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Research article

Biofilm scrubbing for restoration—algae community composition and succession in artificial streams

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Abstract: Photoautotrophic biofilms play a pivotal role in self-purification of rivers. We took advantage of the biofilm's cleaning capacity by applying artificial stream mesocosms, called algae turf scrubber^{TM} (ATS), to reduce the nutrient load of a highly eutrophicated backwater in Vienna (Austria). Since purification strongly depends on benthic algae on the ATS, we focused on the algae community composition and succession. Estimation of coverage, photographic documentation for micromapping, species identification and pigment analyses were carried out. Already one week after exposition, 20–30 different taxa were recorded, suggesting a rapid colonization of the substrate. In total around 200 taxa were identified, mainly belonging to Chlorophyta, Bacillariophyceae and Cyanoprokaryota. Nonmetric multidimensional scaling implied that season and succession strongly influenced species composition on the ATS and a minimum turnover of 0.28 indicates a development towards a more stable community at the end of experiments. We measured maximum biomass production of ~250 g m^{-2} in June and August and during a period of 5 months nearly 19 kg ha^{-1} phosphorus could be removed. ATS systems proved to retain nutrients and produce algae biomass in an environmentally friendly and cost effective way and thus support restoration of highly eutrophicated water bodies.

Keywords: Phytobenthos; biofilm; algae turf scrubber; eutrophication; ecological engineering

Abbreviations

ATS	Algal turf scrubber™
HSW	Heustadelwasser
chl-a	chlorophyll-a
TO	turnover rate
NMS	Nonmetric multidimensional scaling

1. Introduction

Large impact of humans on natural aquatic ecosystems frequently results in dramatic changes of hydrology and nutrient cycling, commonly going along with eutrophication [1-3]. Highly eutrophicated waters facilitate development of algal blooms and comparably low species diversity [4]. Furthermore, eutrophication causes a series of problems concerning the usability of the affected ecosystems. In freshwater systems used for drinking water supply, mass development of Cyanoprokaryota, such as *Microcystis* spp. and *Aphanizomenon* spp. can cause severe problems [5], because several taxa are known to produce serious toxins [6]. Moreover, algal blooms are sometimes followed by fish kill due to oxygen depletion [7], when algal biomass is degraded. In addition, high algal productivity increases pH, causing a conversion of ammonium to toxic ammonia. Especially waters with already elevated ammonium content, like fish ponds and aquacultures, are highly susceptible to pH-shifts [8]. Another negative effect of algal blooms is a reduced recreation value for humans because of high turbidity, scums drifting on the water surface and malodor.

The reasons for eutrophication, induced by enhanced input or accumulation of nutrients, are highly diverse. Amongst others, disconnection of side arms from the main channel of rivers can favor eutrophication of backwaters due to biomass aggregation and siltation. This is the case at a former backwater of the river Danube, the Heustadelwasser (HSW), located in one of Vienna's very popular recreation areas and tourist attractions—the "Prater". Structural measures, such as regulation of the Danube at the end of the 19th century and construction of the hydropower plant Freudenau in 1998 [9], led to disconnection of the side arm from the main channel and finally to eutrophication. A first set of restoration measures to reduce nutrient levels and prevent fish kill was conducted previously and included the installation of a gravel filter including a chemical phosphorus trap (called Neptunanlage™) in 2007 [10]. These measures showed first positive effects, but further reduction of nutrients and persisting blooms of *Microcystis* spp., passing the gravel filter, was desired.

Reduction of nutrient levels in water bodies can be achieved by application of an algal turf scrubber™ (ATS), a technology first described by Adey & Loveland [11]. The underlying process is comparable to the self-purification process in natural streams, where biofilms are highly efficient in removing inorganic and organic compounds [12]. The photoautotrophic biofilm, developing on the surface of the horizontal ATS, incorporates nutrients from the supplied water, which can be harvested and removed afterwards. Cultivation of periphyton on ATS provides several advantages over suspended algae cultivation, such as easier harvesting [13] and already concentrated biomass. Optimized growth conditions on the ATS fuel algae growth and nutrient uptake, which may allow the benthic community to outcompete planktic species. Furthermore, the application of ATS may enable "luxury uptake" of phosphorus by algae, which means that algae take up more phosphorus than necessary for survival [14], leading to higher phosphorus removal rates from surrounding water

compared to a chemical phosphorus trap alone. Luxury uptake of phosphorus is more likely to occur on surfaces, as provided by ATSs, due to improved light conditions compared to other cultivation systems because higher light intensity leads to rapid polyphosphate accumulation. Moreover, installing wave surge buckets on the ATS can improve growth conditions, because enhanced turbulences of the supplied water reduce boundary layers, which would otherwise limit nutrient and metabolite exchange [11]. Increased reduction of phosphorus is additionally caused by co-precipitation of inorganic phosphate and calcite in the presence of photosynthesizing algae, as observed by Hartley et al. [15].

Growth conditions on the ATS affect the algal community, which in turn regulate the cleaning capacity of ATS systems. It is crucial to know which taxa are present under the prevailing conditions and how their abundance changes during the growth period to predict the cleaning capacity of ATS. Also for the harvesting process, knowledge of the community composition is of interest, as different algal taxa contain manifold valuable compounds, which may be exploited in an additional step. In our study, the algal community on the ATS consisted of a natural selection of local species, an approach previously applied for nutrient removal from natural waters [16], as well as wastewater [17]. In the course of biofilm development, the algae community changes and different stages of succession occur, favoring nutrient uptake or release of the biofilm. First results of our pilot study were published earlier and an optimal harvesting period of 3 weeks was suggested to reach a maximal areal phosphorus removal rate of 19.1 ± 2.5 mg m^{-2} d^{-1} and prevent nutrient release due to detachment of senescent biofilm [18]. The actual study provides a detailed description of the benthic algae community occurring on ATSs and its change during succession. Together with the previously published study [18], focusing on estimation of growth, productivity and composition of the biofilm, we provide for the first time an overall impression of ATS's performance for restoration purposes under a temperate climate regime in Europe. The objective of the present pilot study was to investigate the algal community structure on ATSs temporally, by defining (1) how fast the ATSs get colonized after exposition; (2) which algae taxa dominate the community on the ATSs; (3) how species diversity changes over time. We analyzed the algal community on the ATSs via light microscopy and pigment analysis, as well as visual inspection and digital imaging documentation. The results of our pilot study give insights into on-going processes of benthic algae succession on ATSs and will help improving water cleaning systems based on algae cultivation by optimizing the ATS's nutrient removal efficiency. Water quality improvement with ATS systems has been applied successfully in several fields, like tertiary treatment of secondary sewage, purification of industrially contaminated groundwater or restoration of big streams [19-21]. Compared to other water purification systems, the ATS technology is an economical and environmentally friendly method because no chemicals are needed. Additionally, algal biomass and the retained nutrients can be utilized for other purposes, for example as slow-release organic fertilizer [22] or feedstock for the production of renewable biofuel [23]. The application of ATS for sustainable and environmentally friendly restoration of eutrophic water bodies or improvement of existing nutrient retention systems, as the Neptunanlage™ is another opportunity.

2. Material and methods

2.1. Study site

The HSW is located in the recreation area Prater in Vienna, Austria (longitude 16°25'59'', latitude 48°11'56''). The surface area of the HSW is 1.8 ha and the mean depth is 1.5 m. The mean conductivity from June to September 2011 was 548 ± 217 µS cm^{-1}, total nitrogen and phosphorus ranged from 0.64 to 1.23 mg L^{-1} and from 36 to 67 µg L^{-1}, respectively (Table 1).

Table 1. Characteristics of the water supply for the ATSs (mean values with standard deviations and number of samples) derived from the HSW (June–September 2011).

Parameter	Mean	SD	n	Reference
Water temperature HSW [°C]*	20.74	3.10	16	[10]
pH [$-\log H^+$]	8.31	0.18	16	[10]
Conductivity [µS cm^{-1}]	548	217	16	[10]
Total phosphorus [µg L^{-1}]	50.75	9.74	12	[10]
Total nitrogen [mg L^{-1}]	1.04	0.16	16	[10]
Flow rate [L min^{-1}]	25.23	7.63	100	This study
Wave surge frequency [min^{-1}]	2.71	0.77	100	This study

* corresponds with average air temperature during the whole sampling period [18].

2.2. Experimental setup

An ATS is an artificial streambed, where attached benthic biofilm captures solar energy and retains nutrients from the water surrounding it. The benthic biofilm can be dominated by filamentous green algae, giving it a turf-like appearance. This algal turf can be scrubbed off and the obtained biomass used for further purpose. In order to find a design meeting the requirements at the study site, several vertical and horizontal ATS prototypes were constructed prior to the main experiment. Finally a horizontal system was chosen and four replicates constructed; the ATSs were connected to the existing irrigation system on top of the gravel filter (Figure 1). Each ATS consisted of a horizontal wooden flow lane (dimensions: 2.0 m × 0.8 m), with a slope of about 1%, which was covered with pond liner and equipped with a tipping bucket at the inflow of the ATS. To facilitate growth of periphyton, a polyethylene net (mesh size 4 mm) was attached to the bottom of the flow lane. A 2 cm high wooden bar was placed at the outflow of each ATS to prevent biofilm desiccation, which might have happened during low flow rate. Variations of the flow rate resulted from a temperature dependent operation mode of the Neptunanlage. At higher temperatures more water was pumped through the system to increase the filter effect of the gravel filter. At lower temperature the power of the Neptunanlage was reduced together with the flow rate. The amount of water remaining on the ATS between two tipping events (about 15 L) was comparable to the volume of the tipping buckets (~10 L). The buckets consisted of stainless steel and were facilitated with ball bearings to ensure minimal friction during tipping. Due to the special shape of the buckets and the position of the rotation points, no additional energy supply is necessary for charging and discharging of the buckets.

Tipping frequency of the buckets was 2.71 ± 0.77 times min^{-1} and the flow rate was 25.23 ± 7.63 L min^{-1}. Air temperature and solar radiation were measured as described earlier [18].

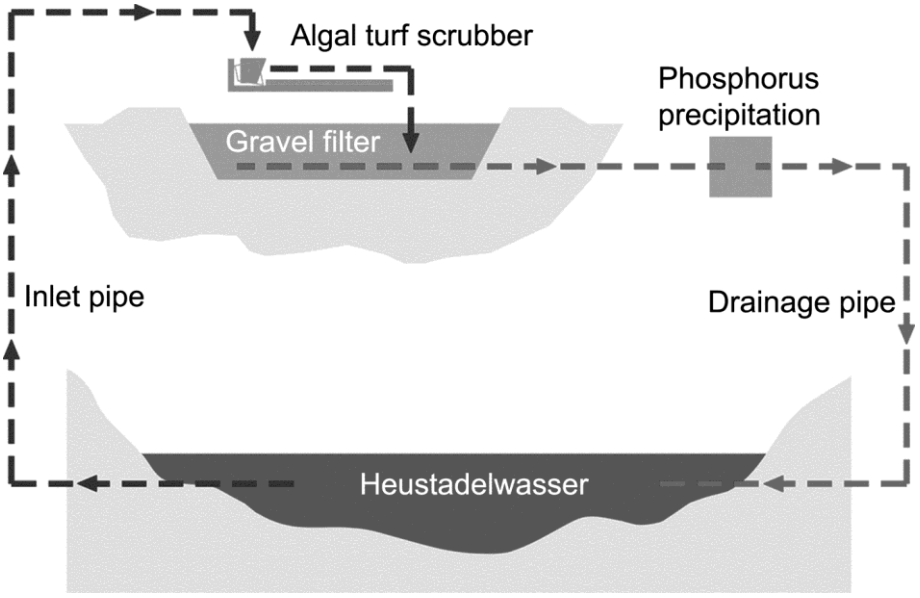

Figure 1. Schematic illustration of the experimental setup and the water circulation (flow direction indicated by arrows; modified after [18]).

Altogether 3 runs were conducted (run 1 from 3.6.2011 to 12.7.2011, run 2 from 12.7.2011 to 9.8.2011 and run 3 from 16.8.2011 to 20.9.2011). Because the Neptunanlage™ had technical problems at the end of run 2, it was excluded from fixed sample microscopy and community pattern analysis. Weekly sampling was carried out for all analysis with a modified Douglas sampler [24]. The algal biomass was scrubbed off the flow lanes with a brush (~3 mm long bristles) and transferred to a collecting bottle with the help of a vacuum flask and a suction unit. Depending on the density of the biofilm 2–4 circular areas of 15 cm^2 each were sampled. To ensure that sampling positions were chosen unsystematically, random numbers from 1–64 were generated with the help of the statistic software R 2.12.1 and a grid with 64 fields was placed on the ATS during sampling (Figure 2). The area (20 cm) around tipping buckets was excluded from the analysis because of heterogeneous conditions, compared to the remaining ATS area. The entire biomass growing on the ATS was harvested every 28–39 d with the help of a squeegee. Before starting the next run, ATS surface was cleaned with brushes and water.

2.3. Biofilm characterization

2.3.1. Visual and photographic biofilm characterization

The total biofilm coverage, green and brown fraction in percentage of the ATS surface, within the used grid (Figure 2), was estimated by naked eye in the field (herein referred to as "field estimation") at every sampling date before quantitative sampling. Additionally estimation was done by digital image processing (herein referred to as "digital estimation"): digital images of the biofilm surface were taken with a single lens reflex camera (Nikon D90 equipped with a circular polarization

filter to minimize reflections of the water surface), photos were rectified with the software ShiftN 3.6 and Photoshop CS6 was used to define brown, green and uncovered areas of the biofilm (Figure 3). A detailed description of the picture processing in Photoshop is provided as supplementary text S1.

Figure 2. Photography of the ATS with the inflow and tipping bucket at the left side and the constructed sampling grid above the flow lane.

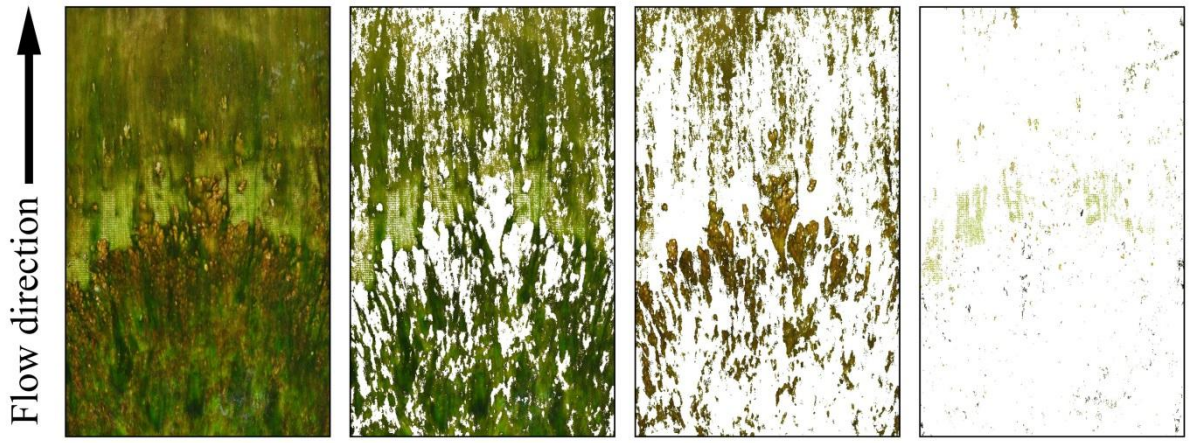

Figure 3. Pictures of the biofilm on the ATS (run 1, 05.07.2011, replicate B) used for digital surface analysis, separated in four layers, from left to right: entire surface (total biofilm), green (mainly green algae), brown (diatoms) and uncovered surface.

2.3.2. Pigments

Chlorophyll-a (chl-a) of the biofilm was quantified spectrophotometrically as described by Mayr et al. [18]. HPLC was used for quantification of additional photosynthetic pigments with an extraction procedure identical to chl-a extraction. After centrifugation, pigments were analyzed by means of a Hitachi LaChrom Elite®-System (composed of a L-2130 pump, a L-2200 autosampler, a column thermostat L-2300 with temperature adjusted to 35 °C, a L-2455 diode array detector and a L-2485 FL-detector; column superspher RP-18 and precolumn LIchrocart rP-18) gradient program after Wright et al. [25]. Pigments were quantified at 440 nm and identified by comparison of the retention times with authentic standards (DHI, Denmark) and spectral data within the VIS range [26]. For the calculation of algal groups based on total chl-a, we used the software package Chemtax 1.95 [27].

2.3.3. Taxonomy

Microscopic analyses were carried out in order to get more precise information on the taxa composition of the biofilm. Each week an area of 30–60 cm^2 was sampled and both live material and diatom samples were identified using compound microscopes (Zeiss Axio Imager M1, Axio Cam MRc5, Axio Vision Release 4.7.2 and Reichert Polyvar, Olympus soft imaging solutions FireWire, Cell^F). Additionally, the intact biofilm was analyzed with the stereo microscope Zeiss Stereo Lumar.V12 (Axio Cam ERc5s, Axio Vision Release 4.8.2) for investigating its 3-dimensional structure. Relative frequency estimation on a semi-quantitative scale from 1 to 5 (1 = occasional, 2 = rare, 3 = common, 4 = frequent, 5 = dominant) was done with live material. For identification and quantification of diatoms, permanent slides were prepared and 500 frustules identified [28]: samples were wet combusted with HCl, HNO_3 + H_2SO_4 and embedded in Naphrax. Identification keys for Bacillariophyceae: [29-32]; Chlorophyta: [33-37]; Chrysophyceae: [38]; Conjugatophyceae: [39-41]; Cyanoprokaryota: [42-44]; Dinophyta: [45]; Xanthophyceae: [46,47]. Macrozoobenthos was not identified in detail.

2.3.4. Community patterns

For characterizing algal diversity on the ATS, species richness (= number of taxa S; alpha diversity) over time was analyzed for all taxa. The Shannon Index H_s was calculated for the diatom communities of run 1 and 3 following the equation:

$$H_s = -\sum p_i \cdot \ln p_i$$

where p_i is the proportion of the number of individuals of taxon *i* to the total of individuals [48]. The Shannon Index H_s considers the number of individuals belonging to one species, additionally to the species richness, and therefore offers more specific information. Additionally, the Shannon-Wiener Evenness E_s was calculated [48,49]:

$$E_s = \frac{H_s}{H_{max}}, \; H_{max} = \ln S$$

Succession of runs 1 and 3 were quantified with the help of species turnover rates. Abundance data of the diatoms and live samples were transformed to presence/absence data and for the whole algal community, the turnover rate (TO) was calculated according to [48]:

$$TO = \frac{(I + E)}{S_t + S_{t+1}}$$

where TO = turnover between the time span *t* and *t+1*, *I* = invasion (number of species present at *t+1* but absent at *t*), *E* = extinction (number of species present at *t* but absent at *t+1*), S_t = community at *t* (number of species present at *t*), S_{t+1} = community at *t+1* (number of species present at *t+1*). TO ranges between 0.0 (= no gain or loss of species) and 1.0 (= complete change of species).

Nonmetric multidimensional scaling (NMS) was performed (PC-ORD 5) [50] with diatom data of runs 1 and 3, consisting of absolute frequencies. An additional NMS was performed with the total algal community. For merging relative frequencies of live samples with absolute diatom frequencies, percentage contribution of diatom taxa was transformed to relative frequencies: <0.4 % = 1; 0.4–2% = 2; 2–10% = 3; 10–50% = 4; >50% = 5 [51]. The results obtained by using only diatom data was comparable to results obtained from the whole dataset. Nevertheless, absolute numbers of diatoms were used exclusively for the final solution because of higher final stability and lower stress. Two species (*Cocconeis pediculus* and *Fragilaria capucina* var. *vaucheriae*) were excluded because they only occurred in run 2. NMS was finally performed with 63 taxa. Sorensen (Bray-Curtis) coefficient was used as distance measure and three dimensions were chosen for the final solution to guarantee sufficient stress reduction and still produce an interpretable result. With the main dataset, 250 runs were performed with a maximum number of 500 iterations for the final solution. Random numbers were used as starting configuration by choosing time of day for the random number seeds. A Monte Carlo test was run (1000 times) and the resulting *p*-value examined to see if a similar final stress have been obtained randomly. The final NMS was performed six times to check for stability of the result and the graph chosen which explained the underlying pattern best. Other statistical analyses were performed using R 2.12.1, SigmaPlot 13.0, and Microsoft Excel 2010.

3. Results

3.1. Visual and photographic biofilm characterization

After an initial colonization phase of 6 d, around 60–90% of the ATS area was covered with biofilm (Figure 4). Digital estimation results supported field estimations, although a lower contribution of diatoms was recorded in the first few days of run 1. Moreover, photo documentation suggested a slower colonization within 18 d of the first run. In the following period, the covered area kept constant, but the biofilm increased in thickness and structural complexity. At the beginning of run 1, the brownish fraction was dominant, indicating a high abundance of Bacillariophyceae, followed by a shift to green within the next weeks, corresponding to dominance of green algae. The contribution of cyanobacteria to one of the two colors could not be assessed by visual biofilm characterization but was probably comparably low, as indicated by HPLC data (Figure 5) and the low abundance recorded during microscopy (unpublished data). Run 2 differed from the first run in several ways. First of all, coverage was slightly higher (up to 100 %; field estimation). Second, it was reached faster and a higher contribution of green algae compared to run 1 was observed already

at the beginning of run 2. Generally diatoms dominated in the second run. The third run was characterized by a strong decline of biofilm at the end of the run, where just 60 % of the area was colonized. Furthermore diatoms were dominating as well, although a larger portion of green algae was measured by digital estimation (Figure 4).

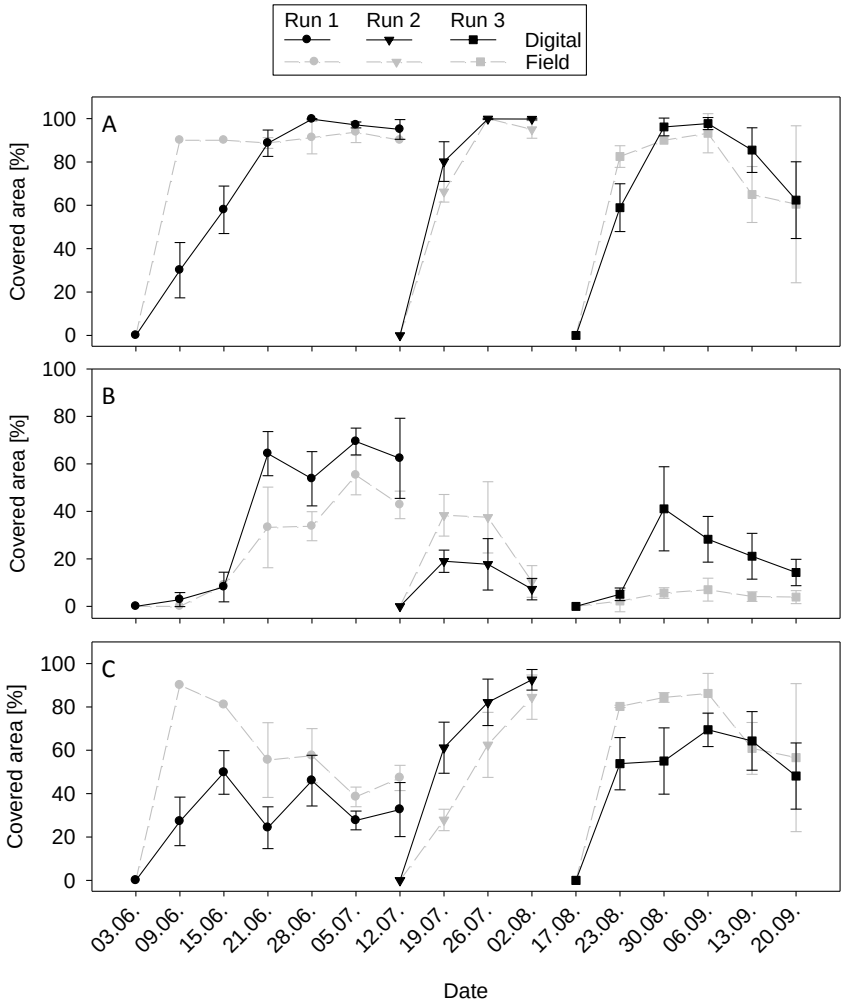

Figure 4. Color estimation of the biofilm surface with the help of digital analysis (black solid lines) compared to estimation carried out in the field (grey dashed lines) by naked eye; A = total covered area, B = green area, C = brown area during the whole sampling period (run 1–3, arithmetic means ± standard deviation, n = 4).

3.2. Pigments

Chl-a data suggested a slow colonization at the beginning of each run (especially run 1), followed by a fast increase after 2–3 weeks (Figure 5, top). The first chl-a maximum of run 1 was 310 ± 90 mg m^{-2} and reached after 4 weeks. A second maximum was recognized in the sixth week and amounted to 355 ± 30 mg m^{-2}. During run 2, the final chl-a peak was reached much faster: after 3 weeks 700 ± 120 mg m^{-2} were measured, which was also the highest value of all runs. The chl-a peak of run 3 was measured after 3–4 weeks. The chl-a content of the ATS's water supply showed just minor deviations for each run and ranged between 21.1 ± 1.5 and 69.1 ± 1.9 µg L^{-1} chl-a.

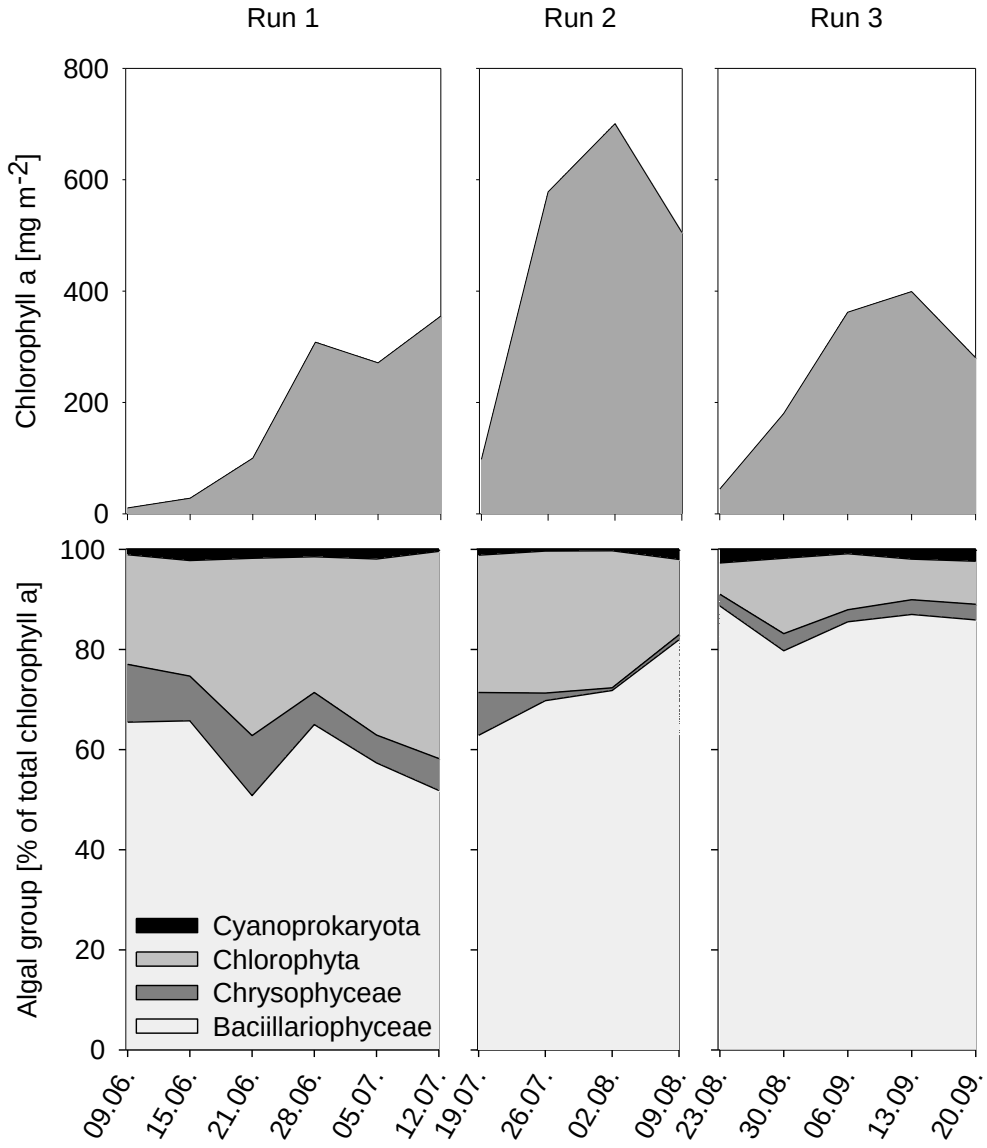

Figure 5. Chl-a measured spectrophotometrically as proxy for algal biomass in run 1–3 (top); dominating algal groups estimated from HPLC analyses in run 1–3 (bottom); (arithmetic means, n = 4).

Four major algal groups were identified by means of HPLC: Bacillariophyceae, Chlorophyta s.l., Chrysophyceae and Cyanoprokaryota (Figure 5, bottom). Bacillariophyceae accounted always for more than 50% of total biomass; in the third run diatoms accounted for around 90%. The second largest group was represented by Chlorophyta (maximum of 41% at the end of run 1), followed by Chrysophyceae, which contributed to up to 12% of chl-a at the beginning of run 1. Thereafter Chrysophyceae declined until the end of run 1 and remained mostly below 7% until the end of the observation period. Cyanoprokaryota had the smallest contribution with less than 3% of the total biomass throughout the investigation period.

3.3. Taxonomy

During the entire sampling period 196 algae taxa were identified, belonging to 9 different phyla (Figure 6). For a detailed list of taxa see table S1. In terms of taxon richness, the ATSs were dominated by Ochrophyta (38% including Bacillariophyceae) followed by Chlorophyta (33%) and Cyanoprokaryota (17%). Rhodophyta and Haptophyta accounted for less than 1% of the richness.

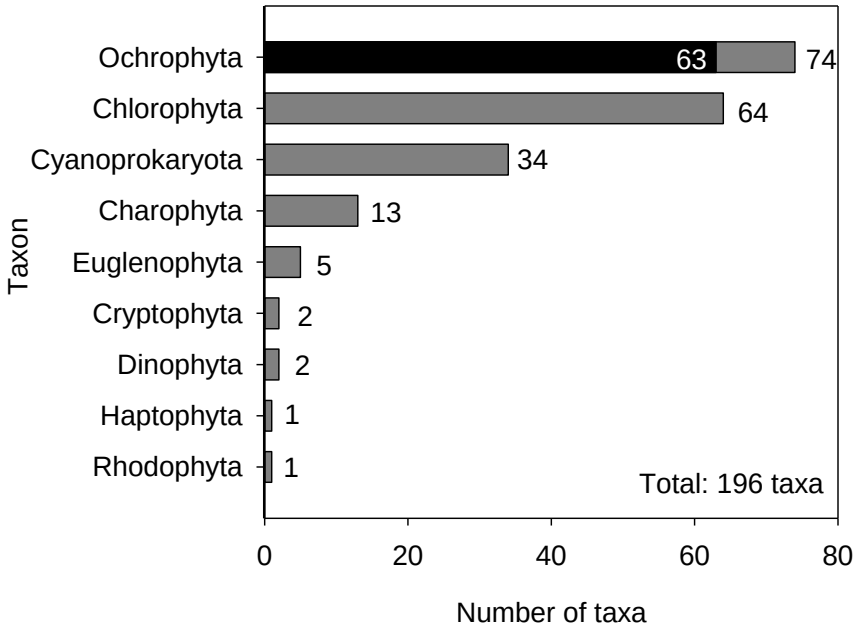

Figure 6. Number of identified algal taxa per phylum (grey bars) and number of Bacillariophyceae within Ochrophyta (black bar), observed on all four ATS™ replicates during the entire sampling period.

Microscopy of the live samples surprisingly showed not only benthic, but also planktonic taxa. Benthic algae were clearly dominating and among green algae filamentous genera such as *Mougeotia*, *Cladophora*, *Spirogyra* and *Oedogonium* were very common. *Mougeotia* spp. occurred during the whole sampling period. The highest abundance of these genera was detected from June to July at chl-a peaks. The following planktonic genera were always present, but only in low numbers: *Scenedesmus/Acutodesmus* (8 species), *Pediastrum* (6 species), *Coelasturm* (6 species), *Tetraedron* (5 species) and *Chlamydomonas* sp.. Species like *Coelastrum astroideum* De Notaris, *Pediastrum duplex* Meyen, *Phacotus lenticularis* (Ehrenberg) Stein, *Coelosphaerium aerugineum* Lemmermann and *Microcystis* spp. were rare but occurred throughout the season. The most common diatom species was *Fragilaria capucina* Desmaziers, which formed huge ribbon-shaped colonies and contributed to ~90% of the diatoms. Other very abundant benthic diatom species were *Diatoma tenuis* C. Agardh, *Achnanthidium minutissima* (Kützing) Czarnecki, *Cymbella caespitosa* (Kützing) Brünn. *Diatoma vulgaris* Bory was also common, but did not occur before the end of run 1. The genus *Cymbella* was represented by 13 species, but just two of them—*C. microcephala* Grunow and *C. caespitosa*—were found in both runs; *C. affinis* Kützing was highly abundant in run 3. Other rare taxa were *Gomphonema*, *Nitzschia* and *Navicula*. The planktonic diatom community included *Cyclotella* (5 species), *Aulacoseira* and *Stephanodiscus*. *Cyclotella ocellata* Pantocsek was very

common and found together with *C. distinguenda* Hustedt and *C. radiosa* (Grunow) Lemmermann at all sampling dates. Rare species were the centric diatom *Stephanodiscus hantzschii* Grunow and the pennate form *Amphipleura pellucida* (Kützing) Kützing. Furthermore, *Chroodactylon ornatum* (C.Agardh) Basson (Rhodophyta) and *Hymenomonas roseola* Stein (Haptophyta) were found on the ATSs. Regarding the fauna on the ATS, we observed only few rotifers, nematodes, Vorticellidae and other ciliates. Additionally, the intact biofilm revealed the presence of *Hydra* sp.; occasionally leeches were noticed on the ATSs during sampling.

3.4. Community patterns

The lowest diversity of 27 taxa occurred at the onset of run 1 and the highest diversity of 68 taxa was observed towards the end of run 3 (Figure 7). Species richness of run 1 was lower compared to run 3, where Bacillariophyceae constituted the most diverse group. Concerning the live samples of run1 and 2, taxa numbers already increased after 1 week, but a decline towards the end of the experiment was found for run 2. In contrast to this finding, a slight overall decline of diversity was observed for run 3 (Figure 7).

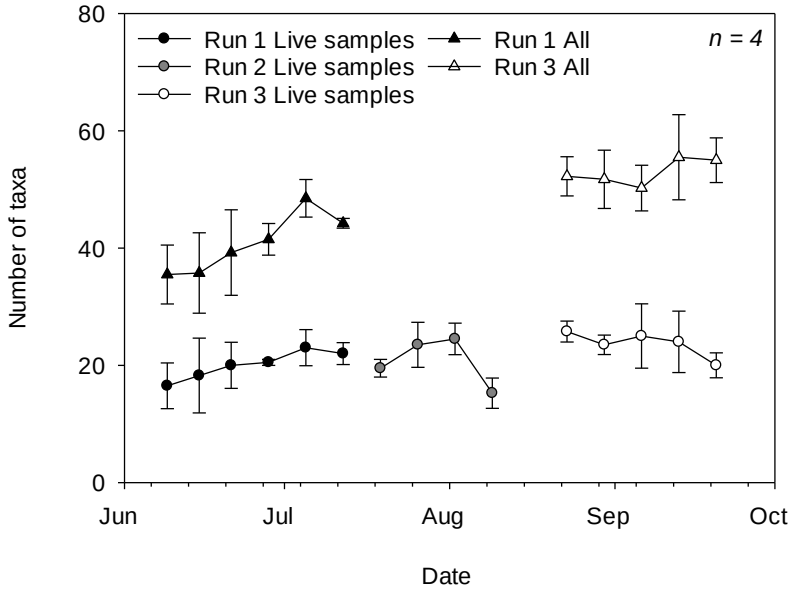

Figure 7. Number of algal taxa observed on the four ATSs during the entire sampling period (All = live samples + diatom samples; arithmetic means ± standard deviations, n = 4).

The Shannon Index H_s calculated for the diatom community ranged from 1.44 ± 0.33 to 2.45 ± 0.16 and was generally higher in run 3 compared to run 1 (Figure 8, top). H_s of the first run showed two peaks and was highest with 2.32 ± 0.10 on the 5th of July. Between those peaks, a major reduction of the diatom community was observed, which was also true for run 3. The highest H_s (2.45 ± 0.16) of run 3 occurred on the 13th of September (Figure 8, top). Evenness E_s calculated for the first run ranged between 0.48 ± 0.08 and 0.71 ± 0.02. It was lowest on the first sampling date. During the third run, E_s showed a smaller variation and ranged between 0.63 ± 0.03 and 0.72 ± 0.06 (Figure 8, center). Generally, E_s and H_s showed a very similar pattern.

The highest TO of the entire sampling period occurred in run 1 already one week after installation of the ATSs and amounted to 0.53 ± 0.04 (Figure 8, bottom). The third run showed a low TO (0.32 ± 0.03) at the beginning, peaked in the third week and decreased to a minimum of 0.28 ± 0.02.

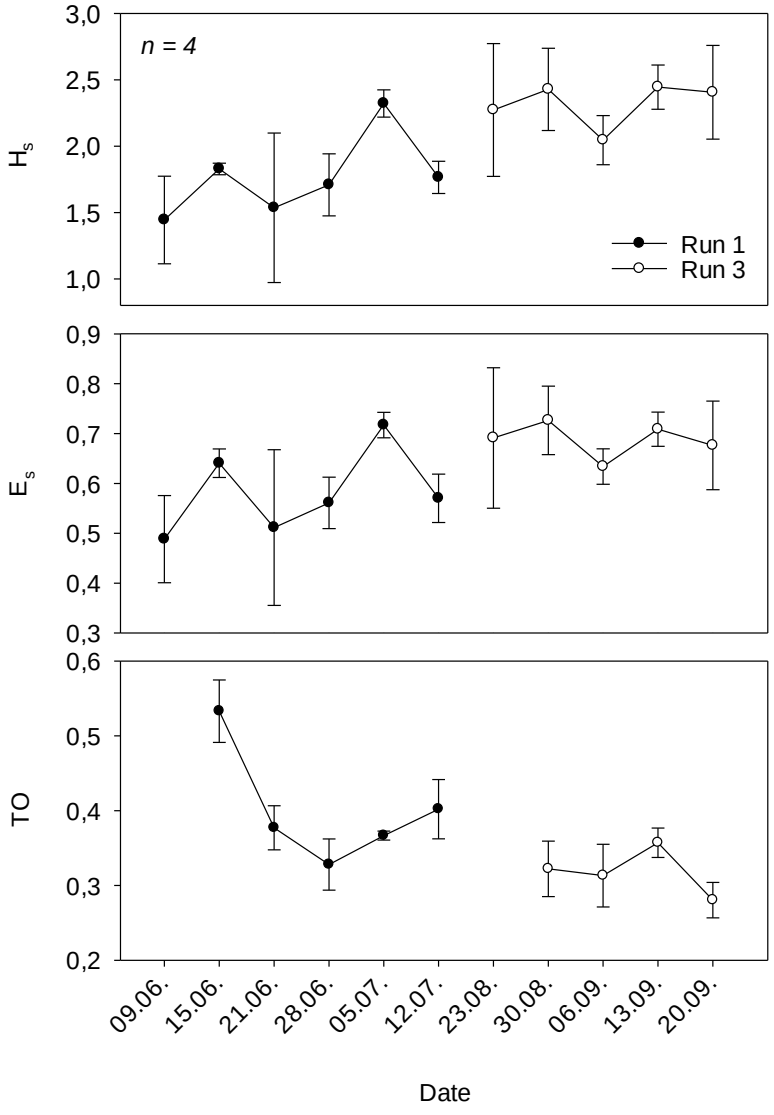

Figure 8. Shannon Index H_s (top), Evenness E_s (center) and turnover TO (bottom) of the diatom community on the ATSs (arithmetic means with standard deviations, n = 4).

The NMS analysis of run 1 and 3 showed the best solution with 3 dimensions, which had a final instability of $<10^{-5}$ and a final stress of 8.58 (p-value 0.0010). NMS results indicated that the first run was more homogenous compared to the third one (Figure 9). The first axis (r^2 = 0.387) separated the algal community found at earlier stages (left side) from the dates towards the end of each run (right side). Axis 2 (r^2 = 0.459) split the first run clearly from the third run. The third axis (r^2 = 0.105) was less important and separated one group (replicate C, run 3) from the remaining data. This group showed a much higher abundance of *Diatoma vulgaris* and *Achnanthidium minutissima*, compared to the second group.

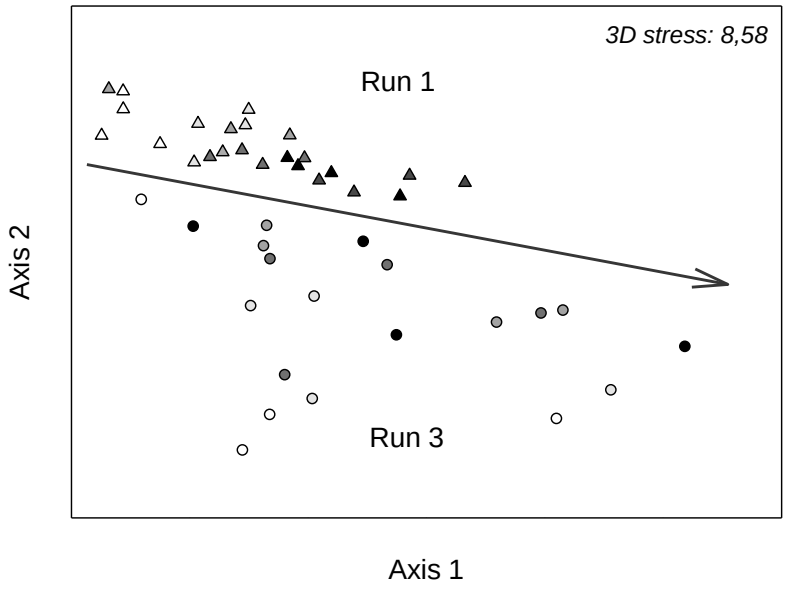

Figure 9. NMS plot, showing ordination of the diatom community, absolute values of run 1 (triangles) and run 3 (circles); earlier dates are represented in bright colors, later ones in dark colors; the black arrow indicates the direction of succession and separates run 1 from run 3.

4. Discussion

The constructed ATS systems are well-suited for algae cultivation and the offered substrate rapidly colonized, which could be shown by the high degree of coverage (70–90%) within one week after installation of the ATS. We found 22 to 33 taxa on the 4 replicates already 5 days after exposition, which means that natural seeding supported a highly diverse algal community. Diatoms dominated the community, regardless of the sampling date. More than 50% of the algae taxa belonged to Ochrophyta, which is consistent with work done by Adey et al. [52]. In the HSW (water supply for biofilms), diatoms reached their first maximum in June and July [10], which obviously promoted the high abundance of diatoms in the biofilms. HPLC results are in line with field estimations, digital estimation and microscopy, but the slightly increased pigment fraction accounting for Chrysophyceae was not confirmed by microscopy. Only one genus of this class was detected by microscopy. Possible reasons for this discrepancy are destruction of Chrysophyte cells during sampling or overestimation of this group by Chemtax. A weak correlation between microscopy and Chemtax results for Chrysophytes was also observed in a shallow eutrophic lake in Estonia [53]. Similar to our study, the authors explained the poor agreement in case of Chrysophytes by low counting precision of this minority group and errors in quantification of minor pigments. Proportions of diatoms and green algae varied between the three runs, which can be attributed to a strong impact of season and succession. Shortly after start of run 1, diatoms dominated the community, which is in accordance to Chen et al. [54] and D'Aiuto et al. [17]. Also in brackish waters, diatoms prevailed during the experiments [55]. In agreement to other studies [17,54], filamentous green algae gained in importance in the following weeks, without losing their dominance. The slight decrease of green algae at the end of the first run was caused by a recovery of diatoms, forming colonies and growing as epiphytes on green algae filaments, a pattern which was not

observed in runs 2 and 3. The second run differed because of faster re-colonization of the ATSs by green algae at the beginning. Run 2 was started directly after harvest and cleaning of run 1 and remaining basal algal elements, attachments or holdfasts from the first run accelerated re-colonization, as observed also in previous studies [16]. Addressing the fauna on the ATS, our observations imply that the algal community on the ATS was not strongly affected by grazing and therefore not top-down controlled. Similar results were documented by Adey et al. [16], who described a more diverse macro-invertebrate community but found no significant effects on the algal productivity by herbivores.

At the onset of each run, high numbers of single-celled algal taxa were recorded, but with increasing age of the biofilm, slow-growing filamentous green algae and other colony forming algae became more abundant. The same pattern was found by D'Aiuto et al. [17], who worked with waste water of a citrus farm in Florida. Even in brackish to salty waters, filamentous and thallus-sheet like green algae were dominant [56]. In the course of succession, a change in vertical community structure from low to high physical stature occurs, leading to competition for substrate surface [57]. The enhanced 3-dimensional structure provided by filamentous algae like *Cladophora glomerata* (L.) Kützing can act as substrate for other organisms [58], enlarging the surface for colonization. A larger surface can lead to raised biomass productivity because of enhanced diatom retention on the ATS, as shown by Adey et al. [52], who applied an artificial 3-dimensional substrate structure extending from a 2-dimensional screen. One drawback of naturally grown 3-dimensional mats might be reduced growth of the colonized algae, followed by lower productivity. Raised structural complexity of diatom and green algal colonies was well documented by the photos taken for micromapping (Figure 3). Chl-a loss was detectable after the third to fifth week and pointed at a beginning detachment of senescent biofilm. Raised density of the algal mats leads to reduced resistance against turbulences, resulting in detachment of the biofilm from the substrate [59]. The detachment of algal mats is based on autogenic succession [60]. It occurs when interspecific competition of algae causes changes in their environment, leading to favorable or inhibiting conditions for other algal species. According to McCormick & Stevenson [61], autogenic factors outweigh seasonal effects in short time processes, which means that autogenic succession was very important on the ATSs within each run. Larson et al. [62] suggest that succession in algal biofilms is largely influenced by environmental or abiotic factors. The seasonal impact on the ATS was not as substantial as autogenic factors in short term, but the recorded change of species composition, abundance and coverage of the ATSs between the three runs showed that seasonal succession had a strong impact in the long term. According to the NMS results, mainly two processes influenced the community development on the ATSs. First, run 1 and 3 are separated, which means that the season played a key role for the community composition (allogenic factors). Second, earlier dates are separated from later ones, indicating that the algal community changed because of the on-going succession (autogenic succession).

Seasonal changes led to varying conditions on the ATS, which probably contributed to increased biodiversity. This in turn promoted enhanced nutrient removal [63]. Comparable patterns were found by Chen et al. [54], who found highest biodiversity during the summer months. In our case the irregular water supply probably had a positive effect on biodiversity on the ATS, but the high biodiversity recorded was also a result of the biofilm filtering effect. Planktonic taxa originating from the HSW were transferred through the pipes onto the biofilm and finally retained. This

observation is underlined by pictures taken with the help of the stereo microscope, showing colonies of *Microcystis* sp. trapped in filamentous colonies of diatoms (Figure 10).

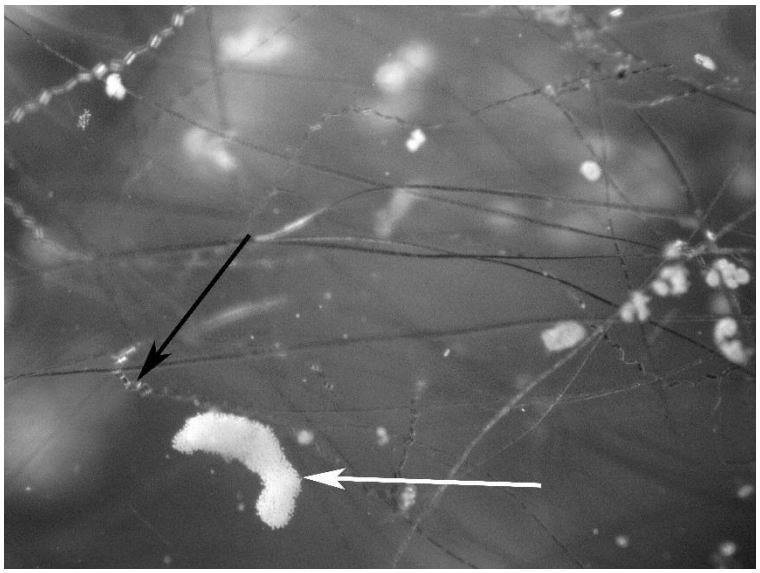

Figure 10. Stereomicroscopic photo of the biofilm, showing colonies of *Microcystis* sp. (white arrow) trapped in filamentous colonies of *Fragilaria* sp. (black arrow).

For describing the biodiversity, the H_s was calculated for diatoms. Based on H_s and E_s, the algae community of run 3 was more diverse and more evenly distributed compared to run 1. These findings are in accordance with increased species richness (Figure 6) and clearly showed that the biofilms became more diverse over time. The calculated evenness E_s for run 1 suggested a stronger dominance of single species compared to run 3, where a more uniform distribution was found. The decline of H_s during the third week of run 3 is explained by a change in algal composition, induced by the amplified growth of filamentous green algae. The enlarged habitat structure (three-dimensional) combined with more substrate types offered facilitated a second increase in richness, as has been already described for bacterial communities [64]. A change of dominance from diatoms to filamentous green algae was also observed in the first run. This shift occurred probably delayed on each ATS replicate, resulting in a high standard deviation of H_s. The TO was highest during the initial colonization of the ATS, followed by a decrease when the established biofilm community started to compete for resources. A lower TO towards the end of the first run indicated major changes in community composition due to aging of the biofilm. TO was lowest at the end of the third run, showing that stability and balance of the algal community increased during the season. One explanation for this could be the increased complexity and heterogeneity within the developing biofilm and a diversified species pool, which makes community dynamics become a composite of differential responses [65].

An important aspect of water supply was the use of tipping buckets, which had several advantages. First, sedimentation of particles and algal cells was enhanced because of pulsed water supply (sedimentation of cells between two tipping events). Second, the flushes of water lead to an optimized nutrient supply by minimizing the boundary layers around algal cells, which otherwise

would limit nutrient and metabolite exchange [11]. Third, pulsed water supply improved irradiance, because particles, entering the system via the water supply, were periodically removed.

One component of the ATS that proved to be essential in our study was the 2 cm high barrier at the outlet of the ATSs. It prevented desiccation of the biofilm, because performance of the Neptunanlage^{TM} was regulated automatically. Depending on water temperature of the HSW, chl-a and particulate organic matter content of the water column [10], the mean flow rate ranged between 16 and 41 L min^{-1} during the observation period (Figure 11).

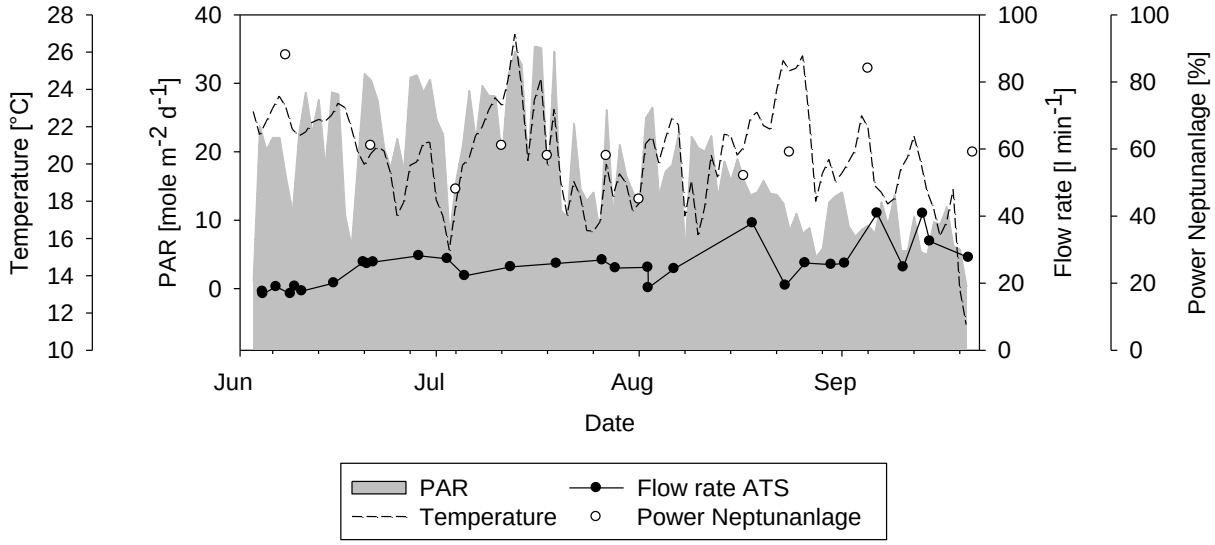

Figure 11. Abiotic parameters (PAR, air temperature above the algae turf scrubbers^{TM} (ATS) and flow rate of the water supply; mean values, n = 4) and performance of the Neptunanlage^{TM} during the observation period [10].

For optimizing productivity, flow rate and wave surge frequency should be regulated according to the nutrient supply [66]. An intermediate wave surge frequency of 17 min^{-1} and raised volumetric flow rate of 95 L min^{-1} can for example result in increased productivity [66]. These values are high compared to our study (mean wave surge frequency 2.71 min^{-1}; mean volumetric flow rate 25.23 L min^{-1}), which means that an optimized flow regime could further enhance productivity and phosphorus removal. The maximal areal removal rate of this study (Table 2) is comparable to phosphorus removal rates of ATS operated by Mulbry et al. [21] (7–45 mg m^{-2} d^{-2}), but removal rates of other studies were higher, most likely due to a higher nutrient load of the water supply [16,20,56]. In our study, enhanced growth of green filamentous algae at the inflow was recognized, which can be attributed to their higher resistance against current velocities. The same pattern along the flow lane was found by D'Aiuto et al. [17], who recognized upstream a filamentous algal matrix. Also in terms of biomass, Chlorophyta dominated in the inflow region [17].

Table 2. Maxima of the biotic parameters on the ATS^{TM} (mean values with standard deviations, n = 4), adapted from Mayr et al. [18].

Parameter	Run 1	Run 2	Run 3
Total phosphorus (mg m^{-2})	261.5 ± 49.9	441.8 ± 69.6	224.0 ± 18.0
Areal removal rate of total phosphorus (mg m^{-2} d^{-2})	9.7 ± 2.6	19.1 ± 2.5	10.6 ± 0.3
Dry mass (g m^{-2})	251.9 ± 44.4	221.0 ± 23.5	249.7 ± 42.7
Ash free dry mass (g m^{-2})	52.0 ± 10.7	68.9 ± 7.0	62.6 ± 5.12
Ash content (g m^{-2})	199.8 ± 34.3	152.2 ± 37.0	187.0 ± 48.3
Chl-a (g m^{-2})	355.1 ± 35.2	700.5 ± 141.1	399.2 ± 132.9

5. Conclusion

The present study suggests that the algal community on ATSs strongly depends on autogenic succession within a few weeks after colonization. In contrast, seasonal impacts shape the community in a time period of several months. For an extended use of the system in temperate regions like Austria, further investigations throughout the growing season from spring to autumn are necessary to assess practicability. At lower latitudes, the ATS technology is also promising during winter [16]. Our results show that harvesting intervals between 3 to 4 weeks are advantageous to prevent detachment and loss of algal biomass in summer. Moreover, the harvesting date is important for the composition of the algal biomass, because the species abundance changes over time due to succession. If required for further applications, biomass composition of biofilms can be modified by altering growth conditions. A higher flow velocity for example could enhance the quantity of green filamentous algae and a different slope of the flow lanes or other kinds of substrate would change the algal community. We were able to show that natural seeding of the ATS is suitable to ensure high species diversity, which probably accelerates the cleaning efficiency of the ATS. Additionally, the natural filtering effect of the algal mats promoted particle retention, which was not achieved by hitherto applied restoration measures. In addition to this study, nutrient analyses of the algal biofilm were carried out [18], and showed that ATS-technology has a great potential to reduce nutrients and to produce algal biomass in a cost-effective and environmental-friendly way. For further improvements, we suggest the application of serial arrangements of ATSs to get higher cleaning efficiencies and investigation of vertical systems as space-saving alternative.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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Supplementary

Text S1. Description of picture processing with Photoshop CS6.
Table S1. List of all taxa found in the biofilm growing on all four algae turf scrubbers from June to September 2011.
Video S1. Operation of the ATS and movement of the tipping bucket in the field.

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