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Oxygen Consumption and Carbon Budget in Groundwater-Obligate and Surface-Dwelling *Diacyclops* Species (Crustacea Copepoda Cyclopoida) Under Temperature Variability

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Abstract: This study explores the metabolic response and carbon budget of two cyclopoid copepod species, *Diacyclops belgicus* Kiefer, 1936 (a stygobitic, groundwater-adapted species) and *Diacyclops crassicaudis crassicaudis* (Sars G.O., 1863) (a stygophilic, predominantly surface-associated species). We measured oxygen consumption rates (OCRs), carbon requirements (CRs), ingestion (I) rates, and egestion (E) rates at 14 °C and 17 °C, representing current and predicted future conditions in the collection habitats of the two species. *Diacyclops belgicus* displayed OCRs (28.15 and 18.32 μL O_2/mg DW × h at 14 and 17 °C, respectively) and carbon budget (CR: 0.14 and 0.10 μg C/mg × d at 14 and 17 °C) lower than those of *D. crassicaudis crassicaudis* (OCR: 55.67 and 47.93 μL O_2/mg DW × h at 14 and 17 °C; CR: 0.3 and 0.27 μg C/mg × d at 14 and 17 °C). However, *D. belgicus* exhibited metabolic rates and carbon requirements comparable to those of other epigean species, challenging the assumption that low metabolic rates are universal among stygobitic species. Temperature variations did not significantly affect the metabolic responses and carbon requirements of the two species, suggesting that they may cope with moderate temperature increases.

Keywords: groundwater; metabolic rates; stygobitic; climate change; copepods; crustaceans; energy budget

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Academic Editor: Simeone Chianese

Received: 20 December 2024
Revised: 8 January 2025
Accepted: 17 January 2025
Published: 20 January 2025

Citation: Di Lorenzo, T.; Tabilio Di Camillo, A.; Iepure, S.; Galassi, D.M.P.; Mori, N.; Simčič, T. Oxygen Consumption and Carbon Budget in Groundwater-Obligate and Surface-Dwelling *Diacyclops* Species (Crustacea Copepoda Cyclopoida) Under Temperature Variability. *Environments* **2025**, *12*, 32. https://doi.org/10.3390/environments12010032

1. Introduction

Groundwater, the Earth's largest freshwater reservoir, possesses distinctive ecological features that set it apart from epigean ecosystems. The lack of light precludes photosynthetic production, making the groundwater community entirely reliant on allochthonous organic carbon inputs or chemolithoautotrophic microbial activity for energy [1]. Groundwater habitats often exhibit limited oxygen availability, with concentrations varying depending on recharge and microbial and metazoan consumption [2]. Despite these constraints, groundwater environments are renowned for their thermal and chemical–physical stability, offering relatively constant conditions over time [2]. This stability, coupled with resource limitations, shapes the unique adaptations and ecological roles of the organisms inhabiting these systems [3].

Stygobitic species (i.e., species that are incapable of completing their life cycle outside aquatic subterranean environments [4]) have evolved to thrive in light-deprived, resource-limited, and stable subterranean environments, showing typical adaptations such as blindness, depigmentation, elongation of sensory organs, low metabolic rates, reduced fertility, and increased longevity [5]. Populations are typically small, reflecting low species abundances in groundwater systems [6]. Stygobitic species exhibit high levels of endemism, with many species being point endemics confined to specific sites [6].

Groundwater communities are often dominated by copepods, which play a pivotal role in these ecosystems [7,8]. With few exceptions, most stygobitic copepods are deposit feeders, consuming detritus and associated microbial biofilms [9]. Their omnivorous diet and ability to exploit diverse organic matter sources further underscore their importance in subterranean food webs, facilitating carbon cycling and contributing to the overall ecological functionality of these ecosystems [10,11].

Groundwater ecosystems are pivotal in the global carbon cycle, serving as a sink for organic matter and sustaining diverse ecosystem services through their communities [12]. Stygobitic copepod activities influence not only the cycling of inorganic and organic matters but also the availability of resources across trophic levels (see, e.g., [13]). Moreover, the presence of epigean copepods in groundwater habitats likely enhances carbon consumption at depth, further altering carbon storage and dynamics in these ecosystems [10]. In concomitance with organic contamination, non-native or generalist species—which often have higher metabolic rates than stygobitic species [14,15]—may disrupt these cycles by displacing native species, temporarily altering groundwater communities [16].

Because of their dominance in groundwater habitats [7], copepods are significant contributors among hypogean crustaceans to biological carbon recycling. Yet their metabolic demands and carbon flux contributions remain poorly understood [14]. They are presumed to have low metabolic rates [15] and a low carbon budget due to the resource-limited and stable nature of their subterranean environments [10]. However, the carbon budget of stygobitic copepods has never been investigated, and the assumption of low metabolic rates has not been systematically tested across life stages and thermal conditions [14,17]. Investigating the carbon requirements, ingestion, and egestion rates of these organisms under varying temperatures is crucial for understanding their ecological roles and their ability to cope with environmental changes, such as rising groundwater temperatures due to climate change [18].

To address these gaps, we focused this study on two cyclopoid species (Crustacea Copepoda): *Diacyclops belgicus* Kiefer, 1936, a stygobitic species occurring in both karst and alluvial aquifers, and *Diacyclops crassicaudis crassicaudis* (Sars G.O., 1863), a facultative groundwater species (stygophilic species [4]). The genus *Diacyclops* within the Cyclopidae family encompasses approximately 150 species and subspecies [19], with 71 species adapted to groundwater habitats [20]. The diversification of *Diacyclops* in groundwater ecosystems likely results from multiple invasions followed by radiation [21].

Diacyclops belgicus is strictly associated with groundwater, inhabiting hyporheic zones, porous aquifers, and caves across the former USSR and Europe [19,22]. The species has been observed in environments where temperatures range between 10 °C and 17 °C [23].

Diacyclops crassicaudis crassicaudis is a eurytopic species found in diverse habitats, including temporary ponds, rain and snowmelt puddles, littoral zones, and psammon of small lakes [24]. It also frequently inhabits groundwater, particularly in karstic aquifers, saturated alluvial groundwater bodies and springs in Italy [25], and hyporheic zone of rivers in Romania and central Spain [8,26], Germany [27], Austria [28], Poland [29,30], the USA [31], Japan [32], and Australia [33]. The species thrives at 9 °C to up 20 °C, tolerating seasonal fluctuations of up to 7 °C [8,30]. *Diacyclop crassicaudis crassicaudis* can complete its life cycle in groundwater but is not confined to this environment [33]. We focused our analyses on the last juvenile stage before the reproductive stage, namely, the fifth copepodid stage (CV). The use of CV copepodids aimed to minimize the confounding effects of age and senescence, which are significant challenges when studying stygobitic copepods, as adults rarely reproduce in laboratory conditions [34]. To evaluate the phenotypic plasticity of these species to thermal changes and its implications for groundwater ecosystems under projected climate change scenarios, we analysed the metabolic rates and carbon budgets of the two species at two temperatures. We hypothesized the following: (1) the metabolic rate and carbon budget of the species would differ, reflecting their distinct ecological traits (stygobitic vs. stygophilic); (2) the metabolic rates and carbon budgets of the two species would vary from 14 °C, representing the minimum temperature of their collection sites, to 17 °C, a temperature projected under future climate change scenarios [35]. To put these assumptions in a broader context, we also compared the metabolic rates of the two species with those of other freshwater cyclopoids by gathering data from the literature.

2. Materials and Methods

2.1. Species Collection and Acclimation

We collected specimens of *D. belgicus* from a borehole (PVC-cased, 16 cm diameter, 14 m deep) in a shallow alluvial aquifer in Italy (Medio Valdarno aquifer; 43°49′02.610″ N, 11°11′59.790″ E). The bore water had a dissolved organic carbon (DOC) concentration of 1.1 mg/L and a mean annual temperature of 15 ± 0.8 °C [17]. We collected individuals of *D. crassicaudis crassicaudis* from a hand-dug, uncased well (45 cm diameter, 6 m deep) in the shallow Quaternary alluvial aquifer of Jarama, Spain (40°23′32.52″ N, 3°30′17.02″ W), with a DOC concentration of 3.2 mg/L and a mean annual temperature of 15 ± 1.0 °C [8,36]. We collected both species using a phraetobiological net with a 63 µm mesh (Figure 1).

Figure 1. Phraetobiological net used to collect *Diacyclops belgicus* (**a**) and *Diacyclops crassicaudis crassicaudis* (**b**).

We transported the specimens to the laboratory in plastic vials filled with bore water within one hour of collection. In the laboratory, we transferred 14–20 juveniles (copepodid stage CV) of each species into 100 mL glass vials filled with bore water (Figure 2).

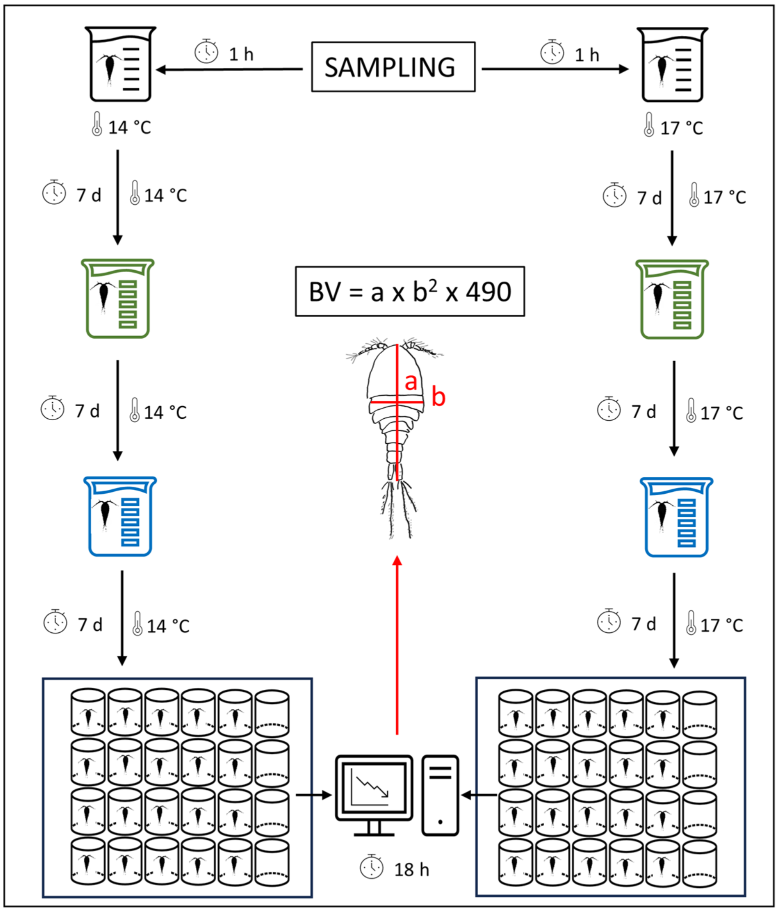


Figure 2. The respirometric setup for oxygen consumption measurements of CV copepodids of *Diacyclops belgicus* and *Diacyclops crassicaudis crassicaudis* at 14 °C and 17 °C. One hour after collection, the specimens were kept in darkness for 21 days, transitioning through three media: 100% bore water (black beaker), a 50% bore and standard water mix (green beaker), and 100% standard water (blue beaker). Individual CV copepodids were placed in 80 µL glass wells with oxygen sensor spots, housed in a microplate, and monitored for oxygen levels over 18 h. At the end of the measurements, the specimens were measured, and their body volume was computed based on body dimensions.

We incubated the specimens in darkness at 14 ± 0.2 °C in a temperature-controlled chamber (Pol-Eko-Aparatura, Wodzisław Śląski, Poland) for seven days without water renewal or additional food (Figure 2). After seven days, we replaced half of the bore water with standard water prepared by remineralizing 1 L of MILLIPORE MILLI-Q® deionized water with 0.06 g $MgSO_4$, 0.096 g $NaHCO_3$, 0.004 g KCl, and 0.06 g $CaSO_4{\cdot}2H_2O$. After another week, we transferred the juveniles into individual 10 mL vials containing standard water and we maintained them in darkness at 14 ± 0.2 °C for an additional seven days to ensure gut-emptying (Figure 2). The number of specimens used in the respirometric trials was dictated by the availability of healthy individuals (actively swimming copepodids) collected from the field. We used 20 individuals of *D. belgicus* and 14 of *D. crassicaudis crassicaudis* at 14 ± 0.2 °C and 20 healthy individuals of *D. belgicus* and 15 of *D. crassicaudis crassicaudis* at 17 ± 0.2 °C.

2.2. Respirometric Trials

We conducted the respirometric trials at two temperatures: 14 ± 0.2 °C and 17 ± 0.2 °C. The 14 °C condition represents the minimum temperature recorded at the collection sites of both species (approximately 1 °C below the annual average temperature), while the 17 °C condition (a 3 °C increase) simulates a worst-case climate change scenario projected for groundwater ecosystems globally over the next century, with an estimated thermal increase of 3–5 °C [35,37]. We measured oxygen consumption rates (OCRs) of the CV copepodids using 80 µL glass wells equipped with oxygen sensor spots (4 mm in diameter) affixed to the base. The wells were arranged in a microplate (Loligo Systems, Viborg, Denmark) and positioned on a Sensor Dish Reader (SDR) equipped with 24 fluorescence-based channels (PreSens Precision Sensing GmbH, Regensburg, Germany). Prior to introducing the specimens, the microplate and SDR were equilibrated in a temperature-controlled chamber at the respective test temperature for at least 12 h [38]. The wells were filled with MILLI-Q remineralized water, and we carefully transferred the copepodids using a ring needle. Four wells were kept empty to serve as control blanks. Once the copepodids were loaded, the wells were sealed, and the system was allowed to stabilize for at least two hours [17].

In the absence of food and conspecifics and given the restricted volume that may pose potential limitations to swimming, our OCR measurements were more representative of standard oxygen consumption rates defined as metabolic rates measured under conditions of quietude, a lack of stress, and abstention from food [39]. We recorded oxygen concentration (in mg/L) every five minutes over an 18-h period. We calculated OCRs by measuring the rate of oxygen concentration change in each well, adjusted for the mean oxygen consumption of control wells. The initial concentration of dissolved oxygen was 6 ± 0.5 mg/L and remained above 80% throughout the trials. All copepods survived the experiments.

At the end of the trials, we anesthetized the individuals with CO_2 and photographed them using a Leica M80 stereomicroscope (Leica Microsystems, Wetzlar, Germany) supplied with a camera at 40× magnification. We determined body length (from the end of the caudal rami to the tip of the cephalic shield; Figure 2) and width (at the widest point of the somite-bearing legs; Figure 2) using ImageJ software vs. 1.53a [40]. We converted these dimensions to dry weight (DW) using a dry-to-wet weight ratio of 0.25 [41] and after assuming the wet weight to be equivalent to biovolume/1.1 [42]. We calculated the biovolume (BV) as follows [41]:

$$\mathrm{BV} = \mathrm{a} \times \mathrm{b}^2 \times \mathrm{CF} \tag{1}$$

where a represents the length (mm), b is the width (mm), and the correction factor (CF) is 490 [43]. We expressed OCRs as $\mu\mathrm{L}\ \mathrm{O_2}/\mathrm{mg\ DW} \times \mathrm{h}$, where DW represents the dry weight of the specimens.

2.3. Carbon Budget Parameters

We estimated the carbon budget of each copepodid in terms of carbon requirement, carbon ingestion rates, and carbon egestion rates, expressed in µg C/ind. × day. To determine the carbon requirement (CR) for the copepodids of both species, we converted the individual oxygen consumption rate (R, in $\mu\mathrm{L}\ \mathrm{O_2}/\mathrm{ind.} \times \mathrm{h}$) to carbon units (C, in µg C/ind. × h). In this conversion, we assumed that 1 mL O_2 mobilizes 0.44 mg of organic carbon for copepod species with a respiratory quotient (RQ) equal to 0.82 based on a mixed diet of proteins, fats, and carbohydrates [44,45]:

$$\mathrm{C} = \mathrm{R} \times 0.44 \tag{2}$$

Afterward, we calculated the carbon ingestion (I) rates, assuming an average digestive efficiency of 70% (U = 0.7) and a gross growth efficiency of 30% (K1 = 0.3), following the methods of Ikeda and Motoda [46] and Bode et al. [47], regardless of species and food availability:

$$I = (R \times C \times RQ) \times 1/(U - K1) \quad (3)$$

$$E = 0.3 \times I, \quad (4)$$

where I is the carbon ingestion rate (µg C/ind. × h), R is the individual oxygen consumption rate (µL O_2/ind. × h), and C equals carbon mass per volume (12 g C per 22.4 L). Finally, we transformed this to µg C/ind. × day.

2.4. Comparative Analyses

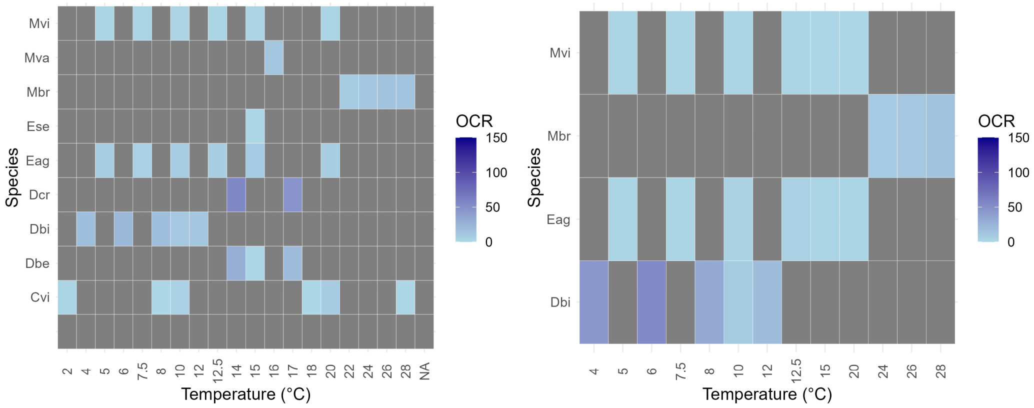
To compare the OCR data from this study with those of other freshwater cyclopoid species, we conducted a literature review using Web of Science. The search employed the following keywords: TS = (“copepod *” OR “Copepoda”) AND TS = (“oxygen consumption *” OR “respiration *” OR “metabolism *”) AND TS = (“Cyclopoida” OR “cyclopoid *”). A thorough manual review of the retrieved papers ensured their relevance and identified additional sources through reference lists. We excluded studies that (1) lacked clear OCR or temperature-specific data; (2) were unpublished or conference papers; (3) reported OCRs under stress without control data; (4) focused on non-freshwater cyclopoids; (5) provided OCRs at the community level; or (6) did not pertain to copepods. For studies with both control and stress-condition data, only the control values were included. Extracted data included author names, publication details, species, ecology, habitat, location, life history traits, dry mass, OCR values, and corresponding temperatures. For graphical OCR data, we used an online graph digitizer https://automeris.io/WebPlotDigitizer (accessed on 17 January 2025). All OCR data were standardized to µL O_2/mg DW × h using a conversion factor of 1.43 [15]. The compiled dataset is reported in Table S1.

We assessed statistical differences in the OCR, CR, and I and E rates using a two-way PERMANOVA (Permutational Multivariate Analysis of Variance) with 999 permutations, an α level of 0.05, and Euclidean distances. PERMANOVA is a non-parametric, distribution-free statistical test that does not require data normality [48]. However, it is sensitive to differences in dispersion, which can confound the results if variances among groups differ significantly [48]. To address this, we evaluated the homogeneity of variance in our data using a PERMDISP (Permutational Analysis of Multivariate Dispersion) test [48]. We initially analysed untransformed data. Afterward, we re-ran the PERMANOVA analysis with log(x + 1)-transformed data. The design included two factors: species (*D. belgicus*, *D. crassicaudis crassicaudis*) and temperature (14 °C, 17 °C). Type III sum of squares accounted for variability across factors and interactions. We also ran a one-way PERMANOVA, with “species” as the only factor, under unrestricted permutation of raw data to test for differences in the dry weight. All analyses were conducted with PERMANOVA+ software v.7 [49], while data visualizations were carried out in R v5.6.3 [50].

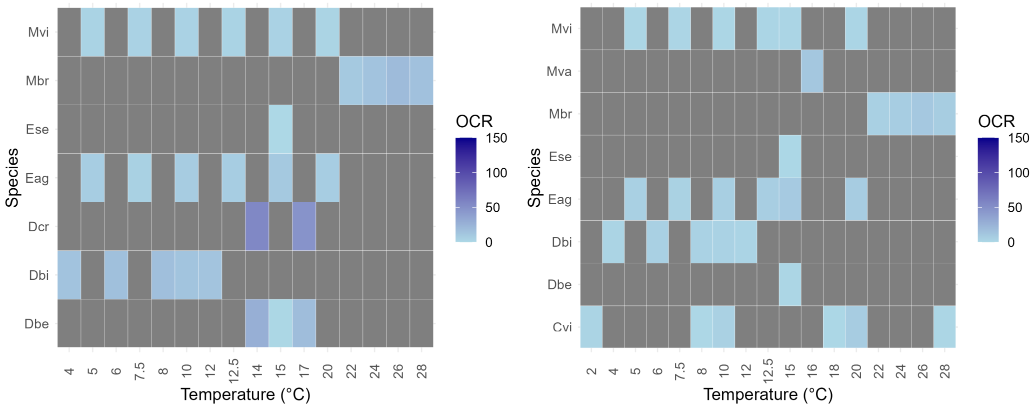
3. Results

Based on the specified criteria, we identified eight studies addressing the metabolic rates of eight freshwater cyclopoid species: *Megacyclops viridis* (Jurine, 1820), *Diacyclops bicuspidatus bicuspidatus* (Claus, 1857), *Microcyclops varicans varicans* (Sars G.O., 1863), *Cyclops vicinus vicinus* Uljanin, 1875, *Diacyclops belgicus* Kiefer, 1936, *Eucyclops agilis agilis* (Koch, 1838), *Eucyclops serrulatus serrulatus* (Fischer, 1851), and *Mesocyclops brasilianus* Kiefer, 1933. These species were collected from ponds, lakes, and groundwater habitats (Table S1). The publications, spanning from 1969 to 2022, provided 234 OCR values at experimental temperatures ranging from 2 °C to 28 °C, determined using field temperatures. Including the OCR data generated in this study, the final dataset consists of 302 OCR measurements for nine freshwater cyclopoid species (Table S1).

The four-panel heatmap (Figure 3) presents the OCRs across a temperature gradient, categorized by life stage: (a) all stages combined, (b) the naupliar stage, (c) the copepodid stage, and (d) the adult stage. The naupliar stage of *D. bicuspidatus bicuspidatus* showed notably higher OCR values compared to copepodid and adult stages (Figure 3b–d), especially at lower temperatures. For other species with stage-specific data, the OCRs were relatively consistent across life stages (Figure 3). Regarding OCR variability with temperature, the OCRs of *M. varicans* and *E. serrulatus* were measured at a single temperature, precluding an evaluation of temperature-dependent changes. In contrast, *M. viridis*, *C. vicinus vicinus*, *M. brasilianus*, and *E. agilis agilis* exhibited relatively stable OCRs across all temperatures and stages (Figure 3). Notably, *D. bicuspidatus bicuspidatus* appeared to display higher OCRs at lower temperatures, particularly in the naupliar stage (Figure 3b).



(a) All stages **(b) Naupliar stage**



(c) Copepodid stage **(d) Adult stage**

Figure 3. A heatmap of oxygen consumption rates (OCRs; μL O_2/mg DW × h) of freshwater cyclopoid species across a temperature gradient: (**a**) all stages; (**b**) nauplii; (**c**) copepodids; (**d**) adults. Colour intensity indicates OCR magnitude. Mvi: *Megacyclops viridis* (Jurine, 1820); Mbr: *Mesocyclops brasilianus* Kiefer, 1933; Ese: *Eucyclops serrulatus serrulatus* (Fischer, 1851); Eag: *Eucyclops agilis agilis* (Koch, 1838); Dcr: *Diacyclops crassicaudis crassicaudis* (Sars G.O., 1863); Dbi: *Diacyclops bicuspidatus bicuspidatus* (Claus, 1857); Mva: *Microcyclops varicans varicans* (Sars G.O., 1863); Cvi: *Cyclops vicinus vicinus* Uljanin, 1875; Dbe: *Diacyclops belgicus* Kiefer, 1936.

The mean and standard deviation of the DW, OCR, CR, and I and E rates for *D. belgicus* and *D. crassicaudis crassicaudis* at the two tested temperatures are reported in Table 1, while the raw data are reported in Table S2.

Table 1. Mean and standard deviation (sd) of dry weight (DW; mg), oxygen consumption rate (OCR; $\mu L\ O_2/mg\ DW \times h$), carbon requirement (CR; µg C/ind × d), ingestion rate (I; µg C/ind × d), and egestion rate (E; µg C/ind × d) of *Diacyclops belgicus* (DBE; stygobitic) and *Diacyclops crassicaudis crassicaudis* (DCR; stygophilic) at two temperatures (14 °C and 17 °C).

SPECIES	DW_14	DW_17	OCR_14	OCR_17	CR_14	CR_17	I_14	I_17	E_14	E_17
mean										
DBE	0.0005	0.0005	28.13	18.32	0.14	0.10	0.33	0.25	0.10	0.08
DCR	0.0005	0.0006	55.67	47.93	0.30	0.27	0.73	0.66	0.22	0.20
ratio	1	0.82	2.0	2.6	2.1	2.7	2.2	2.6	2.2	2.5
sd										
DBE	0.0003	0.0001	16.68	15.64	0.07	0.09	0.05	0.07	0.40	0.29
DCR	0.0001	0.0004	28.22	52.21	0.21	0.25	0.16	0.18	0.28	0.52

The two species did not significantly differ in dry weight (Pseudo-F = 0.22; *p*-value = 0.665). The PERMANOVA analyses run on untransformed and log(x + 1)-transformed data revealed a significant effect of the species factor on their metabolic rates and carbon budgets (Table S3). The oxygen consumption rates, CRs, and I and E rates of the stygophilic species *D. crassicaudis crassicaudis* were approximately double those of the stygobitic *Diacyclops belgicus* at 14 °C and increased to 2.5- to 2.7-fold higher at 17 °C (Table 1). In contrast, the temperature factor did not yield a statistically significant effect, suggesting no substantial variation in the four variables in response to the different temperature conditions (Table S3). Additionally, the interaction between species and temperature (SP × TE) was found to be non-significant, indicating that the combined influence of species and temperature did not affect the measured parameters (Table S3).

4. Discussion

Diacyclops crassicaudis crassicaudis consistently exhibited values double or more than those of *D. belgicus* in terms of oxygen consumption and carbon requirements. This aligns with its ecological classification: the stygophilic *D. crassicaudis crassicaudis* displays metabolic flexibility that facilitates the exploitation of diverse and dynamic habitats, including surface waters, springs, hyporheic zones, and alluvial aquifers prone to seasonal changes in temperature [24,33]. This flexibility supports higher activity levels and resource utilization, which are traits characteristic of generalist taxa [51]. In contrast, the stygobitic *D. belgicus* has a lower metabolic demand consistent with adaptations to resource-limited groundwater environments and a relatively more constant temperature profile [15,34]. Prior research indicates that stygobitic species tend to have lower metabolic rates than surface water species [14,52,53]. Such adaptations likely enhance survival in nutrient-poor settings but impose constraints on activity and growth [54]. However, a few studies have demonstrated that OCRs show no significant variation between species or subspecies of the same genus inhabiting surface and subterranean environments, but they still show differing phenotypic plasticity by responding differently to temperature changes [55,56].

The contrasting metabolic strategies of the stygobitic and stygophilic species have significant implications for carbon cycling in groundwater ecosystems. Stygophilic species like *D. crassicaudis crassicaudis* not only exhibit higher metabolic rates but also demonstrate greater carbon assimilation and egestion, suggesting a more active role in the redistribution of organic matter. These metabolic patterns emphasize the complementary ecological roles of stygobitic and stygophilic fauna in groundwater ecosystems, with stygophilic species contributing to dynamic nutrient fluxes and stygobitic species maintaining baseline ecosystem stability.

Groundwater carbon availability extends beyond surface-derived organic matter and sedimentary carbon stores [57]. Recent studies indicate that active carbon fixation in groundwater ecosystems, with experimental rates ranging from 0.0095 to 0.23 μgC/L × day, overlaps with marine systems and contributes ~0.108 ± 0.069 PgC annually to the global carbon budget [58]. These contributions, coupled with surface-derived detritus and DOC inputs, form the carbon baseline for microbial and metazoan consumers in groundwater [1]. The DOC values in the two aquifers in this study (1.1 mg/L and 3.2 mg/L) represent a substantial organic carbon pool with significant input from the surface. The carbon requirements of both *D. belgicus* and *D. crassicaudis crassicaudis* are negligible relative to this pool, even under high population densities. However, the nutritional quality of available carbon—especially the scarcity of polyunsaturated fatty acids critical for metazoan growth—may limit carbon transfer efficiency [59,60]. Detrital inputs like animal remains and leaf fragments can directly support larger invertebrates, but most DOC requires microbial conversion before transfer to higher trophic levels [1]. Consequently, energetic constraints in groundwater ecosystems may derive more from carbon quality than quantity, depending on the species' food preferences. This poses challenges for high-metabolism species like *D. crassicaudis crassicaudis* in sustaining long-term populations and in being competitive with obligate groundwater specialists.

Contrary to expectations, neither *D. crassicaudis crassicaudis* nor *D. belgicus* exhibited significant OCR or carbon budget changes in response to temperature variations between 14 °C and 17 °C. This contrasts with typical metazoan responses, where metabolic rates increase with temperature following the Arrhenius equation [61]. However, some copepods (including *M. viridis*, *C. vicinus vicinus*, *M. brasilianus*, and *E. agilis agilis* from this study) exhibit temperature-independent metabolism. For example, the calanoid copepod *Eurytemora affinis affinis* (Poppe, 1880) maintains a constant OCR between 5 °C and 17 °C, which is the natural thermal range for this species [62].

The metabolic stability observed in *D. belgicus* and *D. crassicaudis crassicaudis* may reflect adaptation to the thermal ranges typical of their habitats. *Diacyclops belgicus* thrives in temperate groundwater habitats across Europe and into Russia, occupying environments subject to temperature fluctuations, such as wells, interstitial sediments, and caves. It has been observed at temperatures ranging from 10 °C to 17 °C, suggesting a broad thermal tolerance [23,63–65]. The species has been found in aquifers and interstitial groundwater habitats in association with both strict stygobitic species like the syncarid *Antrobathynella stammeri* (Jakobi, 1954) but also with epigean copepod species like *Attheyella (Attheyella) crassa* (Sars G.O., 1863) and *Bryocamptus (Bryocamptus) minutus* (Claus, 1863) [63,66]. *Diacyclops crassicaudis crassicaudis* endures almost the same fluctuations, up to 7 °C [8]. Such relatively large temperature range adaptations may confer an ability to cope with moderate thermal changes in both species but could falter under long-term warming or additional stressors like contamination or competition with invasive species [67,68]. Additional stressors might interact synergistically with temperature fluctuations, overwhelming the physiological capacity of even thermally tolerant species [67]. For example, temperature changes could exacerbate the effects of low oxygen availability, further challenging the survival of the species.

Emerging evidence highlights the detrimental impacts of rising temperatures on subterranean biota [68]. The effects of climate change on groundwater ecosystems are complex and multifaceted, influencing processes from the genetic level to community dynamics and also altering critical ecosystem functions, such as carbon and nitrogen cycling [68]. To anticipate the cascading effects of climate change on groundwater habitats and biota, it is essential to perform long-term experiments involving the exposure of stygobitic species to thermal conditions that deviate significantly from those of their natural habitats. Such investigations would reveal whether they experience thermal stress and whether their oxygen consumption rates and carbon uptake are affected similarly under these conditions.

The OCRs of *D. belgicus* CV copepodids were comparable to or exceeded those of some epigean species at similar temperatures. This finding challenges the assumption that all stygobitic species universally exhibit lower metabolic rates. Elevated OCRs in *D. belgicus* CV copepodids may offer a significant advantage by facilitating rapid molting to reach the reproductive stage. Previous studies indicate that the developmental time from nauplius N1 to N2 in this species is quite prolonged, lasting approximately 60 days at 15 °C [69]. However, developmental durations are not uniform across naupliar and copepodid stages in all copepods [70]. For *D. belgicus*, it is plausible that copepodid stage V transitions to the adult stage in a relatively shorter time, potentially supported by elevated OCRs during this critical phase. Such a strategy may be advantageous in resource-scarce environments, where episodic food availability could drive selective pressures for rapid energy utilization during critical life stages. However, this assumption remains speculative in the absence of detailed life history data. Future studies should investigate stage-specific developmental durations to confirm this hypothesis. Similarly, the high OCRs in *D. bicuspidatus bicuspidatus* nauplii likely reflect the demands of rapid growth and "cold compensation" in low-temperature habitats [71].

The relatively narrow temperature range (14 °C to 17 °C) used in the experiments of this study restricts the ability to generalize the findings to broader thermal gradients. While these temperatures are ecologically relevant for the habitats of *D. belgicus* and *D. crassicaudis crassicaudis*, they do not capture the entire potential metabolic responses under thermal ranges larger than their actual known ranges, particularly those predicted under future climate change scenarios. It would be interesting to investigate variations in OCRs among populations of the same species from different geographical locations with varying temperatures. This could provide valuable insights into the phenotypic plasticity of the species across geographical scales.

Additionally, the methodology for measuring the OCRs of the copepods of this study varied both among species and within the same species. For instance, the OCRs were either measured using pooled samples, with biomass estimated based on weight, or with individual-level measurements (as in this study) using length regression to compute the biomass [14,34]. This methodological discrepancy may have introduced some data variability. In addition, we did not account for the effect of gender in our experiments, but exploring its potential influence could provide new insights and should be considered in future studies.

Despite some methodological discrepancies, this study challenges the notion that all stygobitic species universally exhibit lower metabolic rates than their surface-dwelling counterparts. In a previous study, we found that the stygobitic amphipod *Niphargus longicaudatus* (Costa, 1851) from the Stiffe Cave (Italy) prefers a temperature range much higher than the mean annual temperatures of its collection sites, challenging the notion that stygobitic species are strictly limited to narrow thermal ranges [72]. Future research should explore broader temperature ranges, additional life stages, and other stygobitic and stygophilic copepods from the same localities of this study to better understand these adaptations. Integrating genomic and transcriptomic approaches could further reveal the molecular mechanisms driving metabolic plasticity. Finally, examining the interplay between carbon quality, availability, and nutritional needs is essential to clarify the role of copepods in carbon cycling within groundwater ecosystems.

5. Conclusions

This study sheds light on the metabolic responses and carbon budget parameters of two groundwater-associated *Diacyclops* species, the stygobitic *D. belgicus* and the stygophilic *D. crassicaudis crassicaudis*, under temperature conditions reflective of their natural habitats and future climate scenarios. Contrary to prevailing assumptions, our findings demonstrate that stygobitic species like *D. belgicus* can exhibit metabolic rates comparable to their epigean counterparts during specific life stages, challenging the assumption that low metabolic rates are universal among stygobitic species. The observed thermal stability in the metabolic rates and carbon budget components of *D. belgicus* and *D. crassicaudis crassicaudis* suggests a degree of thermal independence to moderate temperature increase. The thermal adaptability of *D. belgicus* may reflect the ecological strategy of a stygobitic species inhabiting aquifers that experience some degree of temperature variability. If this relative flexibility could confer an advantage in environments subject to moderate thermal changes, further studies should prioritize investigating the physiological limits and adaptive capacity of this species to elucidate how temperatures exceeding 17 °C influence the survival and ecological roles of *D. belgicus* in groundwater ecosystems.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/environments12010032/s1: Table S1: Dataset of 302 oxygen consumption rate measurements for nine freshwater cyclopoid species. Table S2: individual measurements for both *Diacyclops belgicus* (DBE) and *Diacyclops crassicaudis crassicaudis* (DCR), detailing their dry weight (DW; in mg), oxygen consumption rates (OCRs, in μL O_2/ind·h), carbon requirements (CRs, in µg C/ind·d), ingestion rates (I, in µg C/ind·d), and egestion rates (E, in µg C/ind·da). The data are shown for trials conducted at 14 °C and 17 °C. Table S3: The PERMANOVA results for the oxygen consumption rates (OCRs), carbon requirements (CRs), and ingestion (I) and egestion (E) rates of *Diacyclops belgicus* and *Diacyclops crassicaudis crassicaudis* under two temperature conditions (14 °C and 17 °C). The analysis includes the effects of species (SP), temperature (TE), and the interaction between species and temperature (SP × TE). SS represents the sum of squares, MS is the mean square, Pseudo-F is the test statistic, P(perm) indicates the significance level based on permutation tests, and the number of permutations is provided for each test. Significant effects ($p < 0.05$) are in bold.

Author Contributions: Conceptualization, T.D.L.; methodology, T.D.L. and T.S.; validation, N.M., S.I., A.T.D.C. and D.M.P.G.; formal analysis, T.D.L.; investigation, T.D.L. and S.I.; resources, T.D.L.; data curation, T.D.L. and S.I.; writing—original draft preparation, T.D.L.; writing—review and editing, T.S., N.M., A.T.D.C., S.I. and D.M.P.G.; project administration, T.D.L.; funding acquisition, T.D.L. All authors have read and agreed to the published version of the manuscript.

Funding: T.D.L., A.T.D.C., N.M., and D.M.P.G. acknowledge Biodiversa+ DarCo, the European Biodiversity Partnership under the 2021–2022 BiodivProtect joint call for research proposals, co-funded by the European Commission (GA N°101052342) and the following funding organisations: the Ministry of Universities and Research (Italy), Agencia Estatal de Investigación-Fundación Biodiversidad (Spain), Fundo Regional para a Ciência e Tecnologia (Portugal), Suomen Akatemia-Ministry of the Environment (Finland), the Belgian Science Policy Office (Belgium), Agence Nationale de la Recherche (France), Deutsche Forschungsgemeinschaft e.V.-BMBF-VDI/VDE INNOVATION + TECHNIK GMBH (Germany), Schweizerischer Nationalfonds zur Forderung der Wissenschaftlichen Forschung (Switzerland), Fonds zur Förderung der Wissenschaftlichen Forschung (Austria), the Ministry of Education, Science and Sport (Slovenia), and the Executive Agency for Higher Education, Research, Development and Innovation Funding (Romania). T.D.L. also acknowledges support from NBFC to CNR, funded by the Italian Ministry of University and Research, P.N.R.R., Missione 4 Componente 2, "Dalla ricerca all'impresa", Investimento 1.4, Project CN00000033. TS and NM were funded by the Slovenian Research and Innovation Agency (Research programe P1-0255). S.I. acknowledges Biodiversa+, the European Biodiversity Partnership, in the context of the Sub-BioMon-Developing and testing approaches to monitor subterranean biodiversity in the karst project under the 2022–2023 BiodivMon joint call. The project was co-funded by the European Commission (GA N°101052342) and the following funding organisations: the Ministry of Higher Education, Science and Innovation (Slovenia), the Belgian Science Policy (Belgium), the Ministry of Universities and Research (Italy), the National Research, Development and Innovation Office (Hungary), the Executive Agency for Higher Education, Research, Development and Innovation Funding (Romania), and the self-financing partner, the National Museum of Natural History Luxembourg (Luxembourg).

Data Availability Statement: The data are contained within the article or Supplementary Materials.

Acknowledgments: The authors extend their gratitude to Andrea Castaño-Sanchez for her valuable support during the laboratory activities.

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

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