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Research article

Nitrogen retention in stream biofilms – A potential contribution to the self-cleaning capacity

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ABSTRACT

Surface waters are under increasing pressure due to human activities, such as nutrient emissions from wastewater treatment plants (WWTPs). Using the retention of nitrogen (N) released from WWTPs as a proxy, we assessed the contribution of biofilms grown on inorganic and organic substrates to the self-cleaning capacity of second-order streams within the biosphere reserve Vosges du Nord/Palatinate forest (France/Germany). The uptake of N from anthropogenic sources, which is enriched with the heavy isotope ^{15}N, into biofilms was assessed up- and downstream of WWTPs after five weeks of substrate deployment. Biofilms at downstream sites showed a significant positive linear relationship between $\delta^{15}N$ and the relative contribution of wastewater to the streams' discharge. Furthermore, $\delta^{15}N$ substantially increased in areas affected by WWTP effluent (~8.5‰ and ~7‰ for inorganic and organic substrate-associated biofilms, respectively) and afterwards declined with increasing distance to the WWTP effluent, approaching levels of upstream sections. The present study highlights that biofilms contribute to nutrient retention and likely the self-cleaning capacity of streams. This function seems, however, to be limited by the fact that biofilms are restricted in their capacity to process excessive N loads with large differences between individual reaches (e.g., $\delta^{15}N$: −3.25 to 12.81‰), influenced by surrounding conditions (e.g., land use) and modulated through climatic factors and thus impacted by climate change. Consequently, the impact of WWTPs located close to the source of a stream are dampened by the biofilms' capacity to retain N only to a minor share and suggest substantial N loads being transported downstream.

1. Introduction

Wastewater inputs strongly increase nutrient concentrations in aquatic ecosystems (Painter et al., 2020; Martí et al., 2004; Waiser et al., 2011) causing eutrophication and ecosystem degradation (Eisakhani and Malakahmad, 2009), with a documented decline in water quality (Figueroa-Nieves et al., 2014; Howarth et al., 1997). Under projected global change scenarios (Stocker et al., 2013) and resulting weather extremes, aquatic ecosystems are increasingly under pressure, threatening the provision of essential ecosystem services (Danley and Widmark, 2016), such as high quality drinking water. At the same time, in-stream filtration, retention and metabolization of natural and anthropogenic material (self-cleaning) sustain the water quality (Carey and Migliaccio, 2009; Tank et al., 2000; Hamilton et al., 2001; Tank and Dodds, 2003; Hall et al., 2009; Mulholland et al. 2000, 2002). Biofilms contribute to nutrient retention and thus self-cleaning of freshwaters (Sabater et al., 2007) through their high biological activity and availability of sorption sites (Bighiu and Goedkoop, 2021). The activity of biofilms is a function of local pressures, which include regional climate and levels of nutrients and chemicals (Battin et al., 2007). In addition to the aforementioned parameters, the substrates on which biofilms grow greatly influence their composition with important implications on their function (Besemer, 2016). Hereby two main compartments can be differentiated, namely inorganic and organic substrate-associated biofilms, which are often autotrophic and heterotrophic in nature, respectively (Sabater et al., 2007; Okabe et al., 2005). These characteristics may affect their efficiency to retain nutrients and thus their contribution to the self-cleaning of freshwater ecosystems (Besemer, 2016).

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Nitrogen (N) is a common component in wastewater (Carey and Migliaccio, 2009) and an essential nutrient for primary producers (Schulten and Schnitzer, 1997). Consequently, N retention is a key element of eutrophication management (Conley et al., 2009). Therefore, N may be considered a suitable point of reference to estimate the self-cleaning capacity in fresh waters supported by the high affinity of aquatic microorganisms to incorporate and retain this nutrient. In this context, significant advancements have been made to understand N-cycling (Camargo and Alonso, 2006; Rabalais, 2002; Painter, 1970; Mariotti, 1983; Martí et al., 1994; Peterson et al., 2001; Mulholland et al., 2008; Beaulieu et al., 2011). In fact, due to volatilization and preferential use of ^{14}N during biological wastewater treatment, N released from wastewater treatment plants (WWTPs) is known to be characterised by an elevated ratio of heavy stable N isotopes (^{15}N:^{14}N; Freyer and Aly, 1975; Kreitler, 1975; Gormly and Spalding, 1979; Heaton, 1986; Aravena et al., 1993; Kendall et al., 2007; Snider et al., 2010; Merbt et al., 2011; Ribot et al., 2012; Pastor et al., 2013; Koszelnik, 2014). These insights should allow tracing the retention of N from anthropogenic sources via isotope ratios in biofilms (Valiela et al., 2000; Voss et al., 2006; Wigand et al., 2007). Currently, several studies have documented an elevated share of heavy stable N isotopes in biofilm (Merbt et al., 2011; Peipoch et al., 2012; Ribot et al., 2012; Pastor et al., 2013), related to the concentration of ^{15}N-ammonium in wastewater released to streams. These findings suggest a substantial contribution of aquatic biofilms to the self-cleaning capacity of streams. However, biofilms and their contribution to ecosystem functions (e.g. stream self-cleaning or biomass accrual and organic matter decomposition) may also be negatively affected by wastewater and the organic micropollutants it contains (Bundschuh et al., 2011; Burdon et al., 2019; Mor et al., 2019).

Considering this background, the present study investigated the N retention and the functional performance (biomass accrual and leaf litter decomposition) of biofilms grown on inorganic and organic substrates, assuming the type of substrate drives N retention rates (Bastias et al., 2020). The study was performed in two catchments of headwater streams originating in a nature reserve and draining into landscapes with strong anthropogenic impact (over 20% intensive agricultural use or urbanisation, resulting in values below 3 in the land use index (LUI; Feckler et al., 2014) rating different forms of land use, in a positive relationship towards an ecological favourable status, in a single value). We quantified stable isotope ratios of N in biofilms up- and downstream of wastewater effluents on two substrates, namely ceramic tiles (inorganic substrate) and leaves (organic substrate), reflecting the majority of substrates in headwater streams with a forested catchment (Roche et al., 2019). The impact of climatic factors on nutrient retention and the functional performance of these biofilms were approximated by comparing data from seasons with strongly deviating characteristics (e.g., differing wastewater dilution, temperature and sunshine duration). Therefore, we utilized the fundamental hypothesisis that the isotopic ratio of N (δ^{15}N) in both types of biofilms informs about the release of these nutrients from anthropogenic sources. This study attempts to broaden our understanding about this correlation, which, to the best of our knowledge, has not yet been demonstrated in organic substrate-associated biofilms. We expected the difference in δ^{15}N to be more distinct in biofilms from inorganic substrates, since they can only take up dissolved N from the water (Cummins, 1974), whereas in organic substrate-associated biofilms, N could be derived from both water and organic substrate (Cheever et al., 2013; Pastor et al., 2014). Moreover, we hypothesized that the efficiency to retain anthropogenic N is lower during winter, likely driven by a lower microbial activity and higher dilution of wastewater in the receiving streams. We also hypothesized that the general functional activity (biomass accrual and organic matter decomposition) of both biofilms is negatively affected by wastewater, caused by potentially toxic substances like pharmaceuticals or personal care products (e.g. caffeine, cimetidine and ciprofloxacin; Rosi-Marshall et al., 2013; Xin et al., 2021).

2. Material and methods

2.1. Study period and study region

The present study was conducted during summer 2019 (July and August) and winter 2020 (February and March). Artificial substrates were exposed for five weeks in each season, an incubation time that allows sufficient biofilm growth and substrate-availability for the planned measurements (>2.5 mg; Karouna and Fuller, 1992; Englert et al., 2013; Mahler et al., 2020). Two catchments, the Queich and the Wieslauter, were studied, which both originate in the UNESCO biosphere reserve Vosges du Nord/Palatinate forest in southwestern Germany (Fig. 1). The biosphere reserve is a low mountain range in central Europe, characterised by forested hills dominated by pine, beech and spruce. Within each of the two catchments, four stream reaches were selected, with each reach covering a distance of up to 1250 m. Each reach was characterised and analysed by a set of four sampling sites along the stream, with three sample replicates at each site. Of the four reaches per catchment, three received discharge from WWTPs and one served as an unimpacted reference (Table 1).

The four sampled sites within each reach were identified as follows: One site serving as control was located 250 m upstream of the WWTP effluent (site 1; Table 1). The WWTP effluent (site 2) was sampled when well mixed with stream water (deviations in conductivity of cross section <10 μS/cm). Furthermore, two sites further downstream (30–50 m = site 3 and 500–1000 m = site 4) were sampled. Similar distances between sites have been used in reference reaches, although no effluent existed. Informed by a rapid reduction in water nutrient concentrations observed in the data of the first season (summer 2019), the distance of the last sampling site was reduced to a maximum of 500 m downstream of the reference point in the second season (winter 2020).

The LUI (Feckler et al., 2014) was utilized to characterise local anthropogenic pressures. The LUI combines the land use categories "agriculture", "forestry", "stagnant water bodies" and "urban land use" in a 500 m wide zone along the streams in one value, which we aggregated and processed based on the Corine Land Cover (CLC) data set (Copernicus programme, 2018) using Q-GIS version 3.10.4 (QGIS Development Team, 2020). In brief, the share of each land use category was quantified and ranked based on a score system relying on its proportion (not present = 0, 0.1–5.0% = 1, 5.1–12.5% = 2, 12.6–31.3% = 3, 31.4–78.1% = 4 and ≥ 78.2% = 5) and weighted according to its expected impact on habitat quality. Land use types with no or low likelihood to exert pressure on the aquatic ecosystem (forestry and stagnant water bodies) were weighted positive, whereas land use types with an expected negative impact (agriculture and urban land use) were weighted negative (Jansen et al., 2011; Ohe and Goedkoop, 2013; Zhao and Newman, 2006). With this approach, LUIs ranging from −2 to +5 were derived.

All WWTPs discharge their secondary treated wastewater through an unvegetated sewage channel (max. 500 m length) into the receiving stream. All municipal WWTPs technically reduce the phosphorus load (Ministry for Climate Protection, Environment, Energy and Mobility, Rhineland Palatinate, 2021). Caused by technical issues at one WWTP over the entire study period, an unstable and low wastewater contribution at reach 4 affected our data set. Due to the unsteadiness of the WWTP, it was not possible to calculate the impact dimensions or even amount of wastewater from the facility. Since the impact in this reach could not be rated and does not fit the assigned category (i.e., impacted by a WWTP point source or reference) we excluded reach 4 from further analyses, calculations and figures. However, we decided to still report the biomass, physico-chemical, chemical and isotopic measurement results in data tables (Table 3 and in the Supplementary Information) for the sake of transparency. While three WWTPs (reaches 1–3; Wilgartswiesen, Hofstätten, Dahn) receive mainly domestic wastewater and release it into streams with a positive LUI (LUI ≥0), the WWTPs at reach 5 and 6 (A. Sarnstall, Vorderweidenthal) discharge into streams

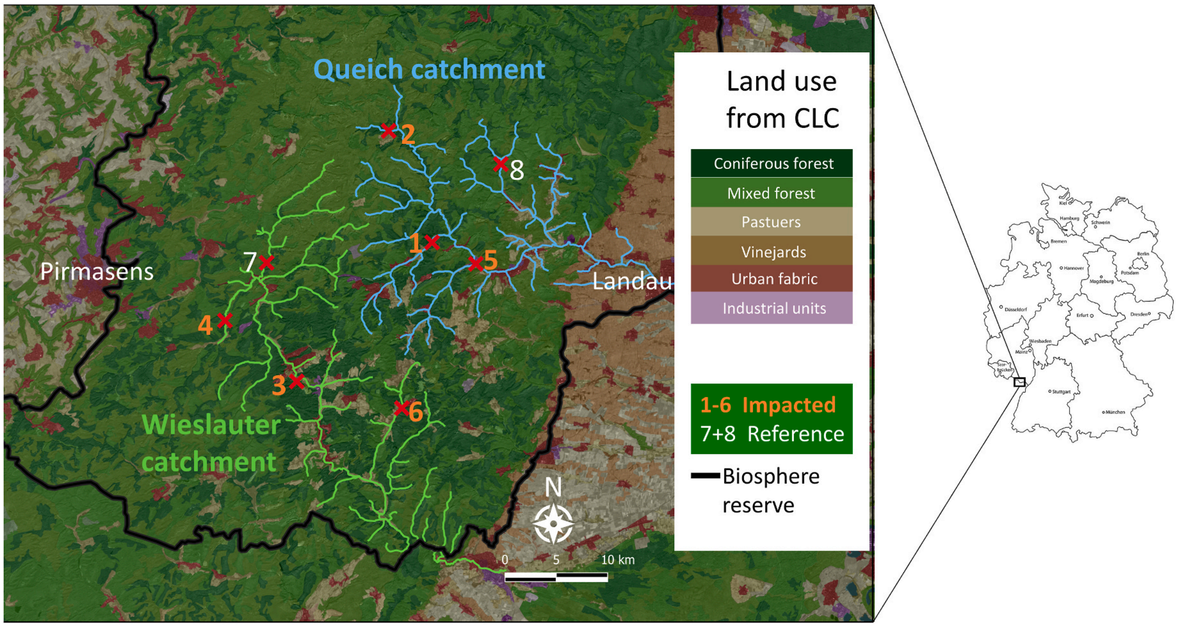

Fig. 1. Locations of the eight studied reaches within the Queich and Wieslauter catchments located in the biosphere reserve Vosges du Nord/Palatinate forest, Southwestern Germany. Black line = border of the biosphere reserve. Impacted reaches (ordered by impact groups: domestic 1–3, others 4–6 and references 7–8, see also Fig. 2): 1 = Wilgartswiesen WWTP, 2 = Hofstätten WWTP, 3 = Dahn WWTP, 4 = Salzwoog WWTP, 5 = A. Sarnstall WWTP, 6 = Vorderweidenthal WWTP. Reference reaches: 7 = Hinterweidenthal, 8 = EERES (Eußerthal). Background relief and colours = Sentinel images and land use after CORINE Land Cover (CLC) (Copernicus programme, 2018). Red cross = effluent position of the respective WWTP or arbitrarily selected reference point in case of reference reaches. Stream network (Ministry for Climate Protection, Environment, Energy and Mobility, Rhineland Palatinate, 2017), refined to relevant catchments.

dominated by urban or agricultural land use in their surrounding (LUI <0; Table 1).

2.2. Physico-chemical water parameters and stream characteristics

Water quality parameters were recorded four times, namely at the initiation and termination of each field study in both seasons (Tab. A 2). The samples were taken at each site in triplicates over one full day in 2019 or as a single sample in 2020, due to the reduced day length in winter 2020. Oxygen saturation, pH, conductivity and velocity were measured midstream and near the surface with a WTW Multi 340i/SET (Wissenschaftlich Technische Werkstätten GmbH, Weilheim, Germany) and a Höntzsch flowmeter (type μO-TAD; Waiblingen, Germany). Simultaneously, mean water depth and stream width were quantified on site. The average contribution of wastewater discharge (quantities calculated as mean wastewater contribution per day from discharge data of the whole year of the respective WWTP; personal communication with WWTPs) to the discharge of the receiving reach was estimated for each season (Tab. A 3). Therefore, the volumetric flow rate for each reach was calculated by multiplying the stream cross-sectional area by the mean velocity of the cross section measured during the respective season. Water temperature was constantly recorded by data loggers (HOBO Pendant Temperature 64 K Onset Computer Corporation, southeast Massachusetts, USA). Water samples (50 mL; n = 3) were analysed for total nitrogen (TN), total phosphorous (TP), total organic carbon (TOC) and chemical oxygen demand (COD), using Macherey Nagel nanocolor® test kits (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany; Tab. A 4). The used kits and their specific measurement range and precision are listed in the Supplementary Information (Tab. A 5).

2.3. Field study and sample preparation

This study focused on biofilms that were grown on unglazed ceramic tiles (4.6 cm × 4.6 cm; inorganic substrate) and leaf litter (organic substrate). Nine sterilized (6 h, 300 °C) tiles were combined as one replicate with a total surface area of 190.44 cm^2, and three replicates were deployed at each sampling site. The nine tiles were stabilised in a cage made of stainless-steel wire (mesh size = 1 cm), without any discernible impact on flow. Each cage was positioned at an angle of approximately 45° in the centre of the stream bed perpendicular to the flow to maximise light exposure. After five weeks (for exact dates see Table 1), the cages were retrieved. Tiles were carefully washed with stream water to remove sediment particles and debris before scraping the biofilm into 50-mL centrifugation tubes, in which they were preserved at −20 °C until further processed.

Pre-weighed black alder (*Alnus glutinosa* (L.) Gaertn.) leaves were deployed in fine mesh bags (mesh size = 1.0 mm; 10 leaves per bag, 7.3 ± 2.5 g standard deviation of n = 192) in triplicates at each sampling site. Leaves were collected from trees in the surroundings of Landau, Germany (49°12′06.0″N 8°08′24.3″E), in autumn 2018 and 2019 shortly before abscission and stored at −20 °C. Freezing may have slightly affected the leaf litter decomposition process, but such effects are generally minor and in the same order as effects that were observed for air-dried leaf litter that is usually used in leaf litter decomposition research (Bärlocher, 1992). For leaf bag preparation in 2020, the leaves were dried at 60 °C, pre-weighed to the nearest 0.01 mg and re-soaked in tap water before putting them in mesh bags. In 2019, the wet weight of the leaves and a dry weight correction factor were used to obtain the initial weight. After five weeks in the streams, the mesh bags were retrieved and the leaves were cleaned of adhering organisms, sand and debris. The exposure duration was selected to ensure sufficient biofilm development on the decomposing leaf samples required for the analyses planned in this study (Englert et al., 2013).

All samples were transported to the laboratory at 4 °C, where they were preserved at −20 °C until freeze-drying (−58 °C) for at least 48 h followed by weighing to the nearest 0.01 mg. The change in total dry weight over the study duration was used to assess biofilm activity in terms of biomass accrual (biofilm associated with inorganic substrate) and leaf litter decomposition (biofilm associated with organic substrate). Prior to its use for isotopic analysis, biofilm material from tiles and leaves (as a combination of leaf material and biofilm growing on this

Table 1
Overview of investigated reaches from both stream systems (Queich and Wieslauter). Number of reach with respective stream system, coordinates, duration of sample exposition (deployment until retrieval), catchment size in km^2 (cumulated; State Office for Environment, Water Management, and Trade Control Rhineland-Palatinate, 2007), land use index (LUI) and land use fractions in % in the aggregated 500 m zone around the effluent with population equivalents and correspondent total N efflux of the concerning WWTP in tons per year (approximated; from measurements in the respective sewer before being discharged 2019; Tab. A 1). Lat. effluent = Latitude coordinate of the effluent position, Long. effluent = Longitude coordinate of the effluent position, Urb. land = Urban land use, Agric. = Agriculture. † = WWTP with technical problems. n.a. = population or equivalent not available, as effluent is form industrial wastewater no such a relation existed. - = N total efflux was not possible to calculate, due to unknown wastewater contribution.

Reach No.	Stream system	Lat. effluent	Long. effluent	Sample exposition summer 2019	Sample exposition winter 2020	Catchment size (km^2)	LUI effluent	Land use in 500 m zone (%)	Population equivalents (number)	N total efflux (tons/year)
1	Queich	49°13′12.7″N	7°53′56.2″E	08.07. - 12.08.	24.02. - 30.03.	23608	2	Agric. = 21.1 Forest = 78.9	9553	≈12.6
2	Queich	49°17′03.1″N	7°51′41.9″E	10.07. - 14.08.	26.02–01.04.	6126	2	Agric. = 14.2 Forest = 85.8	500	≈3.5
3	Wieslauter	49°08′27.8″N	7°46′52.0″E	11.07. - 15.08.	27.02. - 02.04.	140713	0	Agric. = 40.7 Forest = 59.3	5200	≈16.2
4†	Wieslauter	49°10′31.5″N	7°43′09.3″E	11.07. - 15.08.	27.02. - 02.04.	40647	4	Agric. = 0.5 Forest = 99.5	792	–
5	Queich	49°12′32.3″N	7°56′12.4″E	08.07. - 12.08.	24.02. - 30.03.	89847	−2	Urb. land = 25.2 Agric. = 16.7 Forest = 58.1	n.a.	≈24.6
6	Wieslauter	49°07′30.6″N	7°52′23.1″E	12.07. - 16.08.	28.02. - 03.04.	10053	−2	Urb. land = 5.4 Agric. = 48.4 Forest = 46.2	923	≈1.5
7	Wieslauter	49°12′36.6″N	7°45′29.3″E	10.07. - 14.08.	26.02–01.04.	25142	5	Forest = 100	Reference	Reference
8	Queich	49°15′55.2″N	7°57′37.3″E	12.07. - 16.08.	28.02. - 03.04.	13821	5	Forest = 100	Reference	Reference

substrate) was manually ground, homogenised and aliquots were dried at 60 °C for another 48 h. For the isotopic measurements, 2.5 mg of the ground biofilm/leaf material was weighed to the nearest 0.0001 mg from samples at the effluent and 5.5 mg of the ground biofilm/leaf material at the other positions and reference reaches and packed into tin capsules. Elemental contents (as % of dry mass) and isotopic ratios of N were measured using a Flash 2000 HT elemental analyser coupled to a Delta V Advantage isotope ratio mass spectrometer (EA-IRMS, Thermo Scientific, Bremen, Germany). The stable isotope ratio of N (vs. atmospheric air) is expressed using the δ notation in ‰ units (Bond and Hobson, 2012). A reference standard (i.e., casein) was measured in duplicates every ten samples with a precision of ≤0.06‰. The remainder of the ground inorganic substrate-associated biofilm and leaf material was combusted at 550 °C for 5 h to determine the ash free dry mass (AFDM; Benfield, 2007; Tab. A 6).

2.4. Estimation of N retention in biofilms

We estimated the hypothetical retention of anthropogenic N based on the differences in the N concentrations in biofilms and the water column. This estimate sets the contribution of biofilms in N-retention in perspective to the overall nitrogen flux. In principle, the hypothetical N retention (N_{ret}) is hereby expressed as the percent of the anthropogenic N introduced into the stream retained by biofilms normalised to time and area. Since N is also contained in the organic substrate with implications in measurement accuracy of respective biofilms and their contribution to N-retention, the N_{ret} was only calculated for inorganic substrate-associated biofilms deployed at reaches impacted by domestic WWTPs (i.e., reaches 1, 2, 3) using the following equation: $N_{ret=}\frac{\Delta N}{NL}$.

where ΔN is the difference in N between up- and downstream sites expressed as uptake rate per day of N in mg per m^2 by the biofilm and NL is the additional N released from the WWTP in kg per day; ultimately N_{ret} is the percentage of introduced anthropogenic N retained by the biofilm normalised to 1 ha (Table 2). More details are provided in the Supplemental Information (Extended Methods I, also stating uncertainties; Tab. A 3, A 4, A 7).

Table 2
Hypothetical N retention in inorganic substrate-associated biofilms (N_{ret} in % per hectare (ha)) as a function of biofilm N uptake rate in mg per m^2 and day (ΔN biofilm) and N released from WWTPs in kg per day (NL). NL values obtained here are reasonable considering that larger WWTPs release more than 1000 kg N/d (City of Karlsruhe, 2010). (Note: The different scaling of ΔN and NL equal the retention of N in percentage upscaled to ha.)

Season	Reach No.	ΔN biofilm ($mg/m^2/d$)	NL additional nitrogen effluent (kg/d)	N_{ret} (%/ha)
Summer	1	7.02	30.52	0.23
	2	0.66	8.89	0.07
	3	6.43	29.09	0.22
Winter	1	3.24	27.08	0.12
	2	2.64	4.17	0.63
	3	0.60	14.50	0.04

Table 3
Biomass change (mean ± standard deviation) of biofilm and leaf litter during five weeks of exposure in both seasons (summer and winter) as a measure of biofilm activity. Biomass production in case of inorganic substrate-associated biofilm as mass increase in mg/cm^2 and leaf litter decomposition in case of organic substrate-associated biofilm as mass loss in % from total leaf weight before exposure. † = Data for reach 4 (Salzwoog) not further discussed because of technical dysfunctions and shown for completeness. Asterisks denote statistically significant differences to all other sites of the reach (*** = p < 0.001, ** = p < 0.01, * = p < 0 0.05). n.a. = not assessed as samples have been lost or destroyed due to vandalism.

Substrate/ Season	Reach No.	site 1	site 2	site 3	site 4
Inorganic substrate					
Summer	1	0.25 (±0.08)	0.60 (±0.21)	0.23 (±0.07)	0.95 (±0.54)
	2	0.15 (±0.16)	0.15 (±0.02)	0.42 (±0.39)	0.05 (±0.01)
	3	0.32 (±0.06)	1.07 (±0.32)**	0.18 (±0.16)	0.07 (±0.07)
	4†	0.12 (±0.09)	0.38 (±0.12)	n.a.	0.41 (±0.29)
	5	0.73 (±1.02)	0.63 (±0.38)	0.37 (±0.11)	0.73 (±0.44)
	6	0.48 (±0.48)	0.21 (±0.10)	0.44 (±0.36)	0.31 (±0.06)
	7	0.15 (±0.04)	0.54 (±0.51)	0.34 (±0.14)	0.19 (±0.06)
	8	0.24 (±0.01)	0.65 (±0.50)	0.08 (±0.04)	0.13 (±0.02)
Winter	1	0.10 (±0.01)	0.89 (±0.38)*	0.04 (±0.02)	0.29 (±0.10)
	2	0.18 (±0.07)	0.54 (±0.17)**	0.09 (±0.04)	0.04 (±0.01)
	3	0.02 (±0.00)	0.30 (±0.26)	0.07 (±0.02)	0.06 (±0.00)
	4†	0.02 (±0.01)	0.26 (±0.07)	0.28 (±0.08)	0.15 (±0.09)
	5	0.78 (±0.33)	0.21 (±0.26)	0.02 (±0.01)	0.03 (±0.00)
	6	0.06 (±0.04)	0.16 (±0.04)	0.13 (±0.02)	0.11 (±0.04)
	7	0.07 (±0.03)	0.18 (±0.06)	0.19 (±0.09)	0.1 (±0.01)
	8	0.03 (±0.01)	0.15 (±0.02)	0.02 (±0.01)	0.11 (±0.08)
Organic substrate					
Summer	1	87 (±6)	66(±17)	41(±15)	77(±13)
	2	94 (±3)	74(±23)	92(±3)	96(±2)
	3	39 (±7)	63(±12)	37(±16)	64(±19)
	4†	71 (±20)	53 (±27)	n.a.	68(±19)
	5	67 (±17)	47 (±17)	88 (±2)	34 (±29)
	6	42 (±44)	93 (±3)	94 (±2)	75 (±35)
	7	66 (±21)	n.a.	50 (±38)	60 (±35)
	8	68 (±22)	79 (±19)	74 (±17)	89 (±1)
Winter	1	71 (±20)	46 (±15)	47 (±11)	41 (±4)
	2	63 (±3)	38 (±7)	84 (±1)	53 (±12)
	3	63 (±14)	63 (±5)	36 (±5)	48 (±11)
	4†	58 (±5)	41 (±17)	62 (±13)	42 (±12)
	5	40 (±12)	66 (±40)	n.a.	46 (±20)
	6	n.a.	51 (±23)	89 (±4)	41 (±12)
	7	48 (±18)	50 (±8)	54 (±4)	43 (±18)
	8	80 (±0)	52 (±10)	n.a.	35 (±15)

2.5. Data analysis

The data and residuals were checked for homoscedasticity and normal distribution through visual inspection. For hypothesis testing, the $\delta^{15}N$ of the biofilm/leaf material, biomass as dry weight, as well as chemical and physical measurements in the water column were analysed within single reaches to avoid pseudoreplication (Hurlbert, 1984) using analysis of variance (ANOVA; Chambers et al., 1992; Tab. A 2, A 3, A 8 and A 9) followed by pairwise t-tests and a p-value adjustment after Benjamini and Hochberg (1995). In the case of non-normally distributed data, a Kruskal-Wallis test (Hollander et al., 2013) followed by a pairwise Wilcoxon rank-sum test (Wilcoxon, 1945; Mann and Whitney, 1947) was used instead.

Linear regression analyses were applied to examine the relationship between the percentage wastewater contribution at the respective effluent and the measured $\delta^{15}N$ as well as biomass change (biomass production or leaf decomposition). In the case of inorganic substrate-associated biofilm, additional regressions between accumulated dry mass and TN as well as $\delta^{15}N$ were run. A principal component analysis (PCA; Grice, 2001; Legendre and Legendre, 2012) was used to explore the clustering of reaches based on stream characteristics. Herein the means of TN, TP, TOC, COD, oxygen saturation, pH, conductivity and flow velocity of the effluent per season were compared. A second PCA was conducted with isotope ratios to graphically display the differences and correlation of the isotopic discrimination of N ($\delta^{15}N$) and water quality parameters (TN, TP, TOC, COD). The term "significant(ly)" is used exclusively for statistical significance ($p \leq 0.05$) in the remainder of this manuscript. For statistics and figures, R version 3.6.2 was used (R Core Team, 2019).

3. Results

3.1. Physico-chemical water parameters

Triplicate measurements in summer 2019 revealed that wastewater-impacted reaches showed twice as high values of the measured physico-chemical parameters (except for pH, oxygen saturation and flow velocity) relative to reference reaches (Tab. A 2). At reaches impacted by domestic WWTPs, upstream sites deviated significantly from effluent sites, with lower values at the effluent sites regarding oxygen saturation (~20%; $p < 0.001$, F-value = 41.77, degrees of freedom (df) = 1) and higher values in temperature (~25%; $p = 0.002$; F-value = 14.59; df = 1) and conductivity (~100%; $p < 0.001$, F-value = 47.4; df = 1; Tab. A 2). Also, the chemical measures TN, TP, TOC and COD (>30% except TP at reach 3) notably increased at the effluent sites at these reaches (Tab. A 4). Downstream of the effluent, these parameters tended to asymptotically approach values of the upstream site. At all other reaches, no significant differences in the measured physico-chemical parameters have been observed at the effluent, with the exception of a reduced flow velocity and an increased conductivity at reach 5.

The PCA covering all physico-chemical water parameters clustered the reaches into three groups irrespective of the season (Fig. 2). Those groups were (i) reaches impacted by domestic WWTPs (i.e., reaches 1, 2, 3), (ii) reaches impacted by WWTPs from other sources apart from a mainly domestic influence (i.e., reaches 5 and 6), and (iii) reference reaches without WWTP input (i.e., reaches 7 and 8). The first principal component (PC1) in summer 2019 (Fig. 2 a) explained 52% and in winter 2020 (Fig. 2b) 42% of the variance in the data. In the case of the summer data set, the main variables driving the PC1 are TOC, TN and COD, which are all pointing in a similar direction and are mainly associated with domestic WWTPs. PC1 of the winter data set, however, was mainly associated with TP and oxygen saturation, which have a reciprocal relation to each other and span the axis. The second principal component (PC2) explained 34% and 27% of the variance for summer and winter, respectively. PC2 from the summer data was dominated by flow velocity, temperature and oxygen saturation, where the latter two directly oppose each other. In winter, the variance of the data was dominated by the flow velocity followed by temperature and TN, where the latter two are also in opposite directions. Loading scores of all PCs presented are reported in Tab. A 10 a and b in the Supplementary Information.

3.2. Nitrogen stable isotopes in biofilms

Both types of biofilms showed a significant linear relationship between the calculated share of wastewater below the WWTP effluent and

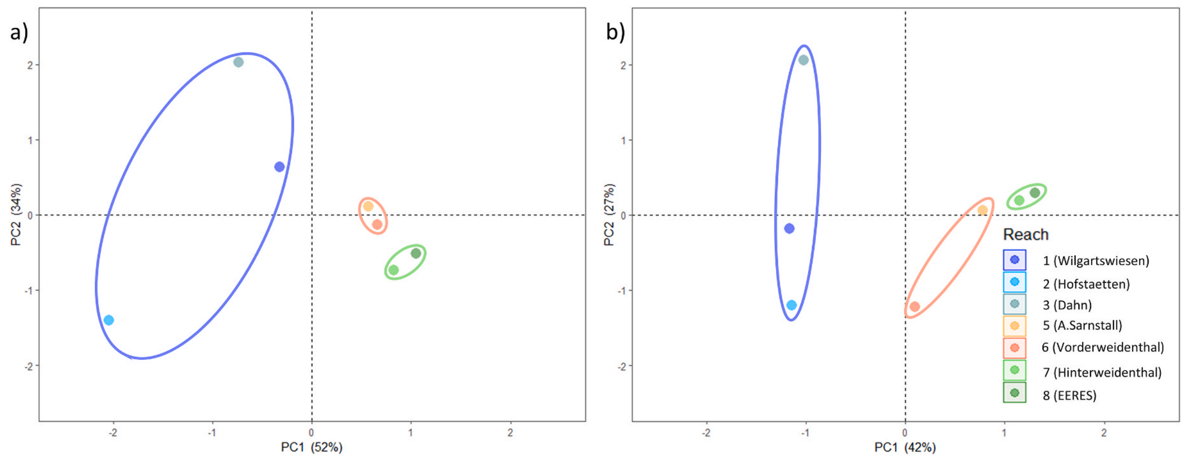

Fig. 2. Ordination by principal component analysis (PCA), of the respective reach and season, a) summer 2019 and b) winter 2020, from the measured physico-chemical water parameters: means of total nitrogen (TN), total phosphorous (TP), total organic carbon (TOC), chemical oxygen demand (COD), oxygen saturation, flow velocity, pH, temperature and conductivity in the water column, at the effluent. Cluster: (i) Domestic WWTP in blue = reach 1 (Wilgartswiesen), 2 (Hofstaetten), 3 (Dahn); (ii) Others in orange = 5 (A. Sarnstall), 6 (Vorderweidenthal); (iii) Reference in green = 7 (Hinterweidenthal), 8 (EERES (Eußerthal)); Not shown or included in analyses, due to the impossibility to rate reach 4 as "impacted by a point source" nor as "unimpacted reference", unsuitable for inquiry = 4 (Salzwoog). Ellipsoids include cluster of reaches in the corresponding colours.

the mean $\delta^{15}N$ (global model: $r = 0.68$, adj. $R^2 = 0.5$, $p < 0.001$; for individual models see caption Fig. 3). The range of $\delta^{15}N$ values between maximum (90%) and minimum (0%) wastewater contribution, predicted from the linear regressions, was about 9.32‰ (0.48–9.8‰) and 6.08‰ (−0.48–5.6‰) in inorganic substrate-associated and organic substrate-associated biofilms, respectively (Fig. 3 and Tab. A 3). The wastewater contribution explained about 40% and 55% of the $\delta^{15}N$ in inorganic substrate-associated and organic substrate-associated biofilms, respectively. The mean $\delta^{15}N$ was also related to the distance between the sampling site and WWTP effluent ($r = 0.04$, adj. $R^2 = 0.1603$, $p < 0.001$), which seems a consequence of the simultaneous reduction in TN in the water phase with increasing distance (Fig. A 1).

The second PCA visualizes the correlations of TN, TP, TOC, COD and $\delta^{15}N$ in biofilms on the clustering of reaches and their sampling sites (Fig. 4). PC1 explained 60% of the variance in the data and was associated with TN, TP and $\delta^{15}N$. These parameters showed a similar ordination and were mainly associated with reaches impacted by domestic WWTPs. PC2 explained 18% of the variance and was influenced by COD, which was triggered by reaches impacted by industrial WWTPs or have been releasing wastewater into a reach dominated by agriculture (i.e., reaches 5 and 6). The reference reaches were clustered together with little deviations between sites.

At reference reaches, the $\delta^{15}N$ remained low and largely constant among sites, independent of the substrate. At reaches impacted by domestic WWTPs, the $\delta^{15}N$ in biofilms associated with the inorganic substrate increased substantially in both summer and winter at the effluent (site 1 vs. site 2; >8‰, $p < 0.001$, F-value = 436, df = 3; range of p: <0.001–0.013; Fig. 5 and Tab. A 8 for further statistical details). The biofilm associated with the organic substrate accumulated a higher amount of N with regards to $\delta^{15}N$ during summer (+7‰) with significant values in reaches 2 and 3 at the effluent site (reach 2: $p = 0.001$, F-value = 20.3, df = 3; reach 3: $p < 0.0001$, F-value = 104, df = 3), but accumulated less during the winter season (+2‰) in which the TN transported in the water was also lower (Fig. 5, Tab. A 4 and A 11). With increasing distance from the effluent, $\delta^{15}N$ approached levels from upstream sections. Reaches impacted by other sources, such as industrial

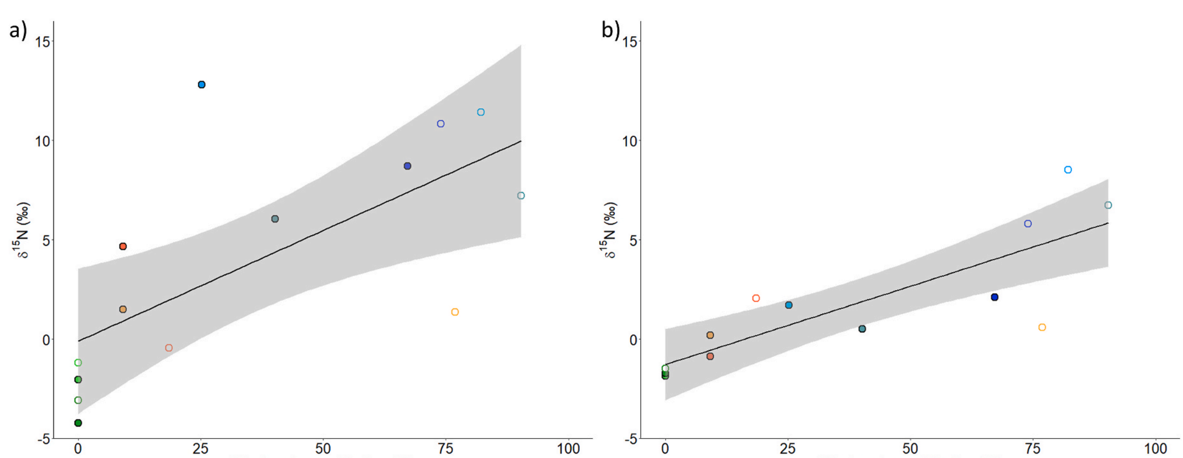

Fig. 3. Linear regression between the mean $\delta^{15}N$ in a) inorganic substrate-associated biofilm and b) organic substrate-associated biofilm and the percent wastewater at the effluent site. Open circles refer to summer 2019, filled circles refer to winter 2020. Colours: (i) Domestic WWTP in blue = reach 1 (Wilgartswiesen), 2 (Hofstaetten), 3 (Dahn); (ii) Others in orange = 5 (A. Sarnstall), 6 (Vorderweidenthal); (iii) Reference in green = 7 (Hinterweidenthal), 8 (EERES (Eußerthal)); Not shown or included in analyses, due to the impossibility to rate reach 4 as "impacted by a point source" nor as "unimpacted reference", unsuitable for inquiry = 4 (Salzwoog). See also Fig. 2 and corresponding legend. Equations of the regression lines: a) $y = 0.11159x - 0.10803$ (adj. $R^2 = 0.4$ $p = 0.007$), b) $y = 0.07894x - 1.28229$ (adj. $R^2 = 0.7$ $p < 0.001$).

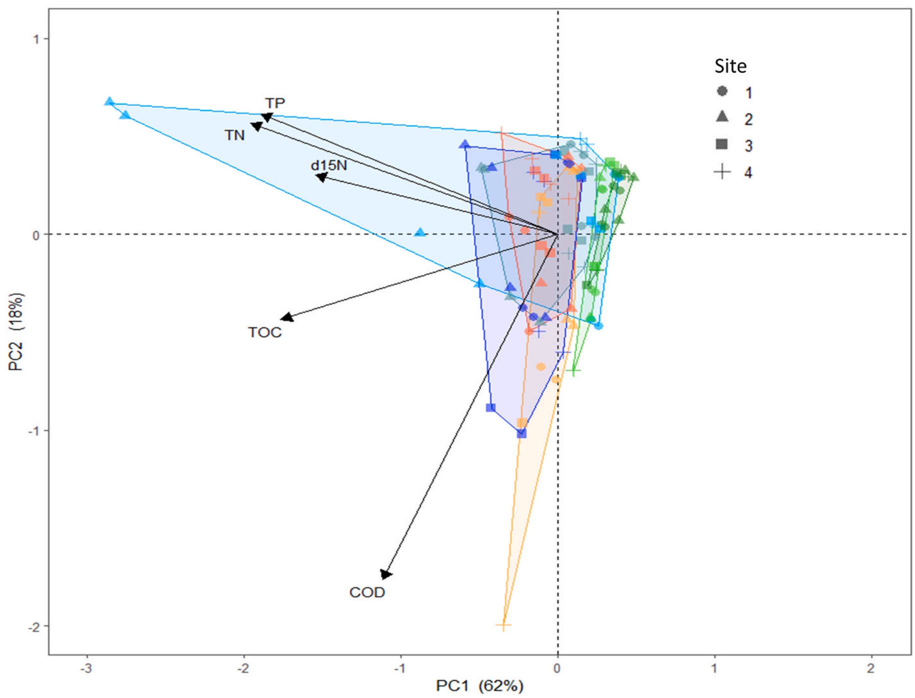

Fig. 4. PCA of the mean $\delta^{15}N$ in biofilm during winter and summer (2019 and 2020) and the means of TN, TP, TOC and COD in the water column, for the eight studied reaches and their four respective sites. Explanation of sites: Site 1 = 250 m above effluent, Site 2 = site of effluent discharge, Site 3 = 30–50 m downstream of effluent, Site 4 = 500–1000 m downstream of effluent. Colours: (i) Domestic WWTP in blue = reach 1 (Wilgartswiesen), 2 (Hofstaetten), 3 (Dahn); (ii) Others in orange = 5 (A. Sarnstall), 6 (Vorderweidenthal); (iii) Reference in green = 7 (Hinterweidenthal), 8 (EERES (Eußerthal); Not shown or included in analyses, due to the impossibility to rate reach 4 as "impacted by a point source" nor as "unimpacted reference", unsuitable for inquiry = 4 (Salzwoog). See also Fig. 2 and corresponding legend.

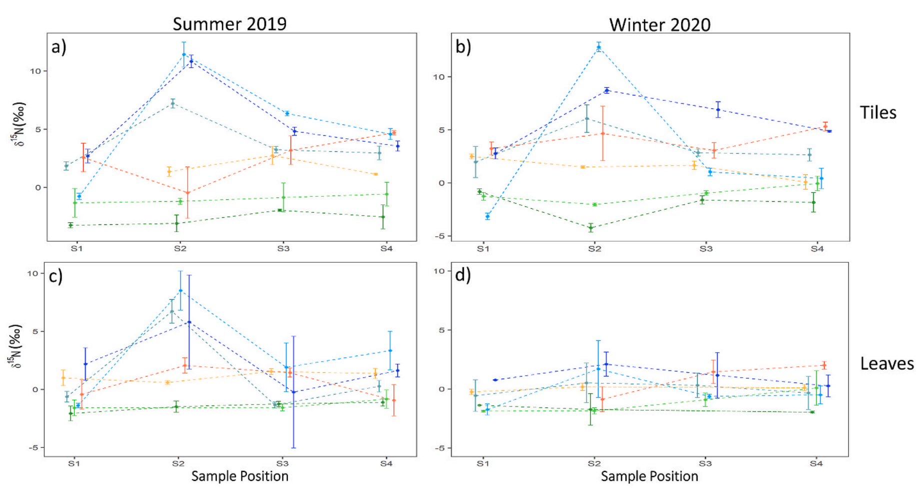

Fig. 5. Results of the mean $\delta^{15}N$ development for both substrates and seasons. Sample position: −250 m = control site, 0 m = effluent site; 30–50 m = total mixture; 500–1000 m = max. distance after effluent. a) inorganic substrate-associated biofilm summer 2019, b) inorganic substrate-associated biofilm winter 2020, c) organic substrate-associated biofilm summer 2019, d) organic substrate-associated biofilm summer 2020. For assignment of colours = see Fig. 2. Not shown or included in analyses, due to the impossibility to rate reach 4 as "impacted by a point source" nor as "unimpacted reference", unsuitable for inquiry = 4 (Salzwoog).

WWTPs or releasing wastewater into a reach dominated by agricultural land use, showed $\delta^{15}N$ values not following a specific pattern.

The hypothetical N retention (N_{ret}) has been be as high as 0.63% of the added N within 1 ha (ha = 10,000 m^2) and suggested some, although minor, contribution of biofilms to N retention (Table 2). Despite the large variances in the underlying calculated values, namely the biofilm N uptake rate (ΔN) and the N released from WWTPs (NL), the present study also pinpoints a substantial variability in N_{ret} between seasons and reaches.

3.3. Primary production and leaf litter decomposition

The inorganic substrate-associated biofilm gained 0.37 ± 0.36 mg/cm^2 (mean ± standard deviation) and 0.18 ± 0.23 mg/cm^2 dry weight across all sites during summer and winter, respectively. Similarly, the activity of the organic substrate-associated biofilm, estimated as leaf mass loss, was with about 68 ± 25% and 53 ± 17% also higher in the summer relative to the winter season, respectively. These differences in biofilm activity between seasons have been significant (inorganic substrate: $p < 0.001$, $\chi^2 = 30.49$, df = 1; organic substrate: p = < 0.001, F-value = 12.28, df = 1). Moreover, a positive linear relationship between percentage wastewater contribution and biomass gain has been found on inorganic substrate (linear model: r = 0.5, adj. $R^2 = 0.28$, $p = 0.049$; Fig. A 2 a). In case of organic substrate, no linear relationship between wastewater contribution and leaf mass loss was evident (Fig. A 2 b). There was no significant difference in biomass gain or loss at the effluent, except for inorganic substrate-associated biofilm with a higher biomass at the effluent in winter at reaches 1, 2 and 5 and in summer at reach 3 ($p = 0.013$, F-value = 0.08, df = 3; range of p: 0.013–0.045; Table 3 and A 9 for further statistical details).

4. Discussion

Our measurements in eight small freshwater streams during two seasons revealed large differences in $\delta^{15}N$ among reaches ranging from −3.25 to 12.81‰, with a substantial increase of $\delta^{15}N$ in inorganic as well as organic substrate-associated biofilms below WWTP effluents. These significant results (Tab. A 8 and A 11) confirm the large deviations found in other studies with $\delta^{15}N$ values ranging from −8.4 to 19.4‰ in dissolved inorganic N and −4 to 16‰ of basal resources (i.e., epilithic biofilms, leaf litter, filamentous algae; Peipoch et al., 2012). Furthermore, the results of the ANOVA on $\delta^{15}N$ in the biofilm biomass from both substrates confirm the direct impact and uptake of N from the WWTPs in contrast to the largely constant ratio in unimpacted reaches (Tab. A 11). The significant differences in $\delta^{15}N$ found in the present study were most pronounced at reaches impacted by domestic WWTPs (ANOVA between site 1 vs. site 2; $p < 0.0001$, F-value = 436, df = 3; range of p: <0.0001–0.0133; Tab. A 8 and A 11), which also exhibited a positive LUI. A similar pattern could not be found for reference reaches (Tab. A 8) that showed a stable and homogenic $\delta^{15}N$ and level of N content among all sites (Fig. 5; Tab. A 8 and A 11), while the reaches impacted by other (i.e., not domestic) WWTPs exhibited a more diverse pattern regarding in $\delta^{15}N$ and thus N retention. In these impacted reaches, the basic N pollution seemed to be dependent on the surrounding anthropogenic land use, as reaches with a higher anthropogenic land use (such as life stock) generally showed a higher $\delta^{15}N$ already upstream of the point source. It is, moreover, evident from the present study that the type of wastewater (domestic vs. industrial) and the dominating land use around the reaches (forestry vs. agriculture) had an indisputable impact on the possibility to trace anthropogenic N in biofilms (cf. Peipoch et al., 2012). This pattern may be best explained by the respective ^{15}N increase caused by WWTPs in the receiving streams (Fig. 5, Tab. A 3, A 4 and A 7). This assumption is supported by earlier studies discovering $\delta^{15}N$ values up to 30‰ in dissolved inorganic N species (NH_4^+, NO_3^-) released from WWTPs (Toda et al., 2002; Ribot et al., 2012; Peipoch et al., 2012). This largely exceeds the range in $\delta^{15}N$ found for the inorganic and organic substrate-associated biofilm samples in the present study. The lower ^{15}N increase in reaches with other (i.e., not domestic) WWTPs is probably due to lower ^{15}N content in the effluent or relatively high background levels in the stream. A more detailed analysis on N-species (e.g., NH_4^+, NO_3^-) and abundance of ^{15}N in the stream water could help with understanding N-cycling and should be included in future studies, but is beyond the scope of this study focusing on retention potential.

Our hypothesis of $\delta^{15}N$ in biofilms associated to inorganic and especially organic substrate, informing about the release of anthropogenic N from WWTPs, is finally underpinned by the significant linear relationship ($r = 0.68$, adj. $R^2 = 0.45$, $p < 0.001$) found between anthropogenic wastewater contribution and elevated $\delta^{15}N$ in both types of biofilms. This result is supported by elevated $\delta^{15}N$ values in biofilms (Merbt et al., 2011; Ribot et al., 2012; Peipoch et al. 2012, 2014), aquatic plants (Wigand et al., 2007), and further studies targeting $\delta^{15}N$ in aquatic systems (Voss et al., 2006; Spruill et al., 2002; Valiela et al., 2000). Therefore, it can be suggested that an elevated $\delta^{15}N$ value in biofilms could be used as indicator for anthropogenic N sources (Painter et al., 2020). This conclusion seems independent of season, as biofilm $\delta^{15}N$ remained constant over time. Furthermore, our expectation of a more distinct difference in $\delta^{15}N$ in inorganic substrate-associated biofilms seems to be confirmed by the estimations of the linear regressions. This is probably caused by the fact that the biofilms from the organic substrate also cover the $\delta^{15}N$ of the allochthonous leaf substrate itself and not only the fraction of the N in the water. However, despite this possible "dilution" of the $\delta^{15}N$ values in organic substrate samples, a significant increase was detected, which highlights the unambiguity of the effect.

This increased content of total N in biofilms below the WWTPs resulted in a hypothetical N_{ret} of up to 0.63% in biofilms suggesting only a minor contribution to self-cleaning. Moreover, N_{ret} varied substantially between seasons and reaches (Table 2), making it difficult to diagnose if the retention capacity is generally higher during summer, although elevated $\delta^{15}N$ values and N content in summer seem to support this assumption (Fig. 5, Tab. A 4 and A 11). The large variations in N_{ret} can partially be explained by reaching the N-uptake maxima of biofilms, making it impossible to benefit from a further increase in available N. Our extrapolations (N_{ret}) consequently support the assumption that stream nutrient retention is reduced at high nutrient loads (Martí et al., 2004; Mulholland et al., 2008). The differences in N_{ret} as well as the deviations in the uptake rate in biofilms ΔN between reaches and seasons moreover indicate that retention, even of essential nutrients, in streams is a versatile and dynamic process (Hauck, 1973; Heaton, 1986; Ifabiyi, 2008). There are many factors influencing the retention process such as flow, turbulence and surface properties (Krsmanovic et al., 2021; Anlanger et al., 2021; Painter et al., 2020), temperature (Guasch et al., 1995), light (Romaní et al., 2004) and water chemistry (e.g., pH, chemicals of anthropogenic origin; Flemming, 1995; Guasch et al., 1995; Corcoll et al., 2012; Mahler et al., 2020). Analysing data from the Linx II study in headwater streams across the United States, Grant et al. (2018) imposed a physical upper limit of the N removal rate, governed by turbulent mass transfer and thus indirectly by flow velocity (Anlanger et al., 2021). However, flow velocity (m/s) is rather constant among the sites in the present study (i.e., varying for most records by a factor of 2 around 1 m/s) suggesting that in our case N-uptake in biofilms is mainly limited by biological processes (Nepf, 2011). These biological processes include the balance between nitrification and denitrification (Hall et al., 2009; Ribot et al. 2012, 2017), the general health and composition of the involved organisms, their activity, ability to grow, to reproduce (Besemer, 2016; Krsmanovic et al., 2021) and their adaption to pollution (Wijeyekoon et al., 2004). Especially the dissimilatory processes such as nitrification and denitrification play an important role in N-cycling (Hauck, 1973; Tank et al., 2000; Mulholland et al., 2000). These dissimilatory processes, together with the composition of N species (e.g., NH_4^+, NO_3^-), when differently retained, might have further contributed to anthropogenic N reduction by biofilms (Mulholland et al., 2000; Mulholland et al., 2008). Therefore, the N-retention values reported in the present study might be a conservative estimate. Despite the variability and complexity of the system, the retention capacity of biofilms may be expanded by increasing physical and ultimately biological heterogeneity as part of renaturation programs (Ryhiner et al., 1994; Sabater et al., 2007; Schmidt et al., 2019; Anlanger et al., 2021).

Biofilm communities are the first trophic level in aquatic systems to interact with dissolved substances such as nutrients, organic matter and toxicants, that are partly also accumulated. The significant linear relationship between percentage of wastewater contribution and biomass gains of biofilms associated to the inorganic substrate suggests a direct impact of wastewater on functional parameters, with the direction of effects being in contrast to our hypothesis. This higher functional performance, namely biomass gain, is most likely caused by the higher availability of nutrients (e.g., phosphorous and nitrogen) proliferating biofilm growth and prevailing supposedly harmful effects of anthropogenic substances like pharmaceuticals or personal care products (Rosi-Marshall et al., 2013). This positive impact on inorganic substrate was not confirmed for the organic substrate, which may be a consequence of other (unquantified) factors governing leaf litter decomposition (e.g., photochemical degradation, intrinsic characteristics like nutrient content in the substrate itself, or physical effects like velocity driven fragmentation; Coûteaux et al., 1995; Bastias et al., 2018; Bastias et al., 2020), masking effects. Nonetheless, biofilms are providing important information for environmental management (Sabater et al., 2007). In fact, the heavy stable N isotope and its presence when of anthropogenic origin, seems valuable to identify potential sources and estimate retention efficiencies in stream biofilms. While an elevation of $\delta^{15}N$ above 6 or 7‰ point toward manure and other anthropogenic sources, values up to 5‰ seem to indicate some sort of pollution either from non-point sources or of distant origin (Fogg et al., 1998). At the same time, lower $\delta^{15}N$ values point to pristine or near-natural conditions (Kendall and McDonnell, 1998; Mayer et al., 2002; Voss et al., 2006).

Furthermore, biofilms are key elements for the ecological integrity of natural water bodies by contributing to the retention and degradation of a number of substances from anthropogenic origins (Sabater et al., 2002;

Romaní and Sabater, 2001; Mahler et al., 2020) at different efficiencies often determined by surrounding conditions. Our results show that the retention of N in biofilms is not only dependent on the availability of substances (here N) but also on other parameters like urbanised or anthropogenically altered areas (cp. like reaches 5 and 6), which also changes the availability of N species (Hall et al., 2009). These changes on the composition of N species has implications on assimilatory uptake or removal (Ribot et al., 2017), which is however, also influenced by stream size (Peterson et al., 2001; Mulholland et al., 2008). In other words, a (near-)natural land usage around streams (such as forestry) reflects positively on the streams' self-cleaning capacity, with a positive impact on the mitigation of substances of anthropogenic origin in the stream system. Furthermore, a higher surface area and density of active biofilms, not to confound with biofilm thickness, is linked to an elevated capacity to retain N (Badhe et al., 2014), partly compensating for the effect of oversaturation. Accordingly, the proliferation of biofilms and the establishment of good conditions for their growth and activity as well as optimal physical conditions (i.e., turbulence) could further support water quality and ultimately ecological integrity. In the studied ecosystems, however, the contribution of biofilms to N retention is rather limited suggesting local natural processes as insufficient to mitigate the load of N released from anthropogenic sources. Consequently, alternative and more efficient approaches need to be considered to further increase the removal of N in WWTPs or limit the release of N to the sewer. In this context, constructed flow-through wetlands installed between WWTPs and receiving streams were shown to effectively mitigate anthropogenic N pollution in downstream sections (Nichols, 1983; Jansson et al., 1994; Vymazal, 2011; Choudhury et al., 2019).

5. Conclusion

Against the background of the study region, which is part of a biosphere reserve, it can be confirmed that point sources already close to the source of a stream substantially affect the N load. Furthermore, the data visualise the vast distance necessary before N is retained by biota. The fact that biofilms with the highest accumulation of $\delta^{15}N$ in our study are exclusively found in reaches with a positive LUI points toward the importance of a high habitat quality surrounding the streams. Providing a higher quantity and quality of habitats for biofilms, through constructed flow-through wetlands or reduced removal of dead wood as part of environmental management measures or renaturation programs could further support the self-cleaning capacity in streams (Elosegi et al., 2016). Furthermore, measures that reduce the influx of N and other chemicals from wastewater into rivers and streams should be explored to minimise their contamination. Such decentralised mitigation strategies could play important roles for ecosystem services. This seems even more important under projected global change scenarios increasing the pressure on aquatic ecosystems in general and in downstream sections in particular where multiple drivers, including land use and additional point sources, may cumulatively add to the load of N.

CRediT author statement

Thomas Löffler: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization; Eric Bollinger: Formal analysis, Investigation, Writing – review & editing, Visualization; Alexander Feckler: Formal analysis, Writing – review & editing, Visualization; Sebastian Stehle: Conceptualization, Methodology, Validation, Writing – review & editing; Jochen P. Zubrod: Conceptualization, Methodology, Validation, Writing – review & editing; Ralf Schulz: Resources, Writing – review & editing, Project administration, Funding acquisition; Mirco Bundschuh: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition

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.jenvman.2023.118746.

References

Anlanger, C., Risse-Buhl, U., Schiller, D., Noss, C., Weitere, M., Lorke, A., 2021. Hydraulic and biological controls of biofilm nitrogen uptake in gravel-bed streams. Limnol. Oceanogr. 66 (11), 3887–3900. https://doi.org/10.1002/lno.11927.

Aravena, R., Evans, M.L., Cherry, J.A., 1993. Stable isotopes of oxygen and nitrogen in source identification of nitrate from septic systems. Ground Water 31 (2), 180–186.

Badhe, N., Saha, S., Biswas, R., Nandy, T., 2014. Role of algal biofilm in improving the performance of free surface, up-flow constructed wetland. Bioresour. Technol. 169, 596–604. https://doi.org/10.1016/j.biortech.2014.07.050.

Bärlocher, F., 1992. Effects of drying and freezing autumn leaves on leaching and colonization by aquatic hyphomycetes. Freshw. Biol. 28, 1–7.

Bastias, E., Ribot, M., Romaní, A.M., Mora-Gómez, J., Sabater, F., López, P., Martí, E., 2018. Responses of microbially driven leaf litter decomposition to stream nutrients depend on litter quality. Hydrobiologia 806 (1), 333–346. https://doi.org/10.1007/s10750-017-3372-3.

Bastias, E., Bolivar, M., Ribot, M., Peipoch, M., Thomas, S.A., Sabater, F., Martí, E., 2019. Spatial heterogeneity in water velocity drives leaf litter dynamics in streams. Freshw. Biol. 65 (3), 435–445.

Bastias, E., Ribot, M., Bernal, S., Sabater, F., Martí, E., 2020. Microbial uptake of nitrogen and carbon from the water column by litter-associated microbes differs among litter species. Limnol. Oceanogr. 65 (8), 1891–1902. https://doi.org/10.1002/lno.11425.

Battin, T.J., Sloan, W.T., Kjelleberg, S., Daims, H., Head, I.M., Curtis, T.P., Eberl, L., 2007. Microbial landscapes: new paths to biofilm research. Nat. Rev. Microbiol. 5 (1), 76–81.

Beaulieu, J.J., Tank, J.L., Hamilton, Stephen K., Wollheim, Wilfred M., Hall, Robert O., Mulholland, Patrick J., et al., 2011. Nitrous oxide emission from denitrification in stream and river networks. Proc. Natl. Acad. Sci. USA 108 (1), 214–219.

Benfield, E.F., 2007. Decomposition of leaf material. In: Richard Hauer, F., Anthony Lamberti, Gary (Eds.), Hg.): Methods in Stream Ecology, second ed. Academic Press, Amsterdam, Boston, pp. 711–720.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Roy. Stat. Soc. B 57 (1), 289–300.

Besemer, K., 2016. Microbial biodiversity in natural biofilms. In: Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment. Caister Academic Press, Norfolk, S, pp. 63–87.

Bighiu, M.A., Goedkoop, W., 2021. Interactions with freshwater biofilms cause rapid removal of common herbicides through degradation - evidence from microcosm studies. Environ. Sci. Processes Impacts 23 (1), 66–72. https://doi.org/10.1039/D0EM00394H.

Bond, A.L., Hobson, K.A., 2012. Reporting stable-isotope ratios in ecology: recommended terminology, guidelines and best practices. Waterbirds 35 (2), 324–331.

Bundschuh, M., Pierstorf, R., Schreiber, W.H., Schulz, R., 2011. Positive effects of wastewater ozonation displayed by in situ bioassays in the receiving stream. Environ. Sci. Technol. 45 (8), 3774–3780. https://doi.org/10.1021/es104195h.

Burdon, F.J., Munz, N.A., Reyes, M., Focks, A., Joss, A., Räsänen, K., et al., 2019. Agriculture versus wastewater pollution as drivers of macroinvertebrate community structure in streams. Sci. Total Environ. 659, 1256–1265. https://doi.org/10.1016/j.scitotenv.2018.12.372.

Camargo, J.A., Alonso, Á., 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environ. Int. 32 (6), 831–849. https://doi.org/10.1016/j.envint.2006.05.002.

Carey, R.O., Migliaccio, K.W., 2009. Contribution of wastewater treatment plant effluents to nutrient dynamics in aquatic systems: a review. Environ. Manag. 44 (2), 205–217. https://doi.org/10.1007/s00267-009-9309-5.

Chambers, J.M., Freeny, A., Heiberger, R.M., 1992. Analysis of variance; designed experiments. In: Chambers, S.J.M., Hastie, T.J. (Eds.), Chapter 5 of Statistical Models in S. Wadsworth & Brooks/Cole, Pacific Grove, CA.

Cheever, B.M., Webster, J.R., Bilger, E.E., Thomas, S.A., 2013. The relative importance of exogenous and substrate-derived nitrogen for microbial growth during leaf decomposition. Ecology 94 (7), 1614–1625.

Choudhury, M.I., Segersten, J., Hellman, M., Mckie, B.G., Hallin, S., Ecke, F., 2019. Importance of plant species for nitrogen removal using constructed floating wetlands in a cold climate. Ecol. Eng. 138, 126–132. https://doi.org/10.1016/j.ecoleng.2019.07.012.

City of Karlsruhe, 2010. The urban wastewater management of Karlsruhe. Available online at: https://www.karlsruhe.de/b3/bauen/tiefbau/entwaesserung/klaerwerk/HF_sections/rightColumn. (Accessed 6 April 2022).

Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Havens, K.E., et al., 2009. Ecology. Controlling eutrophication: nitrogen and phosphorus. Science (New York, N.Y.) 323 (5917), 1014–1015. https://doi.org/10.1126/science.1167755.

Copernicus programme, 2018. Corine Land Cover. (CLC): European Environment Agency (EEA) under the framework of the Copernicus programme - copernicus@eea.europa.eu. Available online at: https://land.copernicus.eu/pan-european/corine-land-cover/clc2018.

Corcoll, N., Bonet, B., Morin, S., Tlili, A., Leira, M., Guasch, H., 2012. The effect of metals on photosynthesis processes and diatom metrics of biofilm from a metal-contaminated river: a translocation experiment. Ecol. Indicat. 18, 620–631. https://doi.org/10.1016/j.ecolind.2012.01.026.

Coûteaux, M.-M., Bottner, P., Berg, B., 1995. Litter decomposition, climate and liter quality. Trends Ecol. Evol. 10 (2), 63–66. https://doi.org/10.1016/S0169-5347(00)88978-8.

Cummins, K.W., 1974. Structure and function of stream ecosystems. BioScience 24 (11), 631–641. https://doi.org/10.2307/1296676.

Danley, B., Widmark, C., 2016. Evaluating conceptual definitions of ecosystem services and their implications. Ecol. Econ. 126, 132–138. https://doi.org/10.1016/j.ecolecon.2016.04.003.

Eisakhani, M., Malakahmad, A., 2009. Water quality assessment of bertam river and its tributaries in Cameron highlands, Malaysia. World Appl. Sci. J. 7 (6), 769–776.

Elosegi, A., Elorriaga, C., Flores, L., Martí, E., Díez, J., 2016. Restoration of wood loading has mixed effects on water, nutrient, and leaf retention in Basque mountain streams. Freshw. Sci. 35 (1), 41–54.

Englert, D., Zubrod, J.P., Schulz, R., Bundschuh, M., 2013. Effects of municipal wastewater on aquatic ecosystem structure and function in the receiving stream. Sci. Total Environ. 454–455, 401–410. https://doi.org/10.1016/j.scitotenv.2013.03.025.

Feckler, Alexander, Zubrod, Jochen P., Thielsch, Anne, Schwenk, Klaus, Schulz, Ralf, Bundschuh, Mirco, 2014. Cryptic species diversity: an overlooked factor in environmental management? J. Appl. Ecol. 51 (4), 958–967. https://doi.org/10.1111/1365-2664.12246.

Figueroa-Nieves, D., McDowell, W.H., Potter, J.D., Martínez, G., Ortiz-Zayas, J.R., 2014. Effects of sewage effluents on water quality in tropical streams. J. Environ. Qual. 43 (6), 2053–2063. https://doi.org/10.2134/jeq2014.03.0139.

Flemming, H.-C., 1995. Sorption sites in biofilms. Water Sci. Technol. 32 (8), 27.

Fogg, G.E., de Rolston, Decker, D.L., Louie, D.T., Grismer, M.E., 1998. Spatial variation in nitrogen isotope values beneath nitrate contamination sources. Ground Water 36 (3), 418–426.

Freyer, H.D., Aly, A.I.M., 1975. Nitrogen-15 Studies on Identifying Fertilizer Excess in Environmental Systems. Nuclear Research Center, Juelich, Ger.

Gormly, J.R., Spalding, R.F., 1979. Sources and concentrations of nitrate-nitrogen in ground water of the central platte region, Nebraskaa. Ground Water 17 (3), 291–301. https://doi.org/10.1111/j.1745-6584.1979.tb03323.x.

Grant, S.B., Azizian, M., Cook, P., Boano, F., Rippy, M.A., 2018. Factoring stream turbulence into global assessments of nitrogen pollution. Science 359 (6381), 1266–1269.

Grice, J.W., 2001. Computing and evaluating factor scores. Psychol. Methods 6 (4), 430.

Guasch, H., Martí, E., Sabater, S., 1995. Nutrient enrichment effects on biofilm metabolism in a Mediterranean stream. Freshw. Biol. 33 (3), 373–383. https://doi.org/10.1111/j.1365-2427.1995.tb00399.x.

Hall, R.O., Tank, J.L., Sobota, D.J., Mulholland, P.J., O'Brien, J.M., Dodds, W.K., et al., 2009. Nitrate removal in stream ecosystems measured by 15N addition experiments: total uptake. Limnol. Oceanogr. 54 (3), 653–665. https://doi.org/10.4319/lo.2009.54.3.0653.

Hamilton, S.K., Tank, J.L., Raikow, D.F., Wollheim, W.M., Peterson, B.J., Webster, J.R., 2001. Nitrogen uptake and transformation in a midwestern US stream: a stable isotope enrichment study. Biogeochemistry 54 (3), 297–340.

Hauck, R.D., 1973. Nitrogen tracers in nitrogen cycle studies—past use and future needs. J. Environ. Qual. 2 (3), 317–327.

Heaton, T.H.E., 1986. Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: a review. Chem. Geol. Isot. Geosci. 59, 87–102. https://doi.org/10.1016/0168-9622(86)90059-x.

Hollander, M., Wolfe, D.A., Chicken, E., 2013. Nonparametric Statistical Methods. John Wiley & Sons.

Howarth, R.W., Billen, G., Swaney, D., 1997. Regional nitrogen budgets and riverine N and P fluxes for the drainages to the North Atlantic Ocean: natural and human influences. Oceanogr. Lit. Rev. 5 (44), 448.

Hurlbert, S.H., 1984. Pseudoreplication and the design of ecological field experiments. Ecol. Monogr. 54 (2), 187–211.

Ifabiyi, I.P., 2008. Self purification of a freshwater stream in ile-ife: lessons for water management. J. Hum. Ecol. 24 (2), 131–137. https://doi.org/10.1080/09709274.2008.11906109.

Jansen, M., Coors, A., Stoks, R., Meester, L. de, 2011. Evolutionary ecotoxicology of pesticide resistance: a case study in Daphnia. Ecotoxicology 20 (3), 543–551. https://doi.org/10.1007/s10646-011-0627-z.

Jansson, M., Andersson, R., Berggren, H., Leonardson, L., 1994. In: Ambio, S. (Ed.), Wetlands and Lakes as Nitrogen Traps, pp. 320–325.

Karouna, N.K., Fuller, R.L., 1992. Influence of four grazers on periphyton communities associated with clay tiles and leaves. Hydrobiologia 245 (1), 53–64. https://doi.org/10.1007/BF00008728.

Kendall, C., McDonnell, J.J., 1998. Isotope Tracers in Catchment Hydrology. Elsevier, Amsterdam. Hg.

Kendall, C., Elliott, E.M., Wankel, S.D., 2007. Tracing anthropogenic inputs of nitrogen to ecosystems. In: Lajtha, K., Michener, R.H. (Eds.), Stable Isotopes in Ecology and Environmental Science, second ed. Wiley-Blackwell Scientific Publication, pp. 375–449.

Koszelnik, P., 2014. Investigating the source of nitrate in Poland's San river water by analysing the nitrogen and oxygen isotopic ratio. Carpathian J. Earth Environ. Sci. 9 (3), 85–96.

Kreitler, C.W., 1975. Determining the source of nitrate in ground water by nitrogen isotope studies. In: Virtual Landscapes of Texas.

Krsmanovic, M., Biswas, D., Ali, H., Kumar, A., Ghosh, R., Dickerson, A.K., 2021. Hydrodynamics and surface properties influence biofilm proliferation. Adv. Colloid Interface Sci. 288, 102336 https://doi.org/10.1016/j.cis.2020.102336.

Legendre, P., Legendre, L., 2012. Numerical Ecology. Elsevier.

Mahler, B.J., Schmidt, T.S., Nowell, L.H., Qi, S.L., van Metre, P.C., Hladik, M.L., et al., 2020. Biofilms provide new insight into pesticide occurrence in streams and links to aquatic ecological communities. Environ. Sci. Technol. 54 (9), 5509–5519. https://doi.org/10.1021/acs.est.9b07430.

Mann, H.B., Whitney, D.R., 1947. On a test of whether one of two random variables is stochastically larger than the other. In: The Annals of Mathematical Statistics, 50–60.

Mariotti, A., 1983. Atmospheric nitrogen is a reliable standard for natural 15N abundance measurements. Nature 303 (5919), 685–687. https://doi.org/10.1038/303685a0.

Martí, E., Armengol, J., Sabater, F., 1994. Day and night nutrient uptake differences in a calcareous stream, 25. In: Internationale Vereinigung für theoretische und angewandte Limnologie: Verhandlungen, pp. 1756–1760, 3.

Martí, E., Aumatell, J., Godé, L., Poch, M., Sabater, F., 2004. Nutrient retention efficiency in streams receiving inputs from wastewater treatment plants. J. Environ. Qual. 33 (1), 285–293. https://doi.org/10.2134/jeq2004.2850.

Mayer, B., Boyer, E.W., Goodale, C., Jaworski, N.A., van Breemen, N., Howarth, R.W., et al., 2002. Sources of nitrate in rivers draining sixteen watersheds in the northeastern U.S.: isotopic constraints. Biogeochemistry 57 (1), 171–197. https://doi.org/10.1023/A:1015744002496.

Merbt, S.N., Auguet, J.-C., Casamayor, E.O., Marti, E., 2011. Biofilm recovery in a wastewater treatment plant-influenced stream and spatial segregation of ammonia-oxidizing microbial populations. Limnol. Oceanogr. 56 (3), 1054–1064. https://doi.org/10.4319/lo.2011.56.3.1054.

Ministry for Climate Protection, Environment, Energy and Mobility, Rhineland Palatinate (Ministerium für Klimaschutz, Umwelt, Energie und Mobilität, Rheinland Pfalz) (MKUEM) (2021): Struktur und Genehmigungsdirektion Süd, Liste Kläranlagen in Wasserkörpern mit p1-Bewertung. Available online at: https://wasser.rlp-umwelt.de/servlet/is/1300/, accessed on 6.10.2021.

Ministry for Climate Protection, 2017. Environment, Energy and Mobility, Rhineland Palatinate (Ministerium für Klimaschutz, Umwelt, Energie und Mobilität, Rheinland Pfalz) (MKUEM). Stream network Available online at: https://wasserportal.rlp-umwelt.de/servlet/is/2026/#. (Accessed 20 April 2022).

Mor, J.-R., Dolédec, S., Acuña, V., Sabater, S., Muñoz, I., 2019. Invertebrate community responses to urban wastewater effluent pollution under different hydro-morphological conditions. In: Environmental Pollution, pp. 483–492. https://doi.org/10.1016/j.envpol.2019.05.114, 252.

Mulholland, P.J., Tank, J.L., Sanzone, D.M., Webster, J.R., Wollheim, W.M., Peterson, B.J., Meyer, J.L., 2000. Ammonium and nitrate uptake lengths in a small forested stream determined by 15N tracer and short-term nutrient enrichment experiments, 27. In: Internationale Vereinigung für theoretische und angewandte Limnologie: Verhandlungen, pp. 1320–1325, 3.

Mulholland, P.J., Tank, J.L., Webster, J.R., Bowden, W.B., Dodds, W.K., Gregory, S.V., et al., 2002. Can uptake length in streams be determined by nutrient addition experiments? Results from an interbiome comparison study. J. North Am. Benthol. Soc. 21 (4), 544–560.

Mulholland, P.J., Helton, A.M., Poole, G.C., Hall, R.O., Hamilton, S.K., Peterson, Br.J., et al., 2008. Stream denitrification across biomes and its response to anthropogenic nitrate loading. Nature 452 (7184), 202–205. https://doi.org/10.1038/nature06686.

Nepf, H.M., 2011. Flow over and through biota. In: Treatise on Estuarine and Coastal Science. Elsevier, pp. 267–288. S.

Nichols, D.S., 1983. Capacity of natural wetlands to remove nutrients from wastewater. J. (Water Pollut. Control Federation) 495–505.

Ohe, P. C. von der, Goedkoop, W., 2013. Distinguishing the effects of habitat degradation and pesticide stress on benthic invertebrates using stressor-specific metrics. In: The Science of the Total Environment, pp. 480–490. https://doi.org/10.1016/j.scitotenv.2012.12.001, 444.

Okabe, S., Kindaichi, T., Ito, T., 2005. Fate of 14C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Appl. Environ. Microbiol. 71 (7), 3987–3994.

Painter, H.A., 1970. A review of literature on inorganic nitrogen metabolism in microorganisms. Water Res. 4 (6), 393–450. https://doi.org/10.1016/0043-1354(70)90051-5.

Painter, K.J., Brua, R.B., Spoelstra, J., Koehler, G., Yates, A.G., 2020. Fate of bioavailable nutrients released to a stream during episodic effluent releases from a municipal wastewater treatment lagoon. Environ. Sci. Processes Impacts 22 (12), 2374–2387. https://doi.org/10.1039/D0EM00315H.

Pastor, A., Peipoch, M., Cañas, L., Chappuis, E., Ribot, M., Gacia, E., et al., 2013. Nitrogen stable isotopes in primary uptake compartments across streams differing in nutrient availability. Environ. Sci. Technol. 47 (18), 10155–10162. https://doi.org/10.1021/es400726e.

Pastor, A., Compson, Z.G., Dijkstra, P., Riera, J.L., Martí, E., Sabater, F., et al., 2014. Stream carbon and nitrogen supplements during leaf litter decomposition: contrasting patterns for two foundation species. Oecologia 176 (4), 1111–1121. https://doi.org/10.1007/s00442-014-3063-y.

Peipoch, M., Martí, E., Gacia, E., 2012. Variability in δ 15 N natural abundance of basal resources in fluvial ecosystems: a meta-analysis. Freshw. Sci. 31 (3), 1003–1015. https://doi.org/10.1899/11-157.1.

Peipoch, M., Gacia, E., Pastor, A., Ribot, M., Riera, J.L., Sabater, F., Martí, E., 2014. Intrinsic and extrinsic drivers of autotrophic nitrogen cycling in stream ecosystems: results from a translocation experiment. Limnol. Oceanogr. 59 (6), 1973–1986. https://doi.org/10.4319/lo.2014.59.6.1973.

Peterson, B.J., Wollheim, W.M., Mulholland, P.J., Webster, J.R., Meyer, J.L., Tank, J.L., et al., 2001. Control of nitrogen export from watersheds by headwater streams. Science (New York, N.Y.) 292 (5514). https://doi.org/10.1126/science.1056874. S. 86–90.

QGIS Development Team (2020): QGIS. Geographic Information System. Version 3.10.4: Open Source Geospatial Foundation Project. Available online at: https://qgis.org.

R Core Team, 2019. R. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Available online at:, Version 3.6.2. https://www.R-project.org/.

Rabalais, N.N., 2002. Nitrogen in aquatic ecosystems, 2. In: Ambio 31. S. 102–112. Available online at: http://www.jstor.org/stable/4315222.

Ribot, M., Martí, E., Schiller, D. von, Sabater, F., Daims, H., Battin, T.J., 2012. Nitrogen processing and the role of epilithic biofilms downstream of a wastewater treatment plant. Freshw. Sci. 31 (4), 1057–1069. https://doi.org/10.1899/11-161.1.

Ribot, M., Schiller, D. von, Martí, E., 2017. Understanding pathways of dissimilatory and assimilatory dissolved inorganic nitrogen uptake in streams. Limnol. Oceanogr. 62 (3), 1166–1183. https://doi.org/10.1002/lno.10493.

Roche, A., Vennin, E., Bundeleva, I., Bouton, A., Payandi-Rolland, D., Amiotte-Suchet, P., et al., 2019. The role of the substrate on the mineralization potential of microbial mats in A modern freshwater river (Paris basin, France). Minerals 9.

Romaní, Anna M., Sabater, Sergi, 2001. Structure and activity of rock and sand biofilms in a mediterranean stream, 82. In: Ecology, p. 3232. https://doi.org/10.2307/2679846, 11.

Romaní, A.M., Guasch, H., Muñoz, I., Ruana, J., Vilalta, E., Schwartz, T., et al., 2004. Biofilm structure and function and possible implications for riverine DOC dynamics. Microb. Ecol. 47 (4), 316–328. https://doi.org/10.1007/s00248-003-2019-2.

Rosi-Marshall, E.J., Kincaid, D.W., Bechtold, H.A., Royer, T.V., Rojas, M., Kelly, J.J., 2013. Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilms, 23. In: Ecological Applications, pp. 583–593, 3.

Ryhiner, G., Sørensen, K., Birou, B., Gros, H., 1994. Biofilm reactors configuration for advanced nutrient removal, 29. In: Water Science and Technology, p. 111, 10-11.

Sabater, S., Guasch, H., Romaní, A., Muñoz, I., 2002. The effect of biological factors on the efficiency of river biofilms in improving water quality. Hydrobiologia 469 (1), 149–156.

Sabater, S., Guasch, H., Ricart, M., Romaní, A., Vidal, G., Klünder, C., Schmitt-Jansen, M., 2007. Monitoring the effect of chemicals on biological communities. The biofilm as an interface, 387. In: Analytical and Bioanalytical Chemistry, pp. 1425–1434. https://doi.org/10.1007/s00216-006-1051-8, 4.

Schmidt, T.S., Konrad, C.P., Miller, J.L., Whitlock, S.D., Stricker, C.A., 2019. Benthic algal (periphyton) growth rates in response to nitrogen and phosphorus: parameter estimation for water quality models, 55. In: Journal of the American Water Resources Association, pp. 1479–1491. https://doi.org/10.1111/1752-1688.12797, 6.

Schulten, H.-R., Schnitzer, M., 1997. The chemistry of soil organic nitrogen: a review, 26. In: Biology and Fertility of Soils, pp. 1–15. https://doi.org/10.1007/s003740050335, 1.

Snider, M., Spoelstra, J., Schiff, S.L., Venkiteswaran, J.J., 2010. Stable oxygen isotope ratios of nitrate produced from nitrification: 18O-labeled water incubations of agricultural and temperate forest soils. Environ. Sci. Technol. 44, 5358–5364.

Spruill, T.B., Showers, W.J., Howe, S.S., 2002. In: Application of Classification-Tree Methods to Identify Nitrate Sources in Ground Water. USGS Staff–Published Research. S. 20.

State Office for Environment, 2007. Water Management and Trade Control Rhineland-Palatinate (Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht Rheinland-Pfalz) (LUWG). Available online at:. In: Körbes, G. (Ed.), Hydrologischer Atlas Rheiland-Pfalz. Gewässerkundliches Flächenverzeichnis. Unter Mitarbeit von H.-J. Rossel y. Heinrichs. Hg. v. Amtliches Topographisch-Kartographisches Informationssystem (ATKIS) https://wasserportal.rlp-umwelt.de/servlet/is/2025/. accessed on 20.10.2021.

Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., et al., 2013. IPCC, 2013: Climate Change 2013: the Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovern-Mental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, p. 1535.

Tank, J.L., Dodds, W.K., 2003. Nutrient limitation of epilithic and epixylic biofilms in ten North American streams. Freshw. Biol. 48 (6), 1031–1049. https://doi.org/10.1046/j.1365-2427.2003.01067.x.

Tank, J.L., Meyer, J.L., Sanzone, D.M., Mulholland, P.J., Webster, J.R., Peterson, B.J., et al., 2000. Analysis of nitrogen cycling in a forest stream during autumn using a 15 N-tracer addition. Limnol. Oceanogr. 45 (5), 1013–1029. https://doi.org/10.4319/lo.2000.45.5.1013.

Toda, H., Uemura, Y., Okino, T., Kawanishi, T., Kawashima, H., 2002. Use of nitrogen stable isotope ratio of periphyton for monitoring nitrogen sources in a river system, 46. In: Water Science and Technology, pp. 431–435, 11-12.

Valiela, I., Geist, M., McClelland, J., Tomasky, G., 2000. Nitrogen loading from watersheds to estuaries: verification of the Waquoit Bay nitrogen loading model, 49. In: Biogeochemistry, pp. 277–293, 3.

Voss, M., Deutsch, B., Elmgren, R., Humborg, C., Kuuppo, P., Pastuszak, M., et al., 2006. Source identification of nitrate by means of isotopic tracers in the Baltic Sea catchments. Biogeosciences 3 (4), 663–676. https://doi.org/10.5194/bg-3-663-2006.

Vymazal, J., 2011. Constructed wetlands for wastewater treatment: five decades of experience, 45. In: Environmental Science & Technology, pp. 61–69. https://doi.org/10.1021/es101403q, 1.

Waiser, M.J., Tumber, V., Holm, J., 2011. Effluent-dominated streams. Part 1: presence and effects of excess nitrogen and phosphorus in Wascana Creek, Saskatchewan, Canada, 30. In: Environmental Toxicology and Chemistry, pp. 496–507. https://doi.org/10.1002/etc.399, 2.

Wigand, C., McKinney, R.A., Cole, M.L., Thursby, G.B., Cummings, Jean, 2007. Varying stable nitrogen isotope ratios of different coastal marsh plants and their relationships with wastewater nitrogen and land use in New England, USA, 131. In: Environmental Monitoring and Assessment, pp. 71–81. https://doi.org/10.1007/s10661-006-9457-5, 1-3.

Wijeyekoon, S., Mino, T., Satoh, H., Matsuo, T., 2004. Effects of substrate loading rate on biofilm structure. Water Res. 38 (10), 2479–2488. https://doi.org/10.1016/j.watres.2004.03.005.

Wilcoxon, F., 1945. Individual comparisons by ranking methods. In: Reprinted in the Bobbs-Merrill Reprint Series in the Social Sciences, pp. 80–83. S-541 Biometrics Bulletin (1), S.

Xin, X., Huang, G., Zhang, B., 2021. Review of aquatic toxicity of pharmaceuticals and personal care products to algae. In: Journal of Hazardous Materials, 124619. https://doi.org/10.1016/j.jhazmat.2020.124619, 410.

Zhao, Y., Newman, M.C., 2006. Effects of exposure duration and recovery time during pulsed exposures, 25. In: Environmental Toxicology and Chemistry, pp. 1298–1304. https://doi.org/10.1897/05-341r.1, 5.