water at the end of the experiment were determined using the methods described in Section 2.4.2.

2.4. Sludge contaminant profiles and contaminant analyses

The sludge solids in the buckets ($n = 4$ for Alkmaar and Rhenen; $n = 5$ for Mijdrecht) were allowed to precipitate for 1 h, whereafter the overlying water was siphoned off, and the remaining material was poured into a 2 L bottle which was stored at 4 °C overnight. The following day, the overlying water was siphoned off again, and the solids were frozen, freeze dried, ground with a ball grinder, and stored in airtight bottles at room temperature until further analysis.

2.4.1. Metal analysis

To determine the concentration of the metals Al, As, Ag, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se, and Zn in the sludge, samples were digested by transferring 500 mg of DW sludge into a Teflon bomb, to which 10 mL of 65% HNO_3 was added. The samples were heated to 175 °C in 5.5 min and maintained at this temperature for another 4.5 min in a microwave (Multiwave Pro, Anton-Paar), and were then allowed to cool down for 1 h, all according to the EPA 3051A protocol (U.S. EPA., 2007), as advised by the manufacturer (Anton-Paar). The contents of the Teflon bombs were poured into 25 mL volumetric flasks, and the volume was adjusted to 25 mL with ultrapure water. A subsample of 9 mL was taken from each flask, and 1 mL of internal standard (0.5 ml of 20 ppm Yttrium and 0.5 mL of 40 ppm caesiumchloride) was added. As a control, two blank Teflon bombs were added per microwave run. Subsequently, the metal concentrations were measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Optima 8000, ICP-OES: PerkinElmer) (Detection limits can be found in Supplementary Table S1).

2.4.2. Organic contaminant analysis

2.4.2.1. Sample preparation. To extract the organic contaminants of each experimental replicate a QuEChERS method validated in our laboratory was applied. In short, 10 g of DW sludge was added to 50 mL sterile polypropylene (PP) tubes filled with 6 g of anhydrous magnesium sulfate ($MgSO_4$) and 1.5 g of sodium-acetate (NaAc) and spiked with 50 µL of a 200 µg/L stock solution of internal standards. 30 mL of acetonitrile was added, mixed for one hour and subsequently centrifuged for 15 min. The supernatant was then transferred into clean 50 mL sterile PP tubes, and mixed for 15 min with the addition of 300 mg magnesium sulfate ($MgSO_4$), 100 mg primary secondary amine (PSA) and 100 mg octyldecylsilane (C_{18}), whereafter the sample was evaporated to 0.5 mL under a gentle nitrogen flow. Subsequently, 150 mg of active carbon was added to the sample, and the sample was diluted to 15 mL by adding ultrapure water. With these samples, a second solid-phase extraction was performed based on a slightly modified version of Albergamo et al. (2018). Briefly, extraction cartridges (Oasis HLB (60 µg); Waters) were conditioned with 5 mL of methanol, and equilibrated with 5 mL of ultrapure water. The 15 mL sample was loaded on the cartridges and left to dry for 20 min, after which the cartridges were eluted with 2 × 2.5 mL methanol by vacuum. This extract was collected, filtered (0.22 µm disk filter) and evaporated to 0.5 mL. Prior to injection the extracts were diluted 5 times.

2.4.2.2. LC-HRMS analysis. To quantify the concentrations of 133 organic contaminants, a six-point calibration curve was created in ultrapure water spiked to 8 µg/L and serially diluted to obtain six concentration factors, with 250 ng/L being the lowest concentration. Calibration lines displayed r-squared values greater than 0.99. Of the measured compounds, 63 lacked an internal standard, here an external standard calibration was used instead. Consequently, this part of the data could only be used to compare relative concentrations between the simultaneously analysed WWTPs (Supplementary Table S2 for complete compound target list). A liquid chromatography coupled to mass spectrometry (LC-MS) was performed on a Maxis UHPLC-q-ToF system based on a slightly modified version of the instrumental method of Albergamo et al. (2018). In short, MS detection separation of a 20 µL sample in both positive and negative ESI mode was achieved with a core-shell Kinetex biphenyl column (100×2.1 mm, 2.6 µm particle size, and 100 Å pore size, Phenomenex, Utrecht, the Netherlands). The mobile phase consisted of two solutions, i.e. a) ultrapure water with acetic acid (0.05%) and b) methanol. With a flow rate of 0.3 mL/min, the LC-gradient was 0% methanol from 0 to 2 min, increased linearly to 100% at 17 min, and kept equal until 25 min. The system was allowed to re-equilibrate for 7 min before the next injection. Prior to each injection, a 50 µM sodium acetate solution in H2O:MeOH (1:1, v:v) was introduced automatically for *m/z* recalibration of the system during data processing. The column oven was kept at 40 °C. MS and data-independent MS/MS data were acquired with positive and negative ESI in separate runs with a resolving power typically of 30,000–60,000 FWHM. Full-scan MS and MSMS spectra acquired in broad-band collision induced dissociation mode (bbCID) were screened for the accurate masses, retention time (tR), mass accuracy, isotopic fit and MSMS for unambiguous identification of the targeted organic contaminants.

2.4.3. Cu-spiked sludge

For the sludge degradation experiment with Cu-spiked sludge, 3 concentrations of Cu-spiked sludge were prepared by pouring 500 mL of sludge from Rhenen into a single glass bottle per Cu concentration. The bottles were manually swerved, and 5 mL of either deionized water, 3585 µg•L^{-1}, or 7170 µg•L^{-1} $CuCl_2{\cdot}2H_2O$ stock solution was slowly added to each of the bottles. This resulted in a nominal Cu concentration of 200 mg•kg^{-1} (Cu0), 5940 mg•kg^{-1} (CuL) and 11,880 mg•kg^{-1} (CuH) Cu per DW sludge. To ensure even mixing, the bottles with spiked sludge were aerated for 16 h before the start of the experiment. To determine the actual Cu concentration in the sludge, the methods from 2.1.2.a were followed. For the Cu concentration in the overlying water, 9 mL water samples ($n = 16$) were directly analyzed with the ICP-AES after the addition of the internal standards.

2.5. Data analysis

2.5.1. Clustering analysis of chemical data

The concentrations of the 55 contaminants that were present in at least one of the sludge samples were normalised, so that a value of 1 represented the highest measured concentration of that specific contaminant. The 13 sludge samples ($n = 4$ for Alkmaar and Rhenen; $n = 5$ for Mijdrecht) were then clustered based on these normalised concentrations. As we aimed to assess the hierarchical structure of the WWTPs based on the contaminant concentrations, an agglomerative clustering technique (Agnes) was applied on the normalized contaminant concentrations. Agnes grouped the sludge samples sequentially into larger clusters, ending with a single cluster containing all samples. For clustering decisions Ward's criterion was used, which aimed to minimize the within cluster variation of a potential new cluster after merging two lower-tier clusters. To assess which contaminant concentrations were correlated in the sludge samples, the same cluster analysis was applied to the normalized contaminant concentrations. To give an overview of the WWTP contaminant profiles, the resulting dendrograms were presented as a heatmap (Kolde, 2019) of the normalised contaminant concentrations. We acknowledge that, although we analysed 145 contaminants to create the chemical profiles, WWTP sludges contain a plethora of other unknown contaminants. This hampers the possibility to attribute observed effects on sludge degradation to specific measured (groups of) contaminants, as the known, measured contaminants often explain only a small fraction of observed effects when analysed in "Iceberg modeling" studies (Escher et al., 2020; Neale et al., 2017).

2.5.2. Calculation of predicted sludge degradation

To assess if biotic interactions played a role during sludge degradation, the predicted sludge degradation by the two- and three taxa assemblages was calculated, based on the sludge degradation by the single taxa assemblages. If no biotic interactions would occur in the two- or three taxa assemblages, the expected degradation would be the average of the sludge degradation in the corresponding single-taxa assemblages. Therefore, the expected sludge degradation in the two or three-taxa assemblages was calculated by averaging a random sample from one single taxa assemblage with a random sample from another single taxa assemblage, until all samples in those assemblages were assigned (Fugère et al., 2012). This created a new expected assemblage with the same number of replicates as the corresponding single taxa assemblages. The same method was used to assess if the sludge degradation in the Chironomus-Physa assemblage was affected differently by increased Cu concentrations than what would be expected based on both single species assemblages.

2.5.3. Statistical analysis

Differences between locations in sludge dry weight and conductivity at the start of the experiment were assessed with a one-way ANOVA (1w-AOV) and a TukeyHSD post-hoc test. To analyses if conductivity changed over time and was affected by macroinvertebrate assemblage, a two-way ANOVA (2w-AOV) was performed for each location separately, with conductivity as a dependent variable, and time (start, day 3, day 7) and taxa assemblage as independent variables. To assess differences in survival between macroinvertebrate taxa and WWTP locations, a general linear model (GLM) with a binomial function was used, with the number of surviving and deceased individuals as the dependent variable, and taxa and WWTP location as independent variables, including the interaction term. A pairwise analysis of estimated marginal means was performed to calculate p-values between groups.

To determine differences in baseline sludge degradation between WWTP locations, a 1w-AOV was performed with the degradation of the control treatments of the three WWTPs as a dependent variable, and the WWTP Location as an independent variable, followed by a TukeyHSD post-hoc test. Subsequently, to assess differences in sludge degradation between macroinvertebrate assemblages and locations, a 2w-AOV with taxa assemblage and WWTP location and their interaction term as independent variables was performed, also followed by a TukeyHSD post-hoc test. To assess macroinvertebrate assemblage effect on degradation for each location separately, three separate 1w-AOVs with subsequent TukeyHSD post hoc tests were performed for each location, as the interaction term from the previous 2w-AOV was significant. To compare the expected and observed sludge degradation of multi-taxa assemblages, a separate 1w-AOV was performed for each expected-observed assemblage pair with degradation as the dependent variable. Although, some deviations from the assumption of normality occurred, we chose to use ANOVAs as these are robust against these deviations.

To assess the effect of Cu and species assemblage on survival of both *C. riparius* and *P. acuta*, the growth of the *C. riparius* larvae and the sludge degradation, 2w-AOV tests were performed, with Cu concentration, species assemblage and the interaction as independent variables, and either survival of *C. riparius, P. acuta, C. riparius* growth, or degraded sludge as dependent variable. If applicable, these 2w-ANOVAs were followed by a TukeyHSD post-hoc test. To compare the expected and observed sludge degradation of the CP assemblages, a separate 1w-AOV was performed for each expected-observed pair per Cu concentration. To assess the effect of Cu concentration and taxa assemblage on the conductivity in the experiments, two 2w-ANOVAs were performed for the start and end of the experiment, with the Cu concentration and taxa assemblage as well as their interactions as independent variables, and conductivity as a dependent variable. Statistical tests and the clustering analysis were performed in core R 3.6.1 (R Core Team, 2020).

3. Results

3.1. Experimental conditions

Oxygen levels in all experimental replicates were between 3.0 and 8.9 mg/L with a mean of 7.3 ± 1.0 mg/L ± SD. The pH ranged from 6.5 to 7.5 during the experiment, in accordance with OECD guideline 235 (OECD, 2011). Conductivity did vary between locations at the start of the experiment (574 ± 0.9, 869 ± 2.5 and 670 ± 2.4 uS/cm ± SE, for Rhenen, Mijdrecht and Alkmaar respectively) ($F_{2,16}$= 4415, $p < 0.001$), but did not show a distinct pattern over time, whereas the taxa assemblage did have a small effect on conductivity ($F_{8,43}$>3.4, $p < 0.05$ for all locations) (Supplementary Fig. S2). Sludge dry weight at the start of the experiment differed significantly between all locations, being the highest in Rhenen (3.9 DW g/L ± 0.03 SE), followed by Mijdrecht (3.7 DW g/L ± 0.02 SE), and Alkmaar (2.9 DW g/L ± 0.02 SE) ($F_{2,10}$= 396.5, $p <$ 0.05).

3.2. Macroinvertebrate survival and sludge degradation

Survival of *P. acuta* (94–100%) and *Tubificidae* (92–100%) was high, whereas the survival of the *C. riparius* larvae was slightly lower than that of the other two taxa (69–91%) (both $p < 0.001$). Location had a small but significant effect on survival of The survival of the *C. riparius* larvae was also slightly but significantly lower in Rhenen (69%) than in Mijdrecht (91%) and Alkmaar (81%) (both $p < 0.05$).

Baseline sludge degradation without macroinvertebrates differed significantly between the three WWTPs ($F_{2,10}$=10.8, p = 0.003), with Rhenen (Fig. 1a) showing the highest control degradation (18.1 mg ±3.8 SE) (both $p < 0.009$), followed by both Mijdrecht (Fig. 1b) (6.5 mg ± 0.9 SE) and Alkmaar (Fig. 1c) (5.7 mg ± 0.4 SE). Taxa assemblage ($F_{7,89}$=15.9, $p < 0.001$) as well as location ($F_{2,89}$=24.3, $p < 0.001$) had a significant effect on the degradation of sludge, and the effect of taxa assemblage differed per location ($F_{14,89}$=3.4, $p < 0.001$). Generally, macroinvertebrate sludge degradation was lowest at Alkmaar (16.0 mg ± 0.9 SE) (both $p < 0.001$), but did not differ between Mijdrecht (22.4 mg ± 2.2 SE) and Rhenen (24.8 ± 0.9 SE) ($p = 0.13$). Macroinvertebrate assemblages significantly enhanced sludge degradation compared to the control (all $p < 0.001$), with the exception of the tubificids ($p = 0.37$). Yet, when evaluating the individual taxa assemblages per location, for Rhenen, only the sludge degradation by *P. acuta* and the *Chironomus-Physa* combination was marginally higher than that of the control treatment ($p = 0.08$ and $p = 0.06$ respectively). This was mainly caused by a single outlier in the control, as repeating the analysis with exclusion of this outlier resulted in all taxa assemblages, except for the single

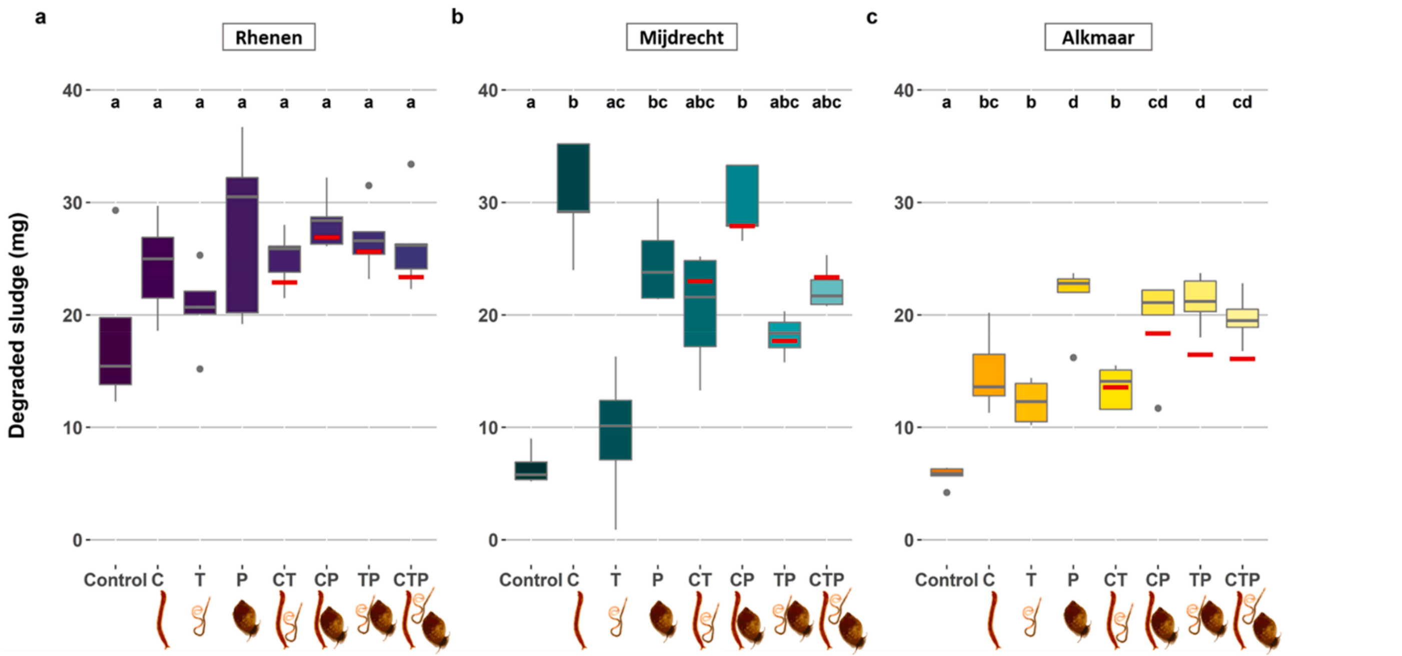

Fig. 1. Degradation (mg) by (combinations of) Chironomus riparius larvae (C), Tubificidae (T), and Physa acuta (P) of sludge from the WWTPs of Rhenen (a), Mijdrecht (b), and Alkmaar (c). Boxes show interquartile range, bold lines represent the median, whiskers indicate the lowest and highest values within a 1.5x interquartile range from the box, dots represent outliers. Letters indicate significant ($p < 0.05$) differences between species (combinations). Two outliers at Mijdrecht (C-treatment: 50.1 mg; CP-treatment: 66.0 mg) are not shown to increase figure readability. Red lines indicate the median of the predicted degradation by multi-taxa assemblages based on single-taxa degradations.

Tubificidae, degrading significantly more sludge than the control ($p < 0.05$). In contrast to Rhenen, for Mijdrecht and Alkmaar the single and two taxa assemblages with either *C. riparius* or *P. acuta* always accomplished a significantly ($p < 0.05$) higher sludge degradation than the corresponding control. Furthermore, the single *Tubificidae* assemblage and *Chironomus-Tubificidae-Physa* assemblage in Alkmaar also degraded significantly ($p < 0.05$) more sludge than the control. However, the observed sludge degradation in the macroinvertebrate multi-taxa assemblages was only higher than the predicted sludge degradation based on the single taxa assemblages for the *Tubificidae-Physa* and *Chironomus-Tubificidae-Physa* combinations in Alkmaar ($F_{1,8}$>3.8, $p < 0.05$), suggesting only limited biotic interactions.

3.3. WWTP sludge contaminant profiles

Of the 133 organic contaminants and 12 heavy metals that were analysed, a total of 44 organic contaminants and 11 heavy metals were present and quantified in the sludge samples of the three WWTPs. The cluster analysis of the chemical data generated an agglomerative coefficient of 0.73 (scale: 0.00–1.00), indicating a decent clustering structure, which confirms that the Ward's criterion was suitable for clustering the sludge samples.

The cluster analysis revealed that the contaminant profiles of the sludge from Rhenen and Mijdrecht were more similar compared to Alkmaar, and moreover, the sludge from the WWTP of Alkmaar contained the highest concentrations of 34 out of the 55 contaminants. Alkmaar was also characterized by several contaminants occurring only at that WWTP, such as the herbicides Dimethanamid-p, Metamitron and Metobrumoron, the insecticide Pyriproxyfen, and the pharmaceuticals Sulfamethazine, Ibuprofen and Carbamazepine (Fig. 2: 1st cluster). Several other contaminants occurred only at Mijdrecht, such as the pesticides Fenuron, Methiocarb and Methyl-pirimifos (3rd cluster). There were no contaminants, however, that were solely measured in sludge from Rhenen although higher concentrations of the antibiotics Clindamycin and Trimethoprim were characteristic for Rhenen (2nd cluster). A large group of contaminants was present in the sludge of all three WWTPs, occurring in the two lowest clusters, which included common metals, such as Zn, Cr, and Ni, but also widely used pharmaceuticals such as Triclosan and Paracetamol (4th and 5th cluster). Also notable is the presence of the pesticides MCPP, 2,6-Dichlorobenxamide and Fipronil at Alkmaar and Mijdrecht, which were absent at Rhenen.

3.4. Macroinvertebrate survival, growth and degradation of Cu-spiked sludge

To evaluate the effects of contaminants on biotic interactions and sludge degradation in more detail, sludge from the WWTP in Rhenen was spiked with Cu and subjected to the invertebrate assemblages. The measured Cu concentrations in the spiked sludge were close to the nominal concentrations (Supplementary Table S3). At the end of the experiment the oxygen concentrations ranged between 5.5 and 8.0 mg/L, and the pH ranged between 6.5 and 7.5 in all treatments. The conductivity was affected by the Cu concentration at the start of the experiment (468 ± 14, 610 ± 7, and 665 ± 12 uS/cm ± SE for Cu0, CuL, and CuH respectively, $F_{2,45}$=82.0, $p = < 0.001$)), and was higher at the end of the experiment (557 ± 21, 719 ± 21 and 773 ± 33 uS/cm ± SE for Cu0, CuL and CuH, $F_{2,36}$=54.5, $p < 0.001$), with species assemblage also having an effect $F_{3,36}$=13.1, $p < 0.001$) (Fig. S3). This was likely due to differences in the degradation of the sludge, and the addition of $CuCl_2$ in the spiked treatments.

Survival of *P. acuta* ($F_{2,18}$=100, $p < 0.001$) was significantly lower at the highest Cu concentration, as no snails survived at this concentration, both with and without the presence of *C. riparius*. The survival of *C. riparius* was only significantly lower at the highest Cu concentration in the presence of snails, as indicated by the significant interaction term ($F_{2,18}$=100, $p < 0.001$) (Fig. 3a, Greek letters). Growth of the *C. riparius* larvae decreased significantly with increasing Cu concentrations in the sludge ($F_{2,16}$=80.7, $p < 0.001$) (Fig. 3b, Latin letters), but was positively affected by the presence of *P. acuta* ($F_{1,16}$=14.1, $p < 0.01$), although only at the low Cu concentration ($p < 0.05$) (Fig. 3b Greek letters). Hence,

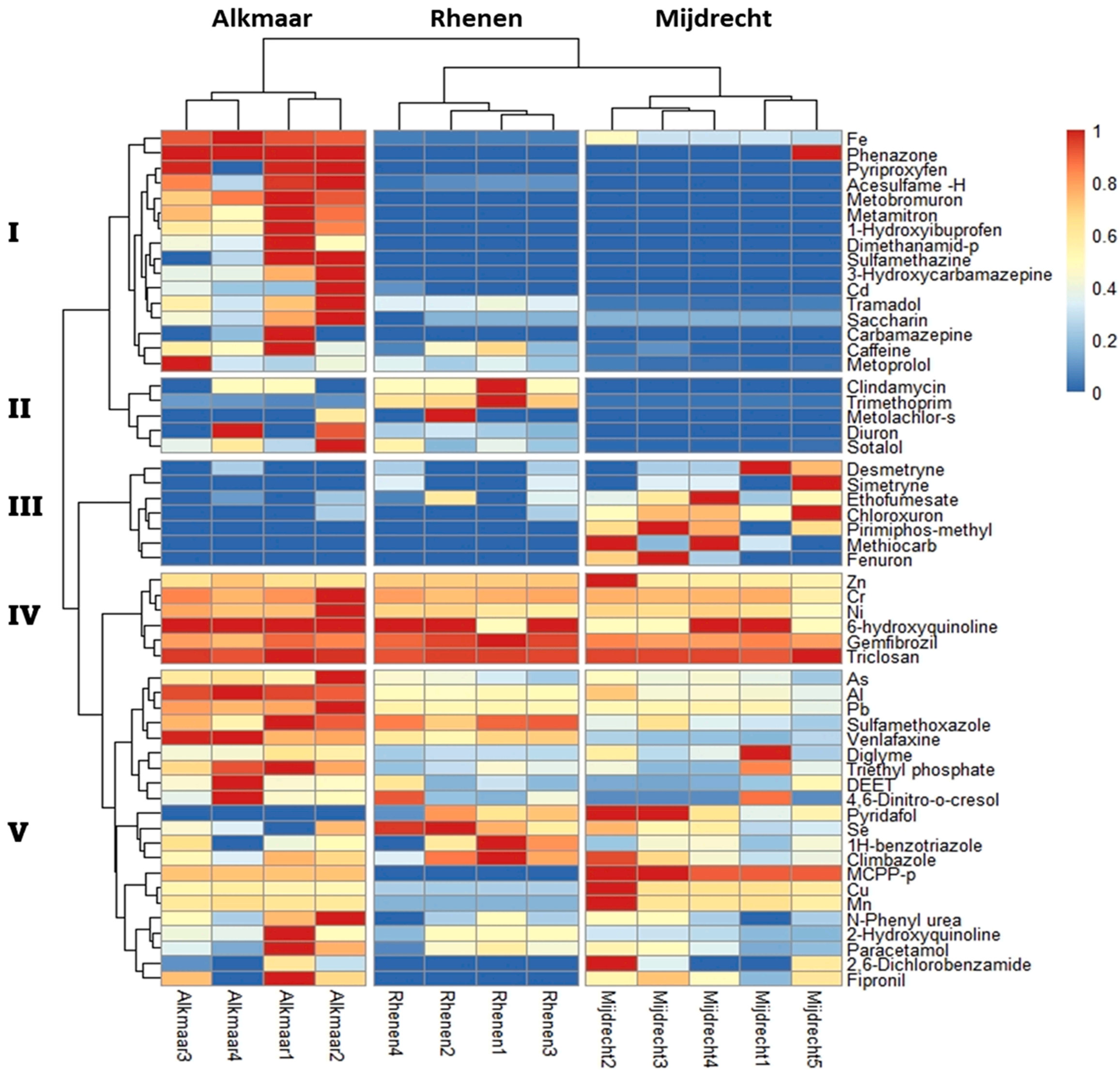

Fig. 2. Heatmap of 11 heavy metals and 44 organic contaminants in the sludge of the three WWTPs. Trees were constructed using Hierarchical clustering with Agglomerative nesting (Agnes - Ward). Contaminant concentrations were normalised, with a value of 1 being the highest observed concentration of that specific contaminant.

positive biotic interactions were manifested at the CuL concentration, and did not occur at Cu0, nor at CuH, likely due to the overall detrimental effect of Cu on *Physa* performance and thus high mortality at this concentration.

Sludge degradation in all species assemblages was significantly lower at the highest Cu concentration ($F_{2,36}$=24.5, $p < 0.001$), compared to the Cu0 and the low Cu concentration (both $p < 0.05$) (Fig. 4, Latin letters). The difference in sludge degradation was also marginally significant ($p = 0.07$) between the Cu0 and CuL concentration. Species assemblage had also a significant effect on sludge degradation ($F_{3,36}$=4.4, $p = 0.01$), but only at the CuL concentration, as the *C. riparius* larvae and the *C. riparius* – *P. acuta* combination accomplished a higher sludge degradation than the control without invertebrates ($p < 0.05$). These effects were not significant though in the non-spiked control sludge and the CuH sludge (Fig. 4, Greek letters). At the highest Cu concentration, this was again likely due to the high *Physa* mortality, and inhibited *C. riparius* larval growth.

The observed sludge degradation by the multispecies assemblage (CP) was only significantly higher than the predicted degradation (predCP) at the CuL concentration ($F_{1,6} = 13.6$, $p = 0.01$) (Fig. 4, indicated by the asterisk). At CuH, no effect on sludge degradation in CP was observed compared to the predicted sludge degradation (predCP), which was likely due to the high *Physa* mortality and low *Chironomus* growth, and the very low sludge degradation in general at this Cu concentration.

4. Discussion

In line with our hypothesis, WWTP contaminant profiles affected sludge degradation by macroinvertebrates. Macroinvertebrates generally enhanced sludge degradation, although the intensity of this enhancement was WWTP and taxa assemblage specific. Sludge degradation by combinations of taxa was, however, only higher than expected in sludges with a higher contaminant load, indicating an effect of biotic interactions.

4.1. WWTP specific sludge degradation

The sludge degradation in absence of macroinvertebrates differed between the three WWTPs. This is not surprising, as no WWTP is exactly alike, due to a difference in incoming volumes and the origins of wastewater with different contaminant loads and profiles, microbial communities, and treatment steps with a range of settings. Elevated

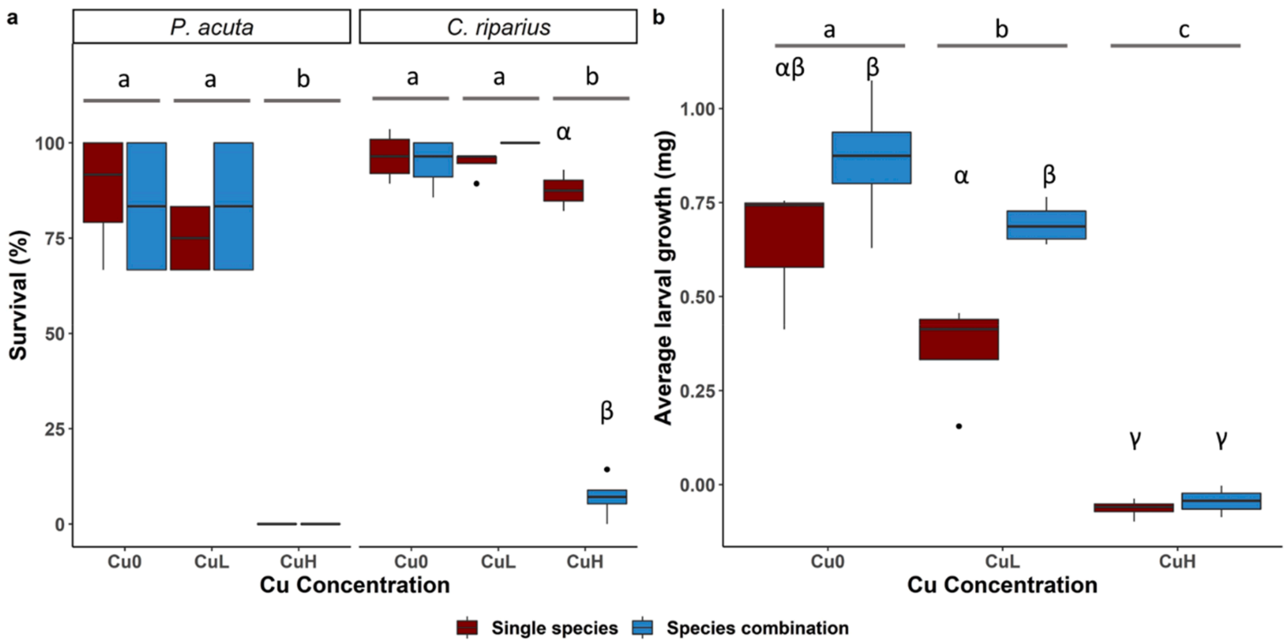

Fig. 3. Survival of P. acuta and C. riparius (%) (a), and growth of C. riparius (mg) (b) exposed to Cu spiked sludge (Cu0: 0.2 $mg{\cdot}g^{-1}$ (control, not spiked), CuL: 4.6 $mg{\cdot}g^{-1}$, CuH: 13.0 $mg{\cdot}g^{-1}$ $Cu{\cdot}DW^{-1}$) in single and multispecies setups. Boxes show interquartile range, bold lines represent the median, whiskers indicate the lowest and highest values within a 1.5x interquartile range from the box, dots represent outliers. Latin letters indicate significant ($p < 0.05$) differences between Cu concentrations, Greek letters indicate significant ($p < 0.05$) differences between single and combined species treatments.

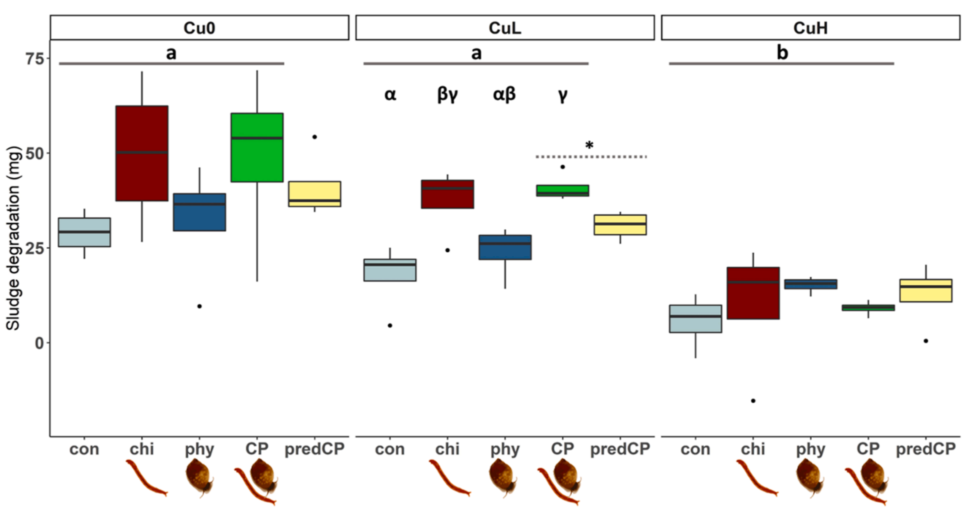

Fig. 4. Sludge degradation (mg) at three concentrations of Cu spiked sediment (Cu0, CuL, CuH) for four species treatments: Control (no invertebrates), C. riparius, P. acuta, C. riparius-P. acuta combination). The predicted sludge degradation for the combination treatment was estimated by averaging the values of the single species treatments. Boxes show interquartile range, bold lines represent the median, whiskers indicate the lowest and highest values within a 1.5x interquartile range from the box, dots represent outliers. Latin letters indicate significant ($p < 0.05$) differences between Cu concentrations, Greek letters indicate significant ($p < 0.05$) differences between species treatments. The asterisk indicates a significant ($p < 0.05$) difference between the observed and predicted sludge degradation in the combined species treatment at the CuL concentration.

contaminant concentrations indeed affect the capability of WWTPs to reduce sludge (Maal-Bared, 2020; Ren, 2004). Hence, the presently observed differences in contaminant profiles and concentrations between the WWTPs may explain the lower sludge degradation in Alkmaar, as it had the highest contaminant load and was characterized by the presence of multiple pesticides, possibly indicating increased pressures from an agricultural origin. The same was the case for Mijdrecht, with multiple pesticides present in the sludge. Rhenen on the other hand contained a lower concentration and number of contaminants and pesticides, allowing for a higher sludge degradation. Macroinvertebrates were able to enhance the degradation of sludge in each WWTP, but the magnitude of this enhancement differed between the WWTPs. As there was only a certain amount of organic matter to be degraded in our experimental setup, a high initial degradation, such as in Rhenen, would limit the contribution of macroinvertebrates to the overall high sludge degradation. Oppositely, when contaminant concentrations in the sludge are high, such as in Alkmaar, the initial sludge degradation of the WWTP, as well as the macroinvertebrate performance may be hampered (Bundschuh et al., 2021), leading to the lowest joint sludge degradation. Macroinvertebrates may have the highest positive impact on sludge degradation at WWTPs like Mijdrecht, where the sludge degradation is relatively low due to an intermediate contaminant profile and load, but where macroinvertebrate performance is not yet greatly affected. Here macroinvertebrates enhanced sludge degradation the most. The results from the Cu-spike sludge experiment are in line with these observations, as the *C. riparius* larvae and *P. acuta* snails enhanced the sludge degradation the most at the intermediate Cu concentration. At the lowest Cu concentration, sludge degradation was already high, and therefore the macroinvertebrates increased the sludge degradation only slightly, while at the highest Cu-concentration, sludge degradation was low, and the macroinvertebrates were severely hampered. Hence, it was at the intermediate

Cu-concentration that macroinvertebrates enhanced sludge degradation the most.

4.2. Positive biotic interactions may occur at moderate contaminant loads

Higher than expected sludge degradation only occurred at the WWTP in Alkmaar, which had the highest contaminant load, as well as in intermediate Cu-spiked sludge from Rhenen. This higher-than-expected sludge degradation could be the result of the altered biotic interactions at these contaminant concentrations. Although the exact mechanisms underlying the observed increase in degradation remain to be unraveled, our results are in line with the predictions of the Stress Gradient Hypothesis (Bertness and Callaway, 1994), which states that in a more stressful environment, the prevalence of positive biotic interactions increases, while the intensity of negative interactions decrease. However, the high mortality and low growth and the low degradation at the highest Cu concentration in the Cu-spiked sediment does emphasize that possible benefits of biotic interactions only occur below a certain contaminant level. As above this level, effects of contaminants on macroinvertebrates may be too severe.

In contrast with studies on the effect of macroinvertebrate interactions or richness on the degradation of natural organic matter and leaves (Costantini and Rossi, 2010; Jabiol et al., 2013; Tonin et al., 2018), we did observe relatively few biotic interactions. This might be due to the homogenous composition of WWTP sludge, whereas leave packs may contain multiple leave species, which have nerves and more woody parts. A more homogenous environment is more prone to competitive interactions, whereas an environment with more substrate variation allows for more niche differentiation and resource partitioning (Townsend, 1989), thus being more prone to positive biotic interactions.

The taxa assemblages degrading more sludge than expected were the *Tubificidae-Physa* and *Chironomus-Tubificidae-Physa* assemblages from Alkmaar, and the *Chironomus-Physa* assemblage in Cu-spiked sludge from Rhenen. One of the causes of the increased sludge degradation in these treatments might be a decrease in movements of the snails (Brown et al., 2012; Gao et al., 2017), which may cause photo- and thigmotactic stimuli that cause retractions of the *Tubificidae* and the *C. riparius* larvae into the sediment (Drewes and Fourtner, 1989; Gresens, 1995). These frequent retractions may result in less time spend on feeding, and therefore a lower sludge degradation. Hence, lower snail activity may result in a higher joint sludge degradation. On the other hand, snails may benefit from the presence of other taxa, as the burrowing and feeding behavior by the *Tubificidae* and *Chironomus* larvae could alter the fungal and bacterial composition of the sludge (Hunting et al., 2012; Poulsen et al., 2014; Samuiloviene et al., 2019), making it a more suitable food source for the snails. These positive interactions may also explain the increased sludge degradation, and growth of the *C. riparius* larvae in the *Chironomus-Physa* assemblage at the CuL concentration. Hence, biotic interactions occurring between these invertebrates partly mitigate the negative impacts of increased contaminant concentrations.

Other positive biotic interactions may have also occurred due to the snail presence, as the snail feces might be a more suitable food for the tubificids (Phipps et al., 1993) and the *C. riparius* larvae than the sludge itself. Additionally, pedal mucus produced by the snails might adsorb a part of the labile contaminants, possibly reducing the contaminant concentration in the overlying water (Jugdaohsingh et al., 1998), thus limiting exposure of the other macroinvertebrates. Another possibility for positive effects on the larval growth is the dampening of intraspecific competition in multi taxa assemblages. Under resource limited conditions, intraspecific competition is often more stringent than interspecific competition, and thus multi-taxa assemblages could dampen the effect of intraspecific competition (Costantini and Rossi, 2010; McKie et al., 2009).

We do acknowledge that further research would be needed to confirm the exact mechanisms underlying the positive effects of multi-taxa assemblages on sludge degradation. Such experiments may try to disentangle the direct behavioural interactions between macroinvertebrates (Gresens, 1995), and indirect interactions such as alterations of the sludge environment by one macroinvertebrate regarding bacterial and fungal mass and composition (Costantini and Rossi, 2010; Graça et al., 1993), and (organic) contaminant concentrations (van der Meer et al., 2022). Ideally this would be accomplished by an experiment in which one species of invertebrate would be allowed to manipulate the sludge for a certain amount of time, removed from the sludge, and introducing another macroinvertebrate into the altered sludge.

4.3. Perspectives: a role for macroinvertebrates in the reduction of the amount of excess WWTP sludge?

While previously the use of *Tubificidae* in WWTPs has garnered most attention, in our setup their sludge degradation was consistently the lowest per unit of DW biomass of the selected taxa, which is in line with our previous work (van der Meer et al., 2021). The present study therefore emphasizes the need to widen the scope of research and to investigate the sludge degradation potential of other (combinations of) macroinvertebrate species, which seems promising on the present experimental scale. An additional advantage of using multiple macroinvertebrates for the degradation of sludge, is the increased combined resilience to contaminants that such a system may have, as positive biotic interactions between the different macroinvertebrates could limit the negative impact of elevated contaminant concentrations on sludge degradation to some extent. Contrary to wastewater treatment steps with *Tubificidae*, no attempts to scale up treatment steps with midges or snails have been made yet. To achieve a successful treatment of sludge with these macroinvertebrates, life-cycle traits and population dynamics have to be taken into account, as to assure consistent reproduction and to prevent negative intraspecific interactions (Cope and Winterbourn, 2004). On the other hand, the *Chironomus* density used in this experiment was still five times lower than some natural population densities (Groenendijk et al., 1998), indicating that sludge degradation rates could even be higher. Due to the high growth rate, high fertility, and short life cycle of both *C. riparius* and *P. acuta,* employing these species in wastewater treatment would result in the production of substantial amounts of invertebrate biomass. This biomass could potentially be harvested, removing additional organic matter. *Chironomus riparius* has the advantage that the terrestrial adult phase allows for a relatively easy collection and harvesting (Reyes-Maldonado et al., 2021). The exuviae left behind after emergence, containing often higher contaminant concentrations, might be skimmed, and collected from the water surface. The solid shells of *P. acuta* would likely allow a separation from sludge and overlying water by sieving. If contaminant concentrations in the harvested biomass are below regulatory limits, this macroinvertebrate mass might be used as a resource for novel products, as is already the case with multiple species of duckweed grown on wastewater (Liu et al., 2021). Although bioaccumulation of contaminants may be limited (van der Meer et al., 2022), care should be still taken to perform regular contaminant analysis on novel products produced with this mass. The successful application of macroinvertebrates in the treatment of wastewater might offer an alternative to the more high-tech energy consuming post-treatment steps such as ozone or UV-treatment. Moreover, the reduction of operational costs, relatively simple techniques, possible production of new biomaterials, and the global occurrence of macroinvertebrates with the appropriate traits, may offer benefits to countries without the infrastructure to build these high-tech post-treatments.

5. Conclusions

The contaminant profiles of WWTP sludges affected sludge degradation by macroinvertebrates, with higher contaminant loads resulting in a lower sludge degradation. Nonetheless, most single and multi-taxa macroinvertebrate assemblages employed in this study enhanced sludge degradation. Moreover, the degradation capacity of multi-species macroinvertebrate assemblages may be more resilient to moderate contaminant loads of WWTP sludge. The present study thus showed that macroinvertebrates are a potential tool for the reduction of excess WWTP sludge, and that using multispecies assemblages of detritivorous macroinvertebrates may increase the resilience of this additional treatment step.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Tom van der Meer reports financial support was provided by Waterschap Rivierenland. Tom van der Meer reports financial support was provided by Hoogheemraadschap de Stichtse Rijnlanden. Tom van der Meer reports financial support was provided by Hoogheemraadschap Hollands Noorderkwartier.

Data Availability

Data are available from the Mendeley data repository (van der Meer et al. 2022): https://data.mendeley.com/datasets/z6ys4b763s/1.

Acknowledgments

This work was supported by three Dutch Water Boards: Hoogheemraadschap Hollands Noorderkwartier, Waterschap Rivierenland, and Hoogheemraadschap de Stichtse Rijnlanden. We would like to thank the employees of the WWTPs in Rhenen, Alkmaar and Mijdrecht for allowing of and assisting in the collection of sludge. Emma Schroeijers and Janna van Burik performed and collected the data for the Cu-spike experiment, Annabel Landman aided the experimental work, and Samira Absalah and Rutger van Hall assisted with the contaminant analysis.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118863.

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