arthropoda

MDPI

Article

Survival and Growth of *Asellus aquaticus* on Different Food Sources from Drinking Water Distribution Systems

Nikki van Bel [1,*], J. Hein M. van Lieverloo [2,3], Antonie M. Verschoor [1], Leonie Pap-Veldhuizen [1], Wim A. M. Hijnen [1,3], Edwin T. H. M. Peeters [4] and Julia Wunderer [3]

[1] KWR Water Research Institute, Groningenhaven 7, 3433 PE Nieuwegein, The Netherlands; anthony.verschoor@wur.nl (A.M.V.); leonie.pap@kwrwater.nl (L.P.-V.)
[2] Viaeterna, Rijksweg 46, 6581 EN Malden, The Netherlands; hein.van.lieverloo@viaeterna.nl
[3] Evides Water Company, Schaardijk 150, 3063 NH Rotterdam, The Netherlands
[4] Aquatic Ecology and Water Quality Management, Wageningen University, Droevendaalsesteeg 3a, 6708 PB Wageningen, The Netherlands; edwin.peeters@wur.nl
* Correspondence: nikki.van.bel@kwrwater.nl; Tel.: +31-30-606-9516

Abstract: Invertebrates, including *Asellidae*, are part of the natural ecosystem of the drinking water distribution system (DWDS) and are known to cause a nuisance to consumers. In addition, recently, the potential role of the species *Asellus aquaticus* (L. 1758) in the regrowth of *Aeromonas* bacteria was published. *Aeromonas* is included in the Dutch drinking water guidelines as a process parameter, and the guideline values are regularly exceeded. Although neither *A. aquaticus* nor *Aeromonas* is associated with health risks, the Evides drinking water utility shows a strong interest in the possible reasons for these exceedances and possible control measures. In surface waters, *Asellidae* feed mainly on decaying leaves that are abundantly present. These food sources are not present in the DWDS. Therefore, we determined suitable food sources for *A. aquaticus* in the DWDS. Laboratory experiments show that *A. aquaticus* individuals survive on biofilm on pipe wall material and loose deposits (sediments) collected from DWDS. Growth and survival rates on these loose deposits were even higher than on the positive control (decaying leaves). As the basis of these loose deposits is inorganic (iron deposits, sand, and pipe particles), the organic matter (living and decaying bacteria, protozoans, fungi, and invertebrates) must be their substrate. These experiments validate hypotheses that *Asellidae* can grow and survive on organic matter in deposits in DWDS.

Keywords: loose deposits; natural organic matter; survival analysis; growth rate; *Asellus aquaticus*; drinking water distribution

Citation: van Bel, N.; van Lieverloo, J.H.M.; Verschoor, A.M.; Pap-Veldhuizen, L.; Hijnen, W.A.M.; Peeters, E.T.H.M.; Wunderer, J. Survival and Growth of *Asellus aquaticus* on Different Food Sources from Drinking Water Distribution Systems. *Arthropoda* **2024**, *2*, 192–211. https://doi.org/10.3390/arthropoda2030015

Academic Editor: Sammy De Grave

Received: 25 April 2024
Revised: 13 June 2024
Accepted: 3 July 2024
Published: 2 August 2024

Correction Statement: This article has been republished with a minor change. The change does not affect the scientific content of the article and further details are available within the backmatter of the website version of this article.

1. Introduction

Freshwater lice of the family *Asellidae* are isopods widely spread in a variety of aquatic habitats such as lakes, streams, pools, and even cave waters across Europe, North America, and parts of Asia [1–3]. *Asellidae* species of the genus *Asellus* and *Proasellus* are also common members of the invertebrate communities in oligotrophic drinking water distribution systems (DWDS) worldwide. They occur in both DWDS with and without a (chlorine) disinfectant residual [4]. Early reports showed that already in the 1880s, they colonized the DWDS in Germany and the Netherlands [4,5], and they contributed substantially to the total biomass of the DWDS [4,6].

The presence of invertebrates in the DWDS is seen by drinking water companies as a nuisance that causes water quality problems, and the presence of *Asellus aquaticus* L. in the DWDS has been known to cause some consumer complaints, mainly when their fecal pellets were found in tap water or when dead specimens clogged water meters [4,7]. In addition, a large number of *A. aquaticus* in the DWDS coincide with discoloration of the water and visible fecal pellets, and the consumers' trust in drinking water may be affected by this. Despite these problems, invertebrates in drinking water are not known to cause

health issues, but drinking water utilities often prefer to control *A. aquaticus* numbers. The influence of *A. aquaticus* biomass on the microbial drinking water quality in filters in water meters was first published by Gunkel et al. [6]. Recently, the potential role of *A. aquaticus* in the regrowth of *Aeromonas* and heterotrophic plate count (heterotrophic bacteria cultured on nutrient-rich agar plates) in the DWDS was described [7,8], and *Aeromonas* was also found in invertebrates, including *A. aquaticus* [9], emphasizing the relationship between the two. *Aeromonas* is included in the Dutch drinking water guidelines as a so-called process parameter to monitor biological stability [10]. As the Evides drinking water utility has a long-term history of *Aeromonas* exceedances in their non-chlorinated drinking water produced from eutrophic reservoir water, Evides has a strong interest in *Aeromonas* and the possible reasons for its exceedances. The *Aeromonas* exceedances coincided with other water quality and esthetical issues, such as the occurrence of coliforms, increased heterotrophic plate counts, and the presence of *A. aquaticus* and brown water. The niche of *Aeromonas* bacteria in the DWDS is the loose deposits, and they are present in relatively limited numbers in the water phase and biofilm on the pipe wall [11,12]. The presence and concentration of *Aeromonas* seem to be related to the abundance of *A. aquaticus* [7,8] and loose deposits in DWDS. Furthermore, previous studies showed a clear inverse relationship between invertebrate biomass and dissolved organic carbon content (DOC) and the biological stability of finished non-chlorinated drinking water [7,13,14] and indicated that the biopolymer concentration of the drinking water, iron, and *A. aquaticus* are important factors for *Aeromonas* regrowth [8]. Most of the drinking water studies on *A. aquaticus* are observational studies, and their correlation with DOC and the biological stability of the drinking water are not causal. Lower biological stability of the drinking water is often, but not always, linked to invertebrate biomass [7,13,14].

1.1. Distribution of A. aquaticus in DWDS

A. aquaticus can enter the DWDS via several routes, which may include structural deficiencies of clear water reservoirs and DWDS [15], entry from the surface water after pipe breaks or repairs [4,16], and because they pass through the drinking water production plant. For the growth and survival of *A. aquaticus* in the DWDS and propagation of the *A. aquaticus* population, *A. aquaticus* relies exclusively on sexual reproduction [3,4,17,18], and colonization of other (connected) DWDS parts and, therefore, strongly depends on the abilities for growth and reproduction in these systems. European surface water populations show that *A. aquaticus* usually has two complete reproduction cycles per year [19,20], but in the DWDS, this may even be three complete generations per year [21]. Studies showed marked differences between systems with high and low *A. aquaticus* densities and only a few systems with medium densities [13]. Incidentally detected specimens in systems with low densities seem to have migrated from adjacent connected systems but are not able to reproduce in these systems.

Flushing of DWDSs shows that around 88% of the total invertebrate biomass in most DWDS can be made up of *A. aquaticus* [13]. The success of *A. aquaticus* in DWDS can probably be attributed to a high reproduction rate of about 1 individual per m^2 pipe surface per month [22], the absence of predators (no fish, possibly some *Platyhelminthes*) [23], lack of competitors for available food and nutrient sources, resistance to high flow velocities [24] and many hiding places (couplings, hydrants, etc.) that can create niches with favorable conditions. These niches can also protect them against control measures such as flushing (due to the secluded nature of these niches) or chlorination, as the chlorine does not easily diffuse into these secluded areas [4].

Complete mechanical removal of *A. aquaticus* from DWDS has proven to be close to impossible. Removal of *A. aquaticus* by flushing of mains at a velocity of 1 m/s varies between 10% (living specimen) [24] to 84% (supposedly dead *A. aquaticus* specimen) [17]. Flushing with higher velocities hardly increased their removal and is not always possible in pipes with a bigger diameter [4]. Removal by flushing is limited as *Asellidae* can migrate

upstream in DWDS streams and withstand high flow velocities because the flow velocity just above the pipe surface is lower (due to rough surfaces) [4].

More effective control measures include flushing with CO_2 (sedative) at low velocities [6,24], foam or ice pigs, air, and dosage of the biocide permethrin [4]. A long-term study showed that *A. aquaticus* rapidly recolonized the DWDS after the control measure, and the numbers returned to pre-cleaning conditions within a year [13], and only frequent and systematic (costly) flushing programs led to consistently lower *A. aquaticus* numbers after 5 to 7 years [4,25]. Mechanical control of the *A. aquaticus* population in a DWDS is thus mainly possible by intensive and costly flushing programs. Another possibility may be to influence the available food sources for *A. aquaticus* and, therefore, limit their growth and reproduction.

1.2. Food Sources for A. aquaticus

In surface water, *A. aquaticus* primarily feeds on abundantly available (decaying) plant material, algae, periphyton, and detritus [26,27]. Both the caeca (midgut gland) and the hindgut are known to harbor a number of bacterial endosymbionts that produce digestive enzymes and, therefore, enable *A. aquaticus* to digest detritus plant material which, among others, contains lignocellulose [28–30]. The ability to live on nutritionally low-quality leaf litter is believed to be one of the key factors for the successful colonization of freshwater habitats by *A. aquaticus* [31,32].

Also, bacteria and fungi seem to be an essential part of *A. aquaticus*' diet [33–35], and microbial analysis of fecal pellets from *A. aquaticus* showed the presence of a significant amount of fungal material from the two fungi *Alternaria* and *Fusarium* [36]. On the other hand, in caves in Hungary filled with hypogene cave water (originating from the underground), *A. aquaticus* was found to rely on endogenous, chemoautotrophic mat-forming bacteria [37,38], which supports the hypothesis that biofilms on materials, sediments, and fecal pellets form the basis food source in the invertebrate biocoenosis in DWDS [4,13].

Moreover, also scavenging on dead individuals of *A. aquaticus*, as well as cannibalism by predation on living *Asellidae* has been reported [26]. The feeding pattern in juvenile *A. aquaticus* is different from that of adult specimens. Juveniles, but not adults, need to graze on the fecal pellets of adults in the first 25 days [39], doubling the growth rate compared to the provision of just decaying leaves [36]. It is possible, although not reported in the literature, that the fecal pellets provide the juveniles with symbionts to colonize their digestive tracts [26]. Therefore, *A. aquaticus* can be seen as a real omnivore that is highly adaptive to its habitat; however, not all food sources provide the same nutritional value, and this will highly influence its growth.

Food sources for *A. aquaticus* in DWDSs will be different from surface waters, as plant materials are not present, but biofilm is present on all materials of the DWDS. Only a few possible food sources are available in the DWDS: (biofilm on) loose deposits, biofilm on the pipe wall, and compounds in the drinking water itself. The loose deposits are a mixture of organic, such as bacteria, protozoa, fungi, dead invertebrates, and fecal pellets, and inorganic material, such as iron and manganese flocs, sand particles, and other materials that are used in a DWDS. The color of fecal pellets changes depending on the ingested food and can, therefore, help unravel the question of the main food sources in DWDS. Microscopical analysis of fecal pellets collected in DWDS reveals clear colored bands in some pellets, but many pellets are uniform in color, suggesting (but unconfirmed by chemical analyses) the food source: orange (mainly rust), brown (flocs of iron and manganese deposits), beige (asbestos-cement), black (bitumen when shiny, possibly active carbon particles as well) (J. Hein M. van Lieverloo, pers. comms; [40]). Scanning electron microscopy has, however, confirmed the presence of asbestos fibers in a selection of pellets. These contents suggest either the ability of mouthparts to scrape relatively hard materials from surfaces or the ability to process and ingest relatively hard particles. Even though analysis of fecal pellets hints towards possible food sources, to date, little is known about the main food source of *A. aquaticus* in the oligotrophic drinking water environment. The

formation of biofilm on pipe walls and loose deposits is largely influenced by the DOC concentrations in the drinking water. DOC enters drinking water from the production location and can serve as a nutrient for bacteria growing in the water and on the biofilm of the pipe wall or loose deposits. In addition, organic and inorganic particulate matter enters the DWDS, and together with dead bacteria, it is part of the loose deposits in the DWDS. These loose deposits most likely serve as a food source for invertebrates, including *A. aquaticus*. If loose deposits and/or biofilm on the pipe wall are indeed food sources for *A. aquaticus*, then the (dissolved) organic carbon present in the finished drinking water (indirectly) is the substrate for the ecosystem in the DWDS, including invertebrates [13].

Several studies confirm the important role of sediment for *A. aquaticus* in the DWDS. Results of a survey in DWDS of The Netherlands in 1993–1995 showed that sediment volumes correlate positively with the mean invertebrate biomass [13]. In Denmark, living *A. aquaticus* was only found in samples with more than 100 mL/m^3 loose deposit particles, but no correlation was found with the sediment volume [41]. Sediments from DWDS in France were found to be covered with a biofilm consisting of bacteria and fungi [42]. By ingesting the loose deposits (including small invertebrates), *A. aquaticus* also ingests these biofilms and most likely uses a large part of these organisms as a food source. Some of these organisms might also function as symbionts for digesting dead and living substrates [28]. Next to (biofilm on) loose deposits, biofilm growing on pipe walls could be another potential food source for *A. aquaticus* in DWDS. A third possible but less likely food source could be the dissolved organic matter itself, although, to date, it is not known if *A. aquaticus* is able to feed on this directly.

1.3. Study Goal

The goal of this study was to determine suitable food sources for *A. aquaticus* in the oligotrophic drinking water environment of the DWDS. We performed laboratory growth and survival experiments with *A. aquaticus* on food sources available in the DWDS, namely (a) loose deposits, (b) dissolved organic matter, (c) biofilm on pipe material, and preconditioned leaves that acted as a reference. The sediment, biofilm, and dissolved organic matter were taken from two different DWDSs that differed in the number of *A. aquaticus* found in the DWDS. We hypothesized that the differences in *A. aquaticus* number in the two DWDS are caused by differences in the available food sources or the nutritional value of the food sources.

2. Materials and Methods

2.1. Experimental Design

Four experiments were performed at different moments in time that ran for 16 weeks (Experiments A–C) or 6 weeks (experiment D). In Experiments A–C, 54 specimens were used per condition, allowing the detection (using a repeated measures analysis of variance (ANOVA)) of an effect size of 0.4 standard deviations of the mean length after 16 weeks, with a power of 0.8 and an alpha of 0.05. This choice was based on the observed effect size of 0.57–0.58 standard deviations of the mean length after 8 to 16 weeks, respectively, in preliminary experiments.

Three possible food sources for *A. aquaticus* in a DWDS were tested: water, (biofilm on) sediment, and biofilm on the pipe wall. Decaying *Acer pseudoplatanus* leaves (10 × 20 mm) were tested as a positive control. Water, without any other food source, was used as a negative control. As no effect of water type from TP2 and 3 on survival was detected in Experiment A, drinking water from DWDS4 was used in Experiments B–D for practical reasons. HF concentrate, prepared with drinking water, was tested to assess the capability of *A. aquaticus* to feed on suspended particle-associated substrates. The conditions and denominations of each experiment are described below and summarized in Table 1. In each experiment, pretreated *Acer pseudoplatanus* leaf (10 × 20 mm) was included as a positive control.

Table 1. Water and food sources for *A. aquaticus* growth and survival experiments. X: Conditions that belong to the experiment and the 'Code Used in Paper' column.

	Code Used in Paper	Water Source	Food Source and Origin									
			None	PE Pipe without Biofilm	Preconditioned Leaf	Sediment		HF Concentrate		Cultured Biofilm on PE Pipe		Natural Biofilm on PVC Pipe
						TP2	TP3	TP2	TP3	TP2	TP3	TP3
Experiment A (16 weeks, N = 54, growth and survival)	A_Water_2	TP2	X									
	A_Water_3	TP3	X									
	A_Leaf	TP3			X							
	A_Sed_2	TP2				X						
	A_Sed_3	TP3					X					
Experiment B (16 weeks, N = 54, growth and survival)	B_Water_4	DWDS4	X									
	B_Leaf	DWDS4			X							
	B_Sed_2	DWDS4				X						
	B_Sed_3	DWDS4					X					
	B_HF_2	DWDS4						X				
	B_HF_3	DWDS4							X			
Experiment C (16 weeks, N = 54, growth and survival)	C_PE	DWDS4		X								
	C_Leaf	DWDS4			X							
	C_BFC_2	DWDS4								X		
	C_BFC_3	DWDS4									X	
Experiment D (6 weeks, N = 54, survival)	D_PE	DWDS4		X								
	D_Leaf	DWDS4			X							
	D_BFC_3	DWDS4									X	
	D_BFN_3	DWDS4										X

In Experiment A, drinking water (10 mL per well in a 6-well plate) and sediment (10 mL water + 400 µL sediment per well in a 6-well plate) from different treatment plants and DWDS were compared (Table 1). The 6-well plates were incubated in the dark at room temperature (19–20 °C). Every week, the animals were transferred to new 6-well plates with fresh water and sediment or leaf, depending on the condition.

In Experiment B, drinking water (10 mL drinking water per well in a 6-well plate) was supplemented with concentrated drinking water containing particulate-organic carbon (HF concentrate, 115 µL) or sediment (400 µL). Incubation and addition of fresh water and food sources were similar to Experiment A (Table 1).

In Experiment C, biofilm cultured in drinking water from two different treatment plants was tested. Drinking water (10 mL of DWDS4 per well in a 6-well plate) was supplemented with PE material with biofilm cultured in water of TP2 or TP3. Incubation and addition of fresh water and food sources were similar to Experiment A (Table 1).

In Experiment D, cultured biofilm was compared to biofilm present on a drinking water pipe that was excavated from the distribution system ('natural biofilm'). Drinking water (18 mL water of DWDS4 in a small Petri dish) was supplemented with PVC material with natural biofilm from DWDS3 or PE material with biofilm cultured in water of TP3 (Table 1). As this experiment was performed only to study survival, the Petri dishes were incubated for a shorter period (42 days). Every week, the animals were transferred to new Petri dishes with fresh water and—depending on the selected condition—food.

2.2. Origin and Culture System of A. aquaticus Test Organism

Growth and survival experiments were performed with *A. aquaticus*. All individuals used, except for Experiment A, were grown in a culture system. For Experiment A, the animals were ordered from a private supplier in Germany. The culture system consisted of several plastic containers with drinking water, *Acer pseudoplatanus* leaves pretreated as described below, sediment, and *A. aquaticus*. The latter two were flushed from several unchlorinated DWDS of Evides drinking water company (Rotterdam, The Netherlands). The plastic containers were kept in the dark at room temperature (19–20 °C). Every 2–3 weeks, sediment (thawed from a −80 °C freezer as described below) and leaves were added. Water was added once per week or every two weeks to replace evaporated water. For the start of each experiment, small *A. aquaticus* (Experiment A: 3–6 mm, Experiments B–C: 2.5–4.5 mm, Experiment D: not measured; Supplementary Figure S1A) were selected to allow for growth to the expected maximum length of approximately 10 mm. After Experiment A, care was taken to select a smaller *A. aquaticus* specimen at Day 0, as correlation analysis showed that a higher length was correlated with a lower growth rate, although the r^2 of the correlation was low (<0.29; Supplementary Figure S1B).

Individual specimens of *A. aquaticus* were carefully moved from the culture system to a small container with water to remove any attached sediment and water. From here, the *A. aquaticus* specimen was moved to a well in a 6-well plate (Greiner, Experiments A–C; Supplementary Figure S3A) or 6 cm diameter Petri dish (Greiner, Experiment D; Supplementary Figure S3B) in which the survival and growth experiment was performed.

2.3. Treatments: Water and Food Sources

A. aquaticus were fed various food and water sources. Two treatment plants (TP2 and TP3) were selected that differ in the number of *A. aquaticus* that are found in the DWDS (*A. aquaticus* biomass: respectively, 18 mg/m^3 and 0 mg/m^3) [7]. Both TP produce drinking water from the same surface water source [43]. They have largely comparable treatment steps to produce drinking water, and the water quality and biological stability (nutrients in drinking water based on DOC, iron, and AOC-p17/nox values) of the produced drinking water, in general, were comparable during the experimental period. However, other parameters for biological stability show that the drinking water of TP2 is more biologically stable than the drinking water of TP3 [8]. It is hypothesized that the differences in

A. aquaticus numbers in the DWDS are caused by differences in the available food sources in the drinking water or in the biofilm and loose deposits accumulated in the DWDS.

Unchlorinated drinking water was sampled from TP2 and TP3. In TP2 and TP3, chlorine dioxide was added to the produced drinking water before it entered the clear water reservoirs (but is absent in the distributed drinking water as the dosed chlorine dioxide concentration and residence time in the reservoir are carefully balanced). To prevent any effect of a chlorine dioxide residual, water was sampled before the dosage of chlorine dioxide. Drinking water was also sampled from the tap in the distribution system of treatment plant 4 (DWDS4) of drinking water utility Vitens. This drinking water is produced from anaerobic groundwater and treated by anaerobic softening, aeration, and double-layer sand filtration. The water of TP2 and TP3 was sampled weekly in clean jerry cans, stored at 4 °C for a maximum of 24–48 h before it was used to refresh the water in the growth and survival experiments. Drinking water of TP4 was sampled directly before usage.

Loose deposits were flushed from several distribution mains of the drinking water distribution system from TP2 and TP3 and filtered through a 500 µm mesh filter followed by a 30 µm mesh filter. The sediment of the 30–500 µm fraction was used for the experiment. The volume of this fraction was quantified with an Imhoff funnel as previously described [17]. The loose deposits were mixed 1:1 (v/v) with water collected at the flushing site or with water from the corresponding treatment plant and stored at −80 °C until usage in the experiments. Flushing was performed in February 2019 (Experiment A: Rotterdam [TP3; two flushing locations] and Oostburg [TP2; three flushing locations], The Netherlands), December 2019, and January 2020 (Experiment B; Rotterdam, Capelle aan de IJssel and Delft [TP3; three flushing locations] and Oostburg [TP2; three flushing locations], The Netherlands).

Drinking water from TP2 and TP3 was concentrated using the Hemoflow method (mobile crossflow ultrafiltration system) as described previously [44]. During concentration of the drinking water (concentration factor 971× [TP3]) and 1257× [TP2]), different fractions of particulate and/or high molecular organic carbon (PHMOC, including biopolymers) were concentrated in the drinking water, and the concentrations were determined [45]. The addition of the Hemoflow-concentrate (HF concentrate) to the tested drinking water did not result in a visible suspension or precipitates. This fraction is abbreviated to 'HF concentrate' in this study.

Leaves of *Acer pseudoplatanus* that were used for all experiments were collected in Nieuwegein (The Netherlands) at the same moment and dried and stored at room temperature for several days. Before addition to the culture system or usage in Experiments A–D, the leaves were softened and allowed to decay for two weeks in a mixture of drinking water from DWDS4 with 10% v/v surface water from the Lek canal (The Netherlands) as described in the literature [33,34]. Next, leaves were rinsed under a soft flow of tap water to remove loose biofilm and deposits before addition to the culture system. For usage in Experiments A–D, the pretreated leaves were cut (10 × 20 mm).

PE material was sandblasted to create a large surface and to roughen it. Small coupons were cut (1.6 × 3.5 × 0.1 cm, surface area for biofilm formation: 12.22 cm^2), and after flushing with drinking water, 12 PE coupons (146.64 cm^2) were incubated in 900 mL drinking water of TP2 or TP3 for eight weeks at 30 °C to create a biofilm. The water was refreshed weekly. The drinking water used for biofilm formation was sampled in sterile stainless-steel canisters (rinsed in MQ water, autoclaved, and dried at 50 °C overnight) and stored at 4 °C for 24–48 h. To prevent the PE coupons from floating to the surface during biofilm growth, the PE was slightly bent and put in sterilized stainless-steel tea balls during incubation (Supplementary Figure S2A). After eight weeks, the PE was carefully moved from the tea balls to the 6-well plate using sterile tweezers with which only the sides of the coupons were touched. Weekly ATP and surface measurements of 3–5 randomly chosen coupons were performed to determine the amount of biomass in the biofilm that was added to the *A. aquaticus* in Experiments C and D.

As a biofilm composition depends not only on the material and drinking water in which it is formed but also on the hydraulic conditions such as are present in pipes of the DWDS, a natural biofilm is assumed to contain less biofilm or biomass but does contain sediment particles compared to a cultured biofilm in the presence of drinking water. The response of *Asellidae* to two different types of biofilm was tested. In March 2021, a 160 mm PVC pipe was excavated from the distribution system of TP3, filled with the on-site drinking water, and stored at 4 °C until rings of approximately 15 mm were cut. Next, the rings were halved. The part which was originally the top part of the pipe was not used. The halve rings originating from the bottom part of the pipe were cut into four more or less evenly sized coupons. Due to the thickness of the pipe material (3.3 mm) and because special care was taken not to touch the inside of the pipe and disturb the biofilm, sizes of the PVC coupons varied (1.5 × 5 × 0.3 cm, surface area: 20.7 ± 1.2 cm^2; Supplementary Figure S2B). All coupons were stored in drinking water of TP3 at 4 °C until usage. Weekly, the ATP and surface of 3–5 randomly chosen PVC coupons were measured to determine the amount of biomass in the biofilm.

2.4. Analyses

The chemical and microbiological composition of the tested food sources was analyzed using different techniques. Sediment, drinking water, and HF concentrate of Experiments A and B were analyzed once at the start of the experiment, whereas the biofilm composition was analyzed twice (Experiment D, week 0 and 6) or three times (Experiment C, week 0, 8, and 16).

To analyze the biofilm, the biofilm was first released from the PE and PVC coupons by four consecutive sonification rounds (low energy sonification at 40 kHz for 2 min) in sterile drinking water, which was used for the microbiological and chemical analyses described below [46,47].

Adenosine triphosphate (ATP) is a measure of the amount of active biomass and is present in all microorganisms. The total ATP concentration was determined for all food sources as described earlier [48]. The numbers of total and membrane-intact cells were determined using a flow cytometer, as described previously [49]. Total and membrane-intact cells were distinguished using SYBR green and propidium iodide staining.

The Total Organic Carbon (TOC) concentration was determined according to ISO 8245. After acidification of the suspended biofilm sample, the organic carbon was measured using a non-dispersive infrared detector to remove the inorganic carbon. The protein concentration in the biofilm was determined using a Coomassie (Bradford) Protein Assay Kit. Albumin was used as a reference. The carbohydrate concentration in the biofilm was determined using a colorimetric method and spectrophotometry, as described previously [50]. Glucose was used as a reference molecule. Metal concentrations in the biofilm were determined using ICP-MS (Thermo Fisher Scientific XSeries 2). Samples for ICP-MS were conserved by acidifying below pH 2 using nitric acid (HNO_3, Suprapur 65 m%, CAS: 7697-37-2). XRF (X-ray fluorescence) spectrometry was performed to determine the metal concentration in the sediment. This analysis was outsourced to the Laboratory for Multi-Element Analyses (Raamsdonksveer, The Netherlands). The organic carbon composition was determined with LC-OCD (Liquid Chromatography–Organic Carbon Detection) [51]. This analysis was outsourced to DOC Labor GmbH (Karlsruhe, Germany). The terminology of DOC Labor for the various organic carbon fractions was used in this study. DOC does not accumulate during the Hemoflow concentration, whereas PHMOC does [45].

Survival and growth were monitored as follows. After one, two, and four days and during each transfer to a new 6-well plate or Petri dish, survival was determined. The length of each *A. aquaticus* specimen was determined at the start of the experiment and after 1, 2, 4, 8, 12, and 16 weeks (Experiment A) or after 4, 8, and 16 weeks (Experiments B and C). Length was defined as the distance between the distal ends of the cephalon and the pleotelson, excluding the extremities (Supplementary Figure S3C). After transferring a specimen to a new 6-well plate with water and before adding the food source, the 6-well

plate was placed on a mm-grid, and each specimen was photographed. The length of the specimen was analyzed using the Nuance Power PDF Advance software (Adobe Acrobat Pro 10.1.8). Sometimes, negative growth rates were measured. This is most likely caused by technical artifacts during measuring the specimen as the position of the *Asellus* (not horizontal in water, but [slightly] diagonal, or a slightly curved animal) will lead to an underestimation of the length and, thus, growth rate. These values, although including underestimations, were used for further (statistical) analysis. Survival was determined in Experiments A–D and growth in Experiments A–C (Table 1).

2.5. Statistical Analysis

The effect of experimental conditions on survival was analyzed using a parametric, multi-variable Cox Proportional Hazards (CPH [52]) model using the 'survival' package in R software [53]. In the CPH model, survival was compared to the type of water (TP2, TP3, DWDS4), type of food (only water, sediment, HF concentrate, cultured and natural biofilm, PE without biofilm, leaf), and Experiment (A–D).

2.6. Growth Rate

The growth rate was determined as the difference in length between the start of the experiment and Day 28 or earlier upon death of the *A. aquaticus* specimen. It was decided not to determine the growth rate for the entire length of the experiment (16 weeks) as many *A. aquaticus* die in between, therefore polluting the dataset. In addition, it was observed that growth mainly occurred in the first few weeks and leveled off afterward. The length difference was divided by the number of days and expressed as mm/day. As the dataset was not normally distributed, a non-parametric Kruskal–Wallis test with a post hoc Dunn test was performed to test for significant differences in growth rate between the conditions. The "dplyr" and "dunn.test" packages in R software were used to perform these tests.

3. Results

3.1. Growth and Survival of A. aquaticus on Water and Sediment

Survival of *A. aquaticus* was similar for the two drinking water types of TP2 and TP3 (A_Water_2 and A_Water_3; Figure 1A). In the absence of food, 83–89% of *A. aquaticus* were still alive after 16 days, and thereafter, survival decreased rapidly, and within 64–73 days, all *A. aquaticus* had died. Survival on leaves (A_Leaf) and sediment of DWDS2 (A_Sed_2) was higher compared to water without food (Figure 1A). The sediment of DWDS3 (A_Sed_3) led to a low survival rate. This was related to the coverage of the exoskeleton, antennae, and other body parts of *A. aquaticus* with orange/brown-colored, iron-rich sediment due to the chemical or physical properties of the loose deposits (Supplementary Figure S4). There was a large variation in growth within and between the different food sources (Figure 1B). For sediment of DWDS3, the growth rate could not be determined due to the low survival rate. The growth rate on leaves and sediment of DWDS2 were comparable. Both were significantly higher than water from TP2, which in turn was significantly lower than water from TP3. The growth rate showed a weak negative correlation with the length of *A. aquaticus* at the start, as large *A. aquaticus* (approximately > 4–5 mm) showed hardly any growth (Supplementary Figure S1B).

On the water of DWDS4 (B_Water_4; Figure 2A), survival was 11% at Day 36, and all *A. aquaticus* specimens were dead by Day 63. This was significantly worse compared to both sediment conditions (B_Sed_2 and B_Sed_3) and leaves (B_Leaf) but not significantly different from both HF-concentrates (B_HF_2 and B_HF_3). Survival on leaves was significantly higher compared to HF concentrate but lower compared to sediment of DWDS2. The higher survival on the sediment of DWDS3 in this experiment compared to the previous experiment (B_Sed_3 vs. A_Sed_3) was most likely caused by the difference in location from where the sediment was sampled. Sediment from the first experiment contained a higher iron concentration (9.29 µg/L; Supplementary Table S1) compared to this experiment (6.75 µg/L). Growth on water without food was low and differed significantly from growth

on leaves (Figure 2B), which corresponded to the differences in survival (Figure 2A). The significant difference in survival on leaves compared to the sediment of DWDS2 was not visible in the growth rate (Figure 2B; Kruskal–Wallis test with Dunn's test and Bonferroni post hoc test, $p > 0.05$). Growth on the sediment of DWDS 3 was limited and significantly lower than growth on the sediment of DWDS2, which corresponded to the differences in survival. In accordance with the low survival rate for HF concentration, no growth was observed in this food source.

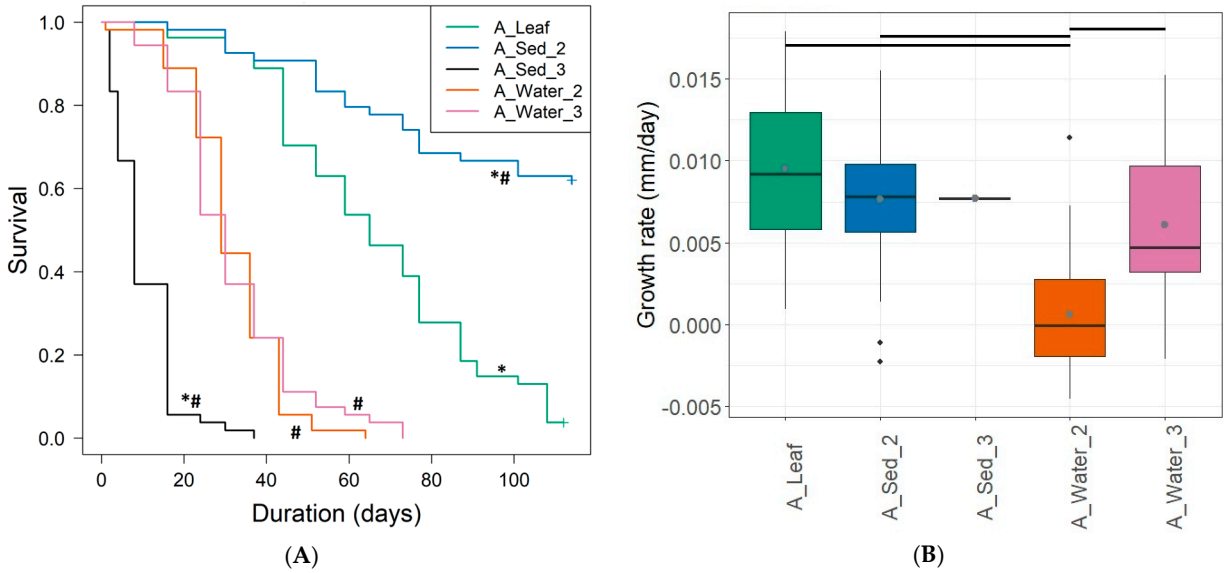

Figure 1. (**A**) Survival of *A. aquaticus* during 16 weeks (112 days) on different food sources in Experiment A: *Acer pseudoplatanus* leaves (A_Leaf), sediment from DWDS2 (A_Sed_2) and DWDS3 (A_Sed_3) and water from TP2 (A_Water_2) and TP3 (A_Water_3). * Survival of *A. aquaticus* is significantly different from A_Water_2 (*) or from A_Leaf (#) (Cox Proportional Hazards model, comparison-wise error: $p < 0.01$). (**B**) The growth rate of *A. aquaticus* between Days 0 and 28. The growth rate of A_Sed_3 was determined for only a few specimens due to the high mortality rate. Horizontal black bars indicate significantly different growth rates between the two conditions at which the bar starts or stops, e.g., A_Leaf vs. A_Water_2 (Kruskal–Wallis test with Dunn's test and Bonferroni post hoc test, $p < 0.05$).

The nutritional value of the drinking water and food sources are shown in Tables 2 and 3 (all data are shown in Supplementary Tables S1–S3). The ATP levels of drinking water of TP2, TP3, and DWDS4 were comparable, as were the ATP levels for the sediment of Experiment A (Experiment B was not analyzed). The three tested drinking waters showed relatively large differences in the organic carbon composition: water from DWDS2 and DWDS3 contained higher concentrations of DOC, biopolymers, LMW acids, and neutrals. These differences were not reflected in the survival of *A. aquaticus*. Differences were observed for various metals, but, except for the high iron content of sediment of DWDS3, they were considered not relevant, which will be discussed later. Drinking water with HF concentrate had higher concentrations of nearly all organic carbon compounds (DOC, CDOC, biopolymers, building blocks, LMW acids, LMW neutrals) compared to drinking water of DWDS4 without the addition of HF concentrate. However, the concentrations of both drinking water with HF concentration were lower than those of drinking water from TP2 and TP3. This corresponds to the survival rates, which are comparable between the drinking water with and without HF concentrate. Besides differences in iron concentrations, the sediment composition differed for various metals, but this could not be clearly linked to survival.

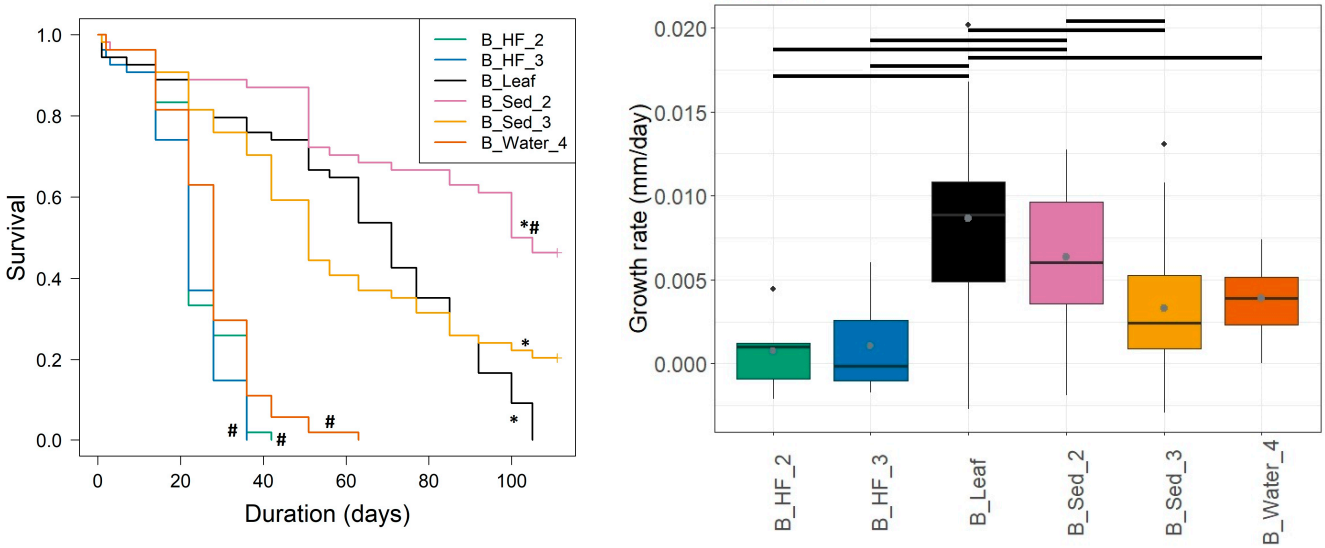

Figure 2. (**A**) Survival of *A. aquaticus* during 16 weeks (112 days) on different food sources in Experiment B: *Acer pseudoplatanus* leaves (B_Leaf), sediment from DWDS2 (B_Sed_2) and DWDS3 (B_Sed_3), water from DWDS4 (B_Water_4) and concentrate from DWDS2 (B_HF_2) and DWDS3 (B_HF_3). Survival of *A. aquaticus* is significantly different from B_Water_4 (*) or from B_Leaf (#) (Cox Proportional Hazards model, $p < 0.05$). (**B**) Growth rate of *A. aquaticus* between Days 0 and 28. Horizontal black bars indicate significantly different growth rates (Kruskal–Wallis test with Dunn's test and Bonferroni post hoc test, $p < 0.05$).

Table 2. ATP concentrations in drinking water and sediment, and organic carbon composition of drinking water of the different locations. ND: not determined.

		Drinking Water			Sediment	
		A_Water_2	**A_Water_3**	**B_Water_4**	**A_Sed_2**	**A_Sed_3**
ATP	(ng/L)	6.5 ± 4.0	3.1 ± 2.0	1–4	5.4×10^4	2.5×10^4
DOC	(mg C/L)	2189	2264	1769	ND	ND
CDOC	(mg C/L)	2008	2146	1563	2146	2146
Biopolymers	(mg C/L)	83	106	<1	106	106
Humic acids	(mg C/L)	1049	1158	1122	1158	1158
Building blocks	(mg C/L)	552	527	262	527	527
LMW Acids	(mg C/L)	16	31	<1	31	31
LMW Neutrals	(mg C/L)	308	325	179	325	325

Table 3. Characterization of cultured (C_BFC_2 and C_BFC_3) and natural (D_BFN_3) biofilm. Average and SD of ATP and flow cytometry measurements in weeks 0, 8, and 16 (Experiment C) or weeks 0 and 6 (Experiment D) with statistical differences (Kruskal–Wallis test with post hoc Dunn's Test en Bonferroni) between the three biofilms. X: statistical difference; -: no statistical difference.

	C_BFC_2	**C_BFC_3**	**D_BFN_3**	**C_BFC_2 vs. C_BFC_3**	**C_BFC_2 vs. D_BFN_3**	**C_BFC_3 vs. D_BFN_3**
Biofilm	**Cultured**		**Natural**			
Water	**TP2**	**TP3**	**DWDS2**			
ATP (pg/cm^2)	$4.7 \times 10^3 \pm 2.8 \times 10^3$	$4.2 \times 10^3 \pm 2.5 \times 10^3$	$5.5 \times 10^2 \pm 3.0 \times 10^2$	-	X	X
FCM, intact (cells/cm^2)	$2.14 \times 10^7 \pm 2.32 \times 10^7$	$1.49 \times 10^7 \pm 2.04 \times 10^7$	$2.21 \times 10^6 \pm 2.63 \times 10^6$	-	X	-
FCM, total (cells/cm^2)	$2.21 \times 10^7 \pm 2.29 \times 10^7$	$1.50 \times 10^7 \pm 2.03 \times 10^7$	$3.22 \times 10^6 \pm 2.11 \times 10^6$	-	-	-

3.2. Growth and Survival of *A. aquaticus* on Cultured and Natural Biofilm

Survival on PE pipe material without biofilm (C_PE) was significantly lower compared to the other conditions. All *A. aquaticus* specimens were dead by Day 49 (Figure 3A). Survival on leaves (C_Leaf) was best and differed significantly from the other conditions, but also, in this condition, none of the *A. aquaticus* survived after 107 days. Survival on biofilm grown in the two different drinking waters (C_BFC_2, C_BFC_3) was nearly identical. The differences in survival were also visible in the growth rate, as growth was only observed on leaves (Figure 3B), which was significantly higher than in the other three conditions.

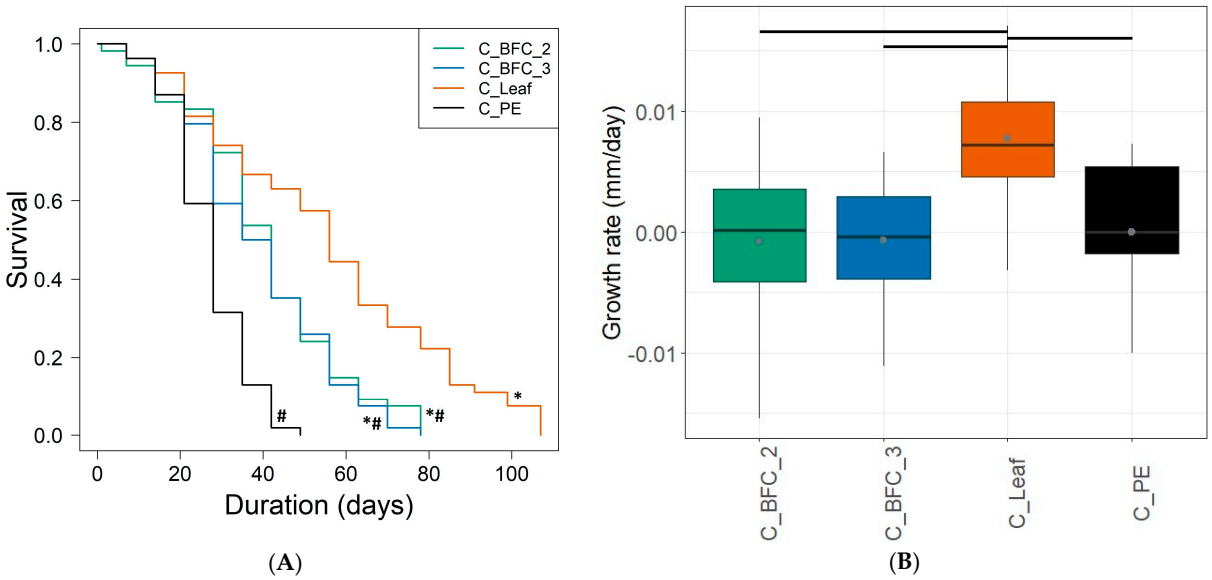

Figure 3. (**A**) Survival of *A. aquaticus* during 16 weeks (112 days) on different food sources in Experiment C: *Acer pseudoplatanus* leaves (C_Leaf), biofilm cultured in drinking water from TP2 (C_BFC_2) and TP3 (C_BFC_3) and PE without biofilm (C_PE). Survival of *A. aquaticus* is significantly different from C_PE (*) or from C_Leaf (#) (Cox Proportional Hazards model, comparison-wise error: $p < 0.0125$). (**B**) Growth rate of *A. aquaticus* between days 0 and 28. Horizontal black bars indicate significantly different growth rates (Kruskal–Wallis test with Dunn's test and Bonferroni post hoc test, $p < 0.05$).

Survival was comparable on PE without biofilm (D_PE), pretreated leaves (D_Leaf), or cultured biofilm (D_BFC_3, Figure 4). Survival in these three conditions was significantly higher than that in natural biofilm (D_BFN_3). The difference between the cultured and natural biofilm indicated that the nutritional value of a cultured and natural biofilm can affect *A. aquaticus* survival.

The ATP levels and numbers of total and intact cells in the two cultured biofilms were approximately ten times higher than in the natural biofilm (Table 3). This was a significant difference, except for the cultured and natural biofilm of TP3 (C_BFC_3) and DWDS3 (D_BFN_3). This difference in biomass in the biofilm matched the survival rates of *A. aquaticus*, with higher survival rates when more biofilm biomass was present.

The metal composition varied between the biofilms, but statistical comparison yielded very limited significant differences between the two cultured and the natural biofilms (Supplementary Tables S2 and S3). A significant difference was only found for lead, manganese, neodymium, rubidium, yttrium, zinc, and zirconium, which were all higher in the natural biofilm compared to the cultured biofilms.

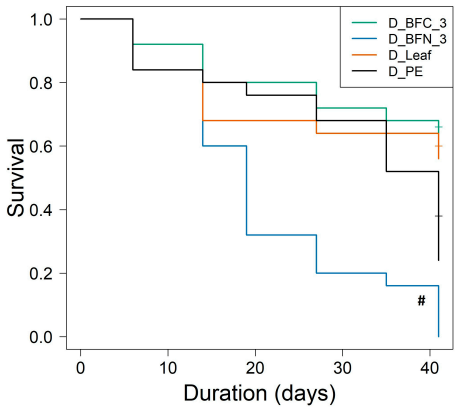

Figure 4. Survival of *A. aquaticus* during 7 weeks (42 days) on different food sources in Experiment D: *Acer pseudoplatanus* leaves (D_Leaf), biofilm cultured in drinking water from TP3 (D_BFC_3), natural biofilm on excavated PVC pipe (D_BFN_3) and PE without biofilm (D_PE). Survival of *A. aquaticus* is not significantly different from D_PE but does differ significantly from D_Leaf (#) (Cox Proportional Hazards model, comparison-wise error: $p < 0.0125$).

4. Discussion

This study tested whether *A. aquaticus* can grow and/or survive on the food sources available in a DWDS: sediment (or loose deposits), organic matter in drinking water, and biofilm on pipe material. The food sources were collected from two different DWDSs with different numbers of *A. aquaticus*. The hypothesis was that the differences in the composition and/or nutritional value of the food sources caused the difference in *A. aquaticus* occurrence in the two DWDS. This study showed that *A. aquaticus* can use sediment (or loose deposits) and biofilm on pipe material as a food source for survival and sometimes growth. As *A. aquaticus* can proliferate fast in a DWDS, the presence of both *A. aquaticus* and suitable food sources can lead to consumer complaints and affect the regrowth of *Aeromonas* and heterotrophic plate count (HPC22) in the drinking water.

As control measures, flushing and chlorination are not very effective, another option for control of *A. aquaticus* numbers in the DWDS can come from improving the biological stability of the drinking water and, therefore, reducing the supply of nutrients into the DWDS ecosystem, although this hypothesis must be proven. The total invertebrate biomass in DWDS was found to be best related to dissolved organic carbon in finished water [6,13,16]. Although it is assumed that *A. aquaticus* cannot directly consume DOC, the formation of biofilm and sediment is (partly) caused by bacteria that feed on DOC and are, in turn, most likely a food source for *A. aquaticus*. It is likely that improving the removal of DOC—and removal of inorganic substrates for bacteria (NH_4)—from finished water during drinking water production will diminish the biomass of invertebrates in DWDS. Next to dissolved organic matter, iron and specific hydraulic conditions in the distribution system are likely to play an important role in biomass formation in drinking water pipes [13,23,41]. These studies, for example, found higher abundance in cast-iron mains than in PVC mains, which in some cases was coupled to higher volumes of loose deposits in the cast-iron DWDS. Also, higher abundance was found in DWDSs, which mainly had PVC mains and more biopolymer and iron accumulation [8]. Characterization and understanding of the DWDS ecosystem offer the opportunity to understand the role of *A. aquaticus*, assist in water-quality management, and therefore lead to long-term control.

4.1. Nutritional Composition Food Sources

The survival and growth of *Asellus* could not be related to microbial and chemical characterization of the various food sources. The only exception is the significant difference in ATP and cell numbers. Although for drinking water standards, the differences between the three drinking waters were sometimes relatively large, the concentrations of the different compounds were well within the tolerance levels of *A. aquaticus* [54–57]. These differences

in drinking water quality are thus not expected to affect survival and thus explain the lack of correlation between the composition of the food source and survival. Larger variations in the chemical composition are likely needed before an effect on survival can be expected. Interestingly, regardless of the differences between drinking water and drinking water supplemented with HF concentrate, survival on HF concentrate was comparable to drinking water alone. The HF concentrate consists of drinking water with concentrated particulate high molecular organic carbon, including DOC and biopolymers. This suggests that the biopolymers, which were identified as one of the differences in drinking water quality between the DWDSs with and without *A. aquaticus* present, is not a direct food source for these invertebrates. This finding does not reject the hypothesis of a previous study [8] that the accumulation of biopolymers in the DWDS, in combination with iron, indirectly affects the absence or presence of *A. aquaticus*. To gain more insight into the effect of the nutritional composition of the food sources on survival, targeted experiments with only one or a few changes in the food sources are required. These studies have been done before and showed that, e.g., *A. aquaticus* can process fungi and algae [27,35] and prefer the fungus *Cladosporium herbarum* over the bacteria *Marmoricola* spp. and *Aquabacterium commune* [58], showing that bacteria in a biofilm can serve as a food source.

4.2. Growth and Survival A. aquaticus

In total, four different growth and survival experiments were performed. The results of the negative controls of these four experiments (water without addition in Experiments A and B and PE material without biofilm for Experiments C and D) and the results of the positive controls (preconditioned leaf) did not differ significantly from each other ($p > 0.05$; Table 4). This good reproducibility between the survival experiments led to our confidence that a comparison of survival on the food sources that were tested in different experiments could be performed. Whereas in the full-scale situation, more *A. aquaticus* are present in DWDS3, and only a few specimens in DWDS2 [7,23]; this was opposite to the results of the survival experiments and also different from the similar survival on the different cultured biofilms. Sediment and biofilm of both locations could sustain *A. aquaticus*, although differences were observed in survival on sediment of DWDS2 and DWDS3. For biofilm cultured in drinking water from two locations, the location was not a discriminating factor. As the basis of sediment is inorganic (rust, iron deposits, sand, and decayed pipe particles), the organic matter in the sediments (living and decaying bacteria, protozoans, fungi, and invertebrates) must be the food source.

The shorter survival of a natural biofilm compared to cultured biofilm could be explained by the fact that 10–12× less biomass was added to natural biofilm than to cultured biofilm (Table 5). However, despite the fact that 2.3–5× more biomass was added as a cultured biofilm than sediment in Experiment A, survival on sediment in Experiment A was better. This confirms that both the nutritional value and the accessibility of the food source are important. From the lower survival on biofilm than sediment (except for the rapid death of A_Sed_3; Figure 5, Table 4), the following conclusions can be drawn: (i) *A. aquaticus* can survive on cultured and natural biofilm, (ii) both biofilm and sediment show better survival than the negative control and (iii) the amount of biomass (in ATP; $5.5 \times 10^2 \pm 3.0 \times 10^2$ pg/cm^2, Table 3) of the natural biofilm is comparable to, and thus representative for, other natural biofilms from different DWDS (8.4×10^1–1.6×10^3 pg/cm^2; [59]), the biofilm on the pipe wall can be seen as a suitable food source.

In this study, both survival and growth were tested, and it seems likely that those two are linked. It was observed that if a specific food source led to a rapid death, the growth rate was also lower compared to other food sources. However, the opposite—a high survival rate combined with a higher growth rate—did not hold for some of the food sources that led to high survival rates (A_Sed_2, A_Leaf, B_Sed_2, B_Leaf, B_Sed_3). This shows that although a food source might be sufficient for survival in our experimental conditions, more nutrients are required for growth.

Table 4. Survival of *A. aquaticus* on different food sources: CPH model of survival experiments. Given is the exponent of the *p*-value (*p*-value: 0 = *p*-value of 0–0.1 (white), 1 = *p*-value of 0.1–0.01 (yellow), 2 = *p*-value of 0.01–0.001 (yellow), 3 = *p*-value of 0.001–0.0001 (red), >3 (green), etc.).

Food Source	**None**			**Leaf**				**Sediment**				**HF – Concentrate**		**PE without Biofilm**		**Biofilm**			
																Cultured			**Natural**
Drinking Water	**TP3**	**TP2**	**DWDS4**	**TP3**	**DWDS4**	**DWDS4**	**DWDS4**	**TP3**	**DWDS4**	**TP2**	**DWDS4**	**DWDS4**	**DWDS4**	**DWDS4**	**DWDS4**	**DWDS4**	**DWDS4**	**DWDS4**	**DWDS4**
Code	**A_Water_3**	**A_Water_2**	**B_Water_4**	**A_Leaf**	**B_Leaf**	**C_Leaf**	**D_Leaf**	**A_Sed_3**	**B_Sed_3**	**A_Sed_2**	**B_Sed_2**	**B_HF_3**	**B_HF_2**	**C_PE**	**D_PE**	**C_BFC_3**	**C_BFC_2**	**D_BFC_3**	**D_BFN_3**
A_Water_3																			
A_Water_2	0																		
B_Water_4	1	0																	
A_Leaf	12	14	15																
B_Leaf	11	13	15	0															
C_Leaf	8	9	12	1	0														
D_Leaf	1	1	2	2	1	1													
A_Sed_3	15	15	15	15	15	15	14												
B_Sed_3	13	14	15	0	0	1	2	15											
A_Sed_2	15	15	15	8	8	11	9	15	6										
B_Sed_2	15	15	15	5	5	8	6	15	4	1									
B_HF_3	4	3	1	15	15	15	4	8	15	15	15								
B_HF_2	3	2	1	15	15	15	3	10	15	15	15	0							
C_PE	1	1	0	15	15	15	3	13	15	15	15	0	0						
D_PE	0	0	0	6	6	4	0	13	7	15	15	2	2	1					
C_BFC_3	1	2	3	7	6	4	0	15	7	15	15	8	7	5	0				
C_BFC_2	1	2	4	6	5	3	0	15	7	15	15	9	8	6	0	0			
D_BFC_3	1	2	3	0	0	0	0	14	1	6	4	5	4	4	1	0	0		
D_BFN_3	4	3	2	15	15	15	4	4	15	15	15	0	0	1	3	7	5	8	

Table 5. Microbial biomass (in ATP) added to the survival experiments. No results are available for the sediment of Experiment B.

Food Source	Condition	Added Biomass (ng ATP)
Sediment	A_Sed_3	10
	A_Sed_2	21.6
Cultured biofilm	C_BFC_3	47.5
	C_BFC_2	52.2
Natural biofilm	D_BFN_3	4.3

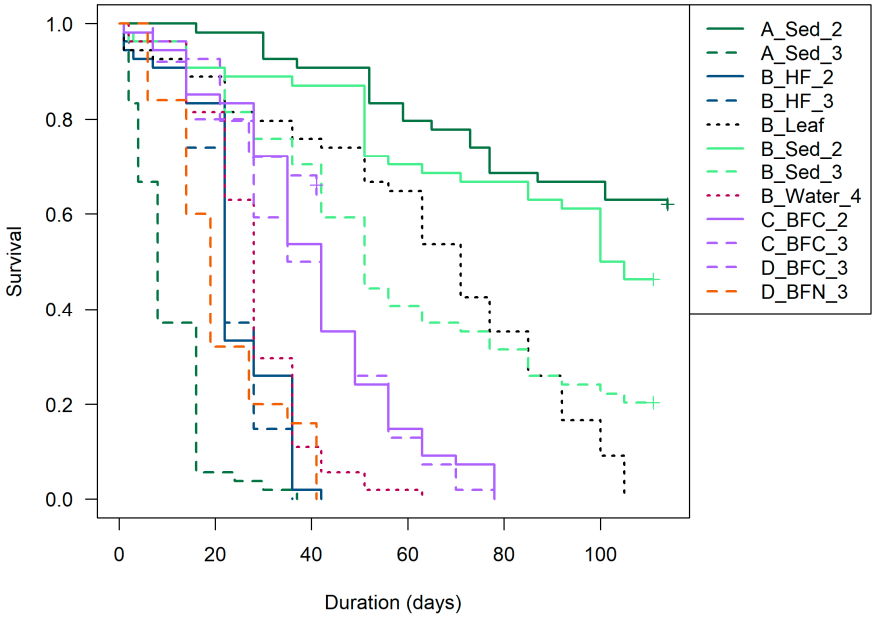

Figure 5. Survival of *A. aquaticus* on all tested food sources. For clarity, only one positive control (B_Leaf) and one negative control (B_Water_4) are included in the graph. +: experiment stopped. Information on statistically significant differences between conditions is given in Table 4.

4.3. Survival and Food Sources of A. aquaticus in the DWDS

It is known from the scientific literature that *A. aquaticus* are grazers, scraping food (and thus nutrients) from surfaces, in addition to ingestion of food sources. This has been demonstrated, for example, for leaf material from which the fungal/biofilm layer is consumed, as well as the leaf itself [33,35,36]. It is assumed that in the DWDS *A. aquaticus* ingests loose sediment, and large particles from the bottom of the pipe wall scrape biofilm from the pipe wall and can travel large distances to obtain enough food. This is consistent with the results from the survival tests measuring best survival on sediment and (cultured) biofilm. Confirmation of sediment as a food source for *Asellus* in the distribution network is demonstrated by fecal pellets from the distribution network in which material used or present in the distribution network is found, including iron, manganese, bitumen, and asbestos [41]. A point of interest here remains that biofilm on the pipe wall may also be qualitatively sufficient as a nutrient source. Although, as mentioned earlier, the biomass concentration of the natural biofilm (in pg/cm^2 ATP) was representative of natural biofilms of other DWDS, the amount administered in the experiments was not sufficient for prolonged survival. This seems to be supported by the fact that *Asellus* survives longer on cultured biofilm, with 10–12× more biomass (Table 5), compared to the natural biofilm.

As the amount of biomass that was added in each experiment differed for each food source, it cannot be concluded what the primary food source is for *A. aquaticus* in the DWDS. ATP showed that with the cultured biofilm, 2.3–5× more biomass was added (based on ATP

and FCM) than the sediment and natural biofilm (Table 5). We hypothesize that the longer survival on sediment, with less biomass, than biofilm, can be explained as sediment is easier to process than biofilm on pipe material. The lower microbial biomass in the natural biofilm compared to the cultured biofilm was considered the most likely explanation for the difference in survival rates. In early studies, the energy budget of individual *A. aquaticus* specimen (at 10 °C) was determined at 8.48 J/day [60]. In our experiments, 4.3–52.2 ng ATP/week, containing 2.6×10^{-7}–3.1×10^{-6} J/week, was added in the form of loose deposits or biofilm. Assuming that a 2 cm^2 leaf has a fresh weight of around 100 mg, this would contain approximately 2200 J/week [60]. Another method to determine the energy budget was recently published [61]. Assuming a wet weight of 1.3 mg for an *A. aquaticus* specimen (length: 3 mm) and a median food intake of 2% per day (wet weight/wet weight), an *A. aquaticus* requires 0.03 mg of food per day. However, one piece of pipe material with biofilm equals 1×10^7 cells/cm^2 or, based on the same study, 9.7×10^{-6} mg biofilm that was fed weekly to the *A. aquaticus*. Although the two calculations do not completely match, it does show a large difference between the offered and required amount of food or energy for sediment and biofilm as food sources. This explains the high survival rates on leaves and the lower survival rate on the other food sources. For loose deposits and biofilm, the available energy is low compared to the requirements of an *A. aquaticus*. It is assumed that *A. aquaticus* can actively move through the pipes of the DWDS in search of food. While doing so, they can scrape biofilm from the pipe wall and ingest sediment from the bottom of the pipe, consuming more nutrients and energy than the limited amount of food that was available in the experimental setup. The addition of a larger amount of food in the experiments will likely allow *A. aquaticus*, if they can consume the food source, to live longer compared to this study. Although this study does provide information on the food sources that *A. aquaticus* can use for survival and growth in the DWDS, the determined survival duration and growth should be considered to be the first exploration into this.

The differences between DWDS2 and DWDS3 with respect to *A. aquaticus* numbers (*A. aquaticus* biomass: respectively, 18 mg/m^3 and 0 mg/m^3; [7]) cannot be fully explained by differences in survival on the different food sources, especially sediment (longer survival on the sediment of DWDS2 than DWDS3). Based on the survival in Experiments A and B, *A. aquaticus* numbers were expected to be high in DWDS2 instead of DWDS3. The reason this did not happen could be the role that the configuration of the DWDS plays. DWDS2 supplies drinking water to a large rural area, and the distribution system has fewer bends, valves, fire hydrants, etc., than the industrial area that is supplied by DWDS3 (pers. comm. Evides). Finally, invertebrate development in DWDS is known to be influenced by other factors such as water composition, the availability of sediment, residence time, flow velocity, dispersal rate, infestation possibilities, and pipe material, notably cast iron [4,62–65].

5. Conclusions

Sediment and biofilm on the pipe walls of the DWDS are suitable food sources for *A. aquaticus* in an oligotrophic drinking water environment, but concentrated organic carbon compounds (including biopolymers) are not.

Microbial and chemical characterization of the food sources showed limited differences and could not be linked to differences in survival or growth, except for differences in the bacterial biomass (as measured with ATP and cell numbers). Targeted experiments are needed for this.

The absence and presence of *Asellus* in, respectively, DWDS2 and DWDS3 could not be explained by the survival rates on either biofilm or loose deposits. Therefore, most likely, other environmental conditions are responsible for this difference.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/arthropoda2030015/s1, Figure S1: *A. aquaticus* length at Day 0 and correlation with growth rate; Figure S2: Cultured and natural biofilm; Figure S3: Experimental setup of growth and survival experiments; Figure S4: Steromicroscopic photo of *A. aquaticus* (condition A_Sed_2), covered in sediment; Table S1: Summary of chemical composition of food sources in Experiment A and B; Table S2. Concentration metals in cultured (C_BFC_2 and C_BFC_3) and natural (D_BFN_3) biofilm, corrected for the concentration in the sterile drinking water used for sonification of the biofilm; Table S3. Characterization of cultured (C_BFC_2 and C_BFC_3) and natural (D_BFN_3) biofilm.

Author Contributions: Conceptualization, N.v.B., W.A.M.H. and A.M.V.; methodology, N.v.B., L.P.-V. and A.M.V.; validation, N.v.B. and L.P.-V.; formal analysis, N.v.B. and A.M.V.; investigation, L.P.-V. resources, L.P.-V., N.v.B., A.M.V. and J.W; data curation, N.v.B.; writing—original draft preparation, N.v.B., J.H.M.v.L. and J.W.; writing—review and editing, N.v.B., J.W., W.A.M.H. and E.T.H.M.P.; visualization, N.v.B.; supervision, N.v.B. and J.W.; project administration, N.v.B. and L.P.-V.; funding acquisition: N.v.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the joint research program (BTO) of the Dutch drinking water companies and by the Evides Water Company in The Netherlands.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to thank Gaby van Doorn-Abelmann for assisting with biofilm culture, Sidney Meijering for preparing the PVC and PE pieces, and Michiel Hootsmans for critically reading the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Sket, B. Distribution of *Asellus aquaticus* (Crustacea: Isopoda: Asellidae) and its hypogean populations at different geographic scales, with a note on Proasellus istrianus. *Hydrobiologia* **1994**, *287*, 39–47. [CrossRef]
2. Verovnik, R.; Konec, M. Chapter 11—*Asellus aquaticus*: A model system for historical biogeography. In *Encyclopedia of Caves*, 3rd ed.; White, W.B., Culver, D.C., Pipan, T., Eds.; Academic Press: Cambridge, MA, USA, 2019; pp. 76–84. [CrossRef]
3. Lafuente, E.; Lürig, M.D.; Rövekamp, M.; Matthews, B.; Buser, C.; Vorburger, C.; Räsänen, K. Building on 150 Years of Knowledge: The Freshwater Isopod *Asellus aquaticus* as an Integrative Eco-Evolutionary Model System. *Front. Ecol. Evol.* **2021**, *9*, 748212. [CrossRef]
4. Van Lieverloo, J.H.M.; Van der Kooij, D.; Hoogenboezem, W. Invertebrates and Protozoa (Free-living) in Drinking Water Distribution Systems. In *Encylopediaof Environmental Microbiology*; Bitton, G., Ed.; John Wiley & Sons: New York, NY, USA, 2002; pp. 1718–1733.
5. Whipple, G.C. *The Microscopy of Drinking Water*; Robert Drummond, Printer: New York, NY, USA, 1899.
6. Gunkel, G.; Michels, U.; Scheideler, M.; Ripl, K. Vorkommen und Bedeutung von Kleintieren in Trinkwasser-Verteilungssystemen—Massnahmen zu deren Regulierung. *Fachber. Wasserversorg.* **2010**, *3R*, 716–724.
7. Ketelaars, H.A.M.; Wagenvoort, A.J.; Peters, M.C.F.M.; Wunderer, J.; Hijnen, W.A.M. Taxonomic diversity and biomass of the invertebrate fauna of nine drinking water treatment plants and their non-chlorinated distribution systems. *Water Res.* **2023**, *242*, 120269. [CrossRef] [PubMed]
8. Hijnen, W.A.M.; Brouwer-Hanzens, A.; Schurer, R.; Wagenvoort, A.J.; van Lieverloo, J.H.M.; van der Wielen, P.W.J.J. Influence of biopolymers, iron, biofouling and *Asellus aquaticus* on Aeromonas regrowth in three non-chlorinated drinking water distribution systems. *J. Water Process Eng.* **2024**, *61*, 105293. [CrossRef]
9. Hijnen, W.A.M.; Reijnen, G.K.; Bos, R.H.M.; Veenendaal, G.; Van der Kooij, D. Lagere *Aeromonas*-aantallen in het drinkwater van pompstation Zuidwolde door verbeterde ontgassing en vernieuwen van het filtergrind. *H_2O* **1992**, *25*, 370–375.
10. Drinkwaterbesluit. Drinking Water Decree 2018 (Drinkwaterbesluit 2018). In *BWBR0030111*; Staatsblad: The Hague, The Netherlands, 2018.
11. van der Wielen, P.W.J.J.; Lut, M.C. Distribution of Microbial Activity and Specific Microorganisms across Sediment Size Fractions and Pipe Wall Biofilm in a Drinking Water Distribution System. *Water Sci. Technol. Water Supply* **2016**, *16*, 896–904. [CrossRef]
12. Liu, G.; Tao, Y.; Zhang, Y.; Lut, M.; Knibbe, W.-J.; van der Wielen, P.; Liu, W.; Medema, G.; van der Meer, W. Hotspots for Selected Metal Elements and Microbes Accumulation and the Corresponding Water Quality Deterioration Potential in an Unchlorinated Drinking Water Distribution System. *Water Res.* **2017**, *124*, 435–445. [CrossRef] [PubMed]

13. van Lieverloo, J.H.; Hoogenboezem, W.; Veenendaal, G.; van der Kooij, D. Variability of invertebrate abundance in drinking water distribution systems in the Netherlands in relation to biostability and sediment volumes. *Water Res.* **2012**, *46*, 4918–4932. [CrossRef] [PubMed]
14. van der Wielen, P.W.J.J.; Bakker, G.; Atsma, A.; Lut, M.; Roeselers, G.; de Graaf, B. A Survey of Indicator Parameters to Monitor Regrowth in Unchlorinated Drinking Water. *Environ. Sci. Water Res. Technol.* **2016**, *2*, 683–692. [CrossRef]
15. Christensen, S.C.B.; Nissen, E.; Arvin, E.; Albrechtsen, H.J. Influence of *Asellus aquaticus* on Escherichia coli, Klebsiella pneumoniae, Campylobacter jejuni and naturally occurring heterotrophic bacteria in drinking water. *Water Res.* **2012**, *46*, 5279–5286. [CrossRef] [PubMed]
16. Christensen, S.C. *Asellus aquaticus and Other Invertebrates in Drinking Water Distribution Systems—Occurrence and Influence on Microbial Water Quality*; Technical University of Denmark (DTU): Lyngby, Denmark, 2011.
17. van Lieverloo, J.H.; Bosboom, D.W.; Bakker, G.L.; Brouwer, A.J.; Voogt, R.; De Roos, J.E. Sampling and quantifying invertebrates from drinking water distribution mains. *Water Res.* **2004**, *38*, 1101–1112. [CrossRef] [PubMed]
18. Birstein, Y.A.; Mercado, A.; Finesilver, R. *Freshwater Isopods (Asellota)*; Israel Program for Scientific Translations: Jerusalem, Israel, 1964.
19. Økland, K.A. Life history and growth of *Asellus aquaticus* (L.) in relation to environment in a eutrophic lake in Norway. *Hydrobiologia* **1978**, *59*, 243–259. [CrossRef]
20. Ridley, M.; Thompson, D.J. Size and Mating in *Asellus aquaticus* (Crustacea: Isopoda). *Z. Für Tierpsychol.* **1979**, *51*, 380–397. [CrossRef]
21. Gunkel, G.; Michels, U.; Scheideler, M. Climate Change: Water Temperature and Invertebrate Propagation in Drinking-Water Distribution Systems, Effects, and Risk Assessment. *Water* **2022**, *14*, 1246. [CrossRef]
22. Ripl, K.; Slavik, I.; Uhl, W.; Michels, U.; Titze, D.; Gunkel, G.; Scheideler, M. Sampling of *Asellus aquaticus* (water lice) in a drinking water distribution system and consequences for drinking water supply. In Proceedings of the Urban Water Management: Challenges and Oppurtunities—11th International Conference on Computing and Control for the Water Industry, Exeter, UK, 5–7 September 2011. CCWI 2011.
23. Wagenvoort, A.; van Asperen, R.; Sandrini, G.; Hijnen, W. Changes in water source cause shift in invertebrate biomass and composition, and regrowth in a non-chlorinated drinking water distribution system. *Water* **2023**, *13*, 732. [CrossRef]
24. Gunkel, G.; Scheideler, M. Wasserasseln in Trinkwasser-Verteilungssystemen—Eintrag, Vorkommen, Bewertung und Bekämpfung der Wasserasseln. *gwf Wasser Abwasser* **2011**, *152*, 308–388.
25. van Lieverloo, J.H.; Van Buuren, R.; Veenendaal, G.; van der Kooij, D. Controlling invertebrates in distribution systems with zero or low disinfectant residual. *Water Supply* **1998**, *16*, 199–204.
26. Marcus, J.; Sutcliffe, D.; Willoughby, L. Feeding and growth of *Asellus aquaticus*_(Isopoda) on food items from the littoral of Windermere, including green leaves of Elodea canadensis. *Freshw. Biol.* **1978**, *8*, 505–519. [CrossRef]
27. Franken, R.J.M.; Waluto, B.; Peeters, E.T.H.M.; Gardeniers, J.J.P.; Beijer, J.A.J.; Scheffer, M. Growth of shredders on leaf litter biofilms: The effect of light intensity. *Freshw. Biol.* **2005**, *50*, 459–466. [CrossRef]
28. Wang, Y.; Brune, A.; Zimmer, M. Bacterial Symbionts in the Hepatopancreas of Isopods: Diversity and Environmental Transmission. *FEMS Microbiol. Ecol.* **2007**, *61*, 141–152. [CrossRef]
29. Liao, A.; Hartikainen, H.; Buser, C.C. Individual level microbial communities in the digestive system of the freshwater isopod *Asellus aquaticus*: Complex, robust and prospective. *Environ. Microbiol. Rep.* **2023**, *15*, 188–196. [CrossRef]
30. Fraune, S.; Zimmer, M. Host-specificity of environmentally transmitted Mycoplasma-like isopod symbionts. *Environ. Microbiol.* **2008**, *10*, 2497–2504. [CrossRef]
31. Zimmer, M.; Danko, J.P.; Pennings, S.C.; Danford, A.R.; Ziegler, A.; Uglow, R.F.; Carefoot, T.H. Hepatopancreatic endosymbionts in coastal isopods (Crustacea: Isopoda), and their contribution to digestion. *Mar. Biol.* **2001**, *138*, 955–963. [CrossRef]
32. Zimmer, M.; Bartholmé, S. Bacterial endosymbionts in *Asellus aquaticus* (Isopoda) and *Gammarus pulex* (Amphipoda) and their contribution to digestion. *Limnol. Oceanogr.* **2003**, *48*, 2208–2213. [CrossRef]
33. Bloor, M.C. Animal standardisation for mixed species ecotoxicological studies: Establishing a laboratory breeding programme for Gammarus pulex and *Asellus aquaticus*. *Zool. Baetica* **2010**, *21*, 179–190.
34. Graca, M.A.; Maltby, L.; Calow, P. Importance of fungi in the diet of Gammarus pulex and *Asellus aquaticus* I: Feeding strategies. *Oecologia* **1993**, *93*, 139–144. [CrossRef]
35. Graca, M.A.; Maltby, L.; Calow, P. Importance of fungi in the diet of Gammarus pulex and *Asellus aquaticus*: II. Effects on growth, reproduction and physiology. *Oecologia* **1993**, *96*, 304–309. [CrossRef]
36. Rossi, L.; Vitagliano-Tadini, G. Role of Adult Faeces in the Nutrition of Larvae of *Asellus aquaticus* (Isopoda). *Oikos* **1978**, *30*, 109–113. [CrossRef]
37. Herczeg, G.; Balázs, G.; Biró, A.; Fišer, Ž.; Kralj-Fišer, S.; Fišer, C. Island and Rensch's rules do not apply to cave vs. surface populations of *Asellus aquaticus*. *Front. Ecol. Evol.* **2023**, *11*, 1155261. [CrossRef]
38. Berisha, H.; Horváth, G.; Fišer, Ž.; Balázs, G.; Fišer, C.; Herczeg, G. Sex-dependent increase of movement activity in the freshwater isopod *Asellus aquaticus* following adaptation to a predator-free cave habitat. *Curr. Zool.* **2022**, *69*, 418–425. [CrossRef]
39. McCahon, C.P.; Pascoe, D. Culture techniques for three freshwater macroinvertebrate species and their use in toxicity tests. *Chemosphere* **1988**, *17*, 2471–2480. [CrossRef]

40. Screech, Y. Effects of temperature and microplastics concentration on the consumption of microplastic particles by the detrititvore *A. aquaticus*. Bachelor's Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden, 2023.
41. Christensen, S.C.; Nissen, E.; Arvin, E.; Albrechtsen, H.J. Distribution of *Asellus aquaticus* and microinvertebrates in a non-chlorinated drinking water supply system—Effects of pipe material and sedimentation. *Water Res.* **2011**, *45*, 3215–3224. [CrossRef]
42. Gauthier, V.; Gérard, B.; Portal, J.-M.; Block, J.-C.; Gatel, D. Organic matter as loose deposits in a drinking water distribution system. *Water Res.* **1999**, *33*, 1014–1026. [CrossRef]
43. Hijnen, W.A.M.; Schurer, R.; Bahlman, J.A.; Ketelaars, H.A.M.; Italiaander, R.; van der Wal, A.; van der Wielen, P.W.J.J. Slowly Biodegradable Organic Compounds Impact the Biostability of Non-Chlorinated Drinking Water Produced from Surface Water. *Water Res.* **2018**, *129*, 240–251. [CrossRef]
44. Veenendaal, H.R.; Brouwer-Hanzens, A.H. *TECHNEAU: A Method for the Concentration of Microbes in Large Volumes of Water*; KWR Watercycle Research Institute: Nieuwegein, The Netherlands, 2007.
45. van der Wielen, P.W.J.J.; Brouwer-Hanzens, A.J.; Italiaander, R.; Hijnen, W.A.M. Initiating guidance values for novel biological stability parameters in drinking water to control regrowth in the distribution system (Open Access). *Sci. Total Environ.* **2023**, *871*, 161930. [CrossRef]
46. van der Kooij, D.; Bakker, G.L.; Italiaander, R.; Veenendaal, H.R.; Wullings, B.A. Biofilm Composition and Threshold Concentration for Growth of *Legionella pneumophila* on Surfaces Exposed to Flowing Warm Tap Water without Disinfectant. *Appl. Environ. Microbiol.* **2017**, *83*, e02737-16. [CrossRef]
47. Magic-Knezev, A.; van der Kooij, D. Optimisation and significance of ATP analysis for measuring active biomass in granular activated carbon filters used in water treatment. *Water Res.* **2004**, *38*, 3971–3979. [CrossRef]
48. van der Wielen, P.W.; van der Kooij, D. Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. *Water Res.* **2010**, *44*, 4860–4867. [CrossRef]
49. van der Wielen, P.W.J.J.; van der Kooij, D. Nontuberculous mycobacteria, fungi, and opportunistic pathogens in unchlorinated drinking water in the Netherlands. *Appl. Environ. Microbiol.* **2013**, *79*, 825–834. [CrossRef]
50. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
51. Huber, S.A.; Balz, A.; Abert, M.; Pronk, W. Characterisation of aquatic humic and non-humic matter with size-exclusion chromatography—Organic carbon detection—Organic nitrogen detection (LC-OCD-OND). *Water Res.* **2011**, *45*, 879–885. [CrossRef] [PubMed]
52. Cox, D.R. Regression Models and Life-Tables. *J. R. Stat. Soc. Ser. B (Methodol.)* **1972**, *34*, 187–220. [CrossRef]
53. Therneau, T. A Package for Survival Analysis in R. R Package Version 3.7-0. 2024. Available online: https://CRAN.R-project.org/package=survival (accessed on 4 April 2024).
54. Migliore, L.; de Nicola Giudici, M. Toxicity of heavy metals to *Asellus aquaticus* (L.) (Crustacea, Isopoda). *Hydrobiologia* **1990**, *203*, 155–164. [CrossRef]
55. Maltby, L.; Snart, J.O.H.; Calow, P. Acute toxicity tests on the freshwater isopod, *Asellus aquaticus* using $FeSO_4$. $7H_2O$, with special reference to techniques and the possibility of intraspecific variation. *Environ. Pollut.* **1987**, *43*, 271–279. [CrossRef] [PubMed]
56. Martin, T.R.; Holdich, D.M. The acute lethal toxicity of heavy metals to peracarid crustaceans (with particular reference to fresh-water asellids and gammarids). *Water Res.* **1986**, *20*, 1137–1147. [CrossRef]
57. Braginskiy, L.; Shcherban, E. Acute toxicity of heavy metals to aquatic invertebrates at different temperatures. *Hydrobiol. J.* **1978**, *14*, 86–92.
58. Mayer, M. Zur Ernährungsweise von Isopoden in Trinkwasserverteilungssystemen. Ph.D. Thesis, Technischen Universität Berlin, Berlin, Germany, 2013.
59. Learbuch, K.L.G.; Smidt, H.; van der Wielen, P.W.J.J. Water and biofilm in drinking water distribution systems in the Netherlands (Open Access). *Sci. Total Environ.* **2022**, *831*, 154940. [CrossRef]
60. Adcock, J.A. *The Bioenergetics of a Population of Asellus aquaticus (L.) (Crustacea, Isopoda)*; University of Northampton: Northampton, UK, 1975. Available online: https://core.ac.uk/reader/287595900 (accessed on 4 April 2024).
61. Gunkel, G.; Michels, U.; Scheideler, M. Water Lice and Other Macroinvertebrates in Drinking Water Pipes: Diversity, Abundance and Health Risk. *Water* **2021**, *13*, 276. [CrossRef]
62. Shaddock, B. *An Evaluation of Invertebrate Dynamics in a Drinking Water Distribution System: A South African Perspective*; University of Johannesburg (South Africa): Johannesburg, South Africa, 2008.
63. Levy, R.V.; Hart, F.L.; Cheetham, R.D. Occurrence and public health significance of invertebrates in drinking water. *J. AWWA* **1986**, *78*, 105–110. [CrossRef]
64. Gunkel, G. Evaluation of Invertebrates in Drinking Water Networks. *Water* **2023**, *15*, 1391. [CrossRef]
65. Smart, A.C.; Harper, D.M. Life after lakes: The ecology and management of the water distribution network. In *The Ecological Bases for Lake and Reservoir Management*; Harper, D.M., Brierley, B., Ferguson, A.J.D., Phillips, G., Eds.; Springer: Dordrecht, The Netherlands, 1999; pp. 379–386.