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Optimal Method for Biomass Estimation in a Cladoceran Species, *Daphnia Magna* (Straus, 1820): Evaluating Length–Weight Regression Equations and Deriving Estimation Equations Using Body Length, Width and Lateral Area

Doyeong Ku ¹, Yeon-Ji Chae ¹, Yerim Choi ¹, Chang Woo Ji ², Young-Seuk Park ³ , Ihn-Sil Kwak ⁴ , Yong-Jae Kim ⁵, Kwang-Hyeon Chang ^{1,*}  and Hye-Ji Oh ^{1,*} 

- ¹ Department of Environmental Science and Engineering, Kyung Hee University, Yongin 17104, Korea; 1920205@naver.com (D.K.); 016399co@naver.com (Y.-J.C.); cyl0124@naver.com (Y.C.)
² Fisheries Science Institute, Chonnam National University, Yeosu 59626, Korea; jichangwoo@gmail.com
³ Department of Biology, Kyung Hee University, Seoul 02447, Korea; parkys@khu.ac.kr
⁴ Department of Ocean Integrated Science, Chonnam National University, Yeosu 59626, Korea; iskwak@chonnam.ac.kr
⁵ Department of Life Science, Daejin University, Pocheon 11159, Korea; yjkim@daejin.ac.kr
* Correspondence: chang38@khu.ac.kr (K.-H.C.); ohg2090@naver.com (H.-J.O.); Tel.: +82-10-8620-4184 (K.-H.C.); +82-10-9203-2036 (H.-J.O.)



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Abstract: Assessing the biomass of zooplankton compensates for the difference between number of individuals and the accumulated body weight of the community, which helps assess aquatic ecosystem food web functions. *Daphnia* are crustaceans that play an intermediate role in biological interactions within food webs. The morphology and body specification of *Daphnia* differ during growth; hence, it is essential to apply species-specific equations to estimate biomass. We evaluated the length–weight regression equations used previously to estimate *Daphnia magna* biomass and conducted regression analyses using various body specifications and biomass measurements taken directly using devices such as a microbalance and microscopic camera. Biomass estimated using an equation from the Environmental Protection Agency was significantly different from the direct measurement: average biomass was lower, indicating that the equation possibly underestimated actual biomass. The biomass of *D. magna* had a higher multiple R^2 value when length was compared with width and area, and a linear regression equation was the most suitable equation for biomass estimation. Because body specifications and biomass are affected by various environmental factors, the development of accurate species-specific biomass estimation equations will contribute to obtaining fundamental data with which the biological responses of zooplankton to aquatic ecosystem changes can be assessed.

Keywords: *Daphnia*; biomass estimation; dry weight; prosome length; carapace width; lateral area; length–weight linear regression

1. Introduction

Zooplankton, as phytoplankton predators, affect the diversity and biomass of phytoplankton and play a role in transmitting energy from the lower to higher trophic level when they are predated by benthic macroinvertebrates and/or fish [1]. They vary in size in a species-dependent manner; generally, individual cladocerans and copepods are larger than rotifers, which affects their biomass [2]. The individual density of zooplankton also varies significantly depending on the classification group: large cladocerans and copepods >1 mm in size may occur at a density of <1 individual per liter, whereas small rotifers may occur at tens of thousands of individuals per liter [3]. Hence, when zooplankton communities are analyzed based on the number of individuals, overestimation or underestimation of

species' roles and contributions to ecosystem function can occur [4]. Biomass, on the other hand, is determined based on the size, generally the body length of each species, so it compensates for the gap between the number of individuals and the accumulated body weight of the biological community, which helps evaluate the functioning of zooplankton within the aquatic food web and enables more accurate relative comparison of their community among several water bodies [5,6].

The biomass of plankton communities can be used to calculate the primary and secondary productivities of water bodies [5]. Quantitative estimates of biomass and the consequent productivity of the plankton community underlying the grazing food web are essential for tracking material and energy flows [7]. Through biomass-based productivity comparisons between organisms at different trophic levels (e.g., phytoplankton–zooplankton–fish), it is possible to determine energy transfer efficiency, which represents an appropriate quantitative index of the circulation of matter in the aquatic food web [2,5,6]. Additionally, biomass is used to better reflect the role of zooplankton as a top-down controller for phytoplankton when using plankton community as an evaluation index for the water quality environment in rivers or lakes [8–11]. The biomass of a plankton community is affected by species composition, the number of individuals and individual size [5,12], and these factors vary depending on environmental changes at a global scale (e.g., climate change) as well as regional and local scales (e.g., habitat connectivity and water quality) [13–17]. Therefore, plankton biomass can be used as fundamental data for the assessment of biological responses to aquatic ecosystem changes. Zooplankton biomass itself is an important indicator of secondary production in marine and coastal ecosystems [5,12]. In freshwater, it has recently been used as a tool to assess the ecosystem health [8]. The biomass proportions of zooplankton functional groups are the main components of multiple metrics for the zooplankton biological integrity index (Z-IBI) [10]. In addition, species-specific zooplankton biomass is essential information required for ecological models such as AQUATOX that predict community dynamics and bioaccumulation of heavy metals in food web [18–20]. However, despite its importance and possible applicability in both ecology and environmental science fields, it remains a challenge to measure directly or estimate accurately the biomass of plankton per unit volume or unit area in lakes and rivers.

Daphnia is a medium to large cladoceran species (0.5–2.0 mm) commonly found in most freshwater ecosystems worldwide. It is the main predator of phytoplankton, causing the clear water phase phenomenon in spring, as well as a food source for fish, especially visual-dependent predators, because of the large size of individuals. Thus, determining the biomass of *Daphnia*, located in the intermediate position between phytoplankton and fish communities, is important for understanding the material and energy transfer processes of the grazing food web [21–23]. As *Daphnia* is too small for direct measurements of either wet or dry weight, its biomass is generally estimated indirectly using known regression equations obtained from the relationship between body length and weight [4,24]. In a length–weight regression equation for *Daphnia*, $y = a \cdot x + b$, x represents the total length from the individual's head except for its apical spine, i.e., the prosome length. Unfortunately, the prosome length of *Daphnia* is characterized by discontinuous changes during the molting process as the individual grows [25]. However, since the body width of *Daphnia* (i.e., the carapace width) changes according to individual nutritional status and body mass during the molting process, it can be considered a more stable factor in *Daphnia* biomass estimation [25]. Additionally, width-based biomass information is available in food chain research because the body width of crustacean species (e.g., cladoceran and copepod species) acts as a controlling factor in predator food selection [26,27].

Previously established length–weight estimation equations used to calculate the biomass of zooplankton, including *Daphnia* species, continue to be supplemented [28–30], and advanced methods, such as taking repeated measurements using a precision balance and image analyses, have been proposed to improve the precision of estimations and measurements [25,29,31]. In the present study, for the aim of suggesting a more accurate

biomass estimation method, we evaluated the suitability of the existing biomass estimation equations using a representative species of *Daphnia*, namely, *Daphnia magna* (Straus, 1820), by comparing biomass estimated using previously presented length–weight regression equations and biomass measured directly using a microbalance. Additionally, through regression analyses between the measured *D. magna* biomass and individual body specifications (i.e., prosome length, carapace width and lateral area), we derived and tried to propose a regression equation that is more suitable for estimating the biomass of *D. magna*.

2. Materials and Methods

2.1. *Daphnia Magna* as the Subject Species

We selected *D. magna*, a common species worldwide and a test species of OECD guidelines for testing chemicals (Test Guideline 211 [32]), as the subject of this study. We cultured *D. magna* individuals provided by the National Institute of Environmental Research of Korea in the laboratory for more than a year prior to the experiment. To secure the number of individuals required for biomass estimation and measurement, egg-bearing females were selected and cultured in 2 L beakers with aerated 21 °C tap water under a 16:8 h light:dark cycle and fed 3 mL of *Chlorella* sp. (3.0×10^5 cell/mL) once a day for two weeks prior to the experiments.

2.2. Estimation and Measurement of *D. Magna* Biomass

Length–weight regression equations for biomass estimation of *D. magna* have been proposed by Dumont et al. and Kawabata and Urabe [17,24]. The United States Environmental Protection Agency (US EPA) suggests that zooplankton biomass, including that of *D. magna*, should be estimated based on the length–weight relationship established by Dumont et al., McCauley and Lawrence et al. in the National Lakes Assessment-Laboratory Operations Manual [4,17,33–35]. In the EPA Standard Operating Procedure for Zooplankton Analysis, a *D. magna*-specific biomass estimation equation is not presented, but it includes an equation for *Daphnia* spp. established by Dumont et al. using pooled *Daphnia* species [17,36]. Accordingly, in the present study, the biomass of each *D. magna* individual was estimated using the length–weight regression equation established by Dumont et al. [17] (Table 1).

Table 1. Length–weight regression equations used to estimate the biomass of *Daphnia magna* (equations from Dumont et al. [17]).

	Equations	Characteristics	Unit
(1)	$W = 1.89 \times 10^{-6} \times L^{2.25}$	Derived using individuals from Donk Lake	W: μg L: μm
(2)	$W = 4.88 \times 10^{-5} \times L^{1.80}$	Derived using individuals from Sambre River	W: μg L: μm
(3)	$\text{Ln}(W) = 1.603 + 2.84 \times \text{Ln}(L)$	Derived using pooled <i>Daphnia</i> species	W: μg L: mm

To estimate an individual's biomass, its length (mm) was measured from the tip of the head to the end of the body, excluding the apical spines, under a microscope (CKX41; Olympus, Japan) according to the EPA manual [35] (Figure 1). Additionally, we measured the carapace width (mm) and lateral area (mm^2) of each individual. To improve the accuracy of measurements, we used a microscope digital camera (FX-500; Olympus, Japan) and an image viewer program (ImageView).

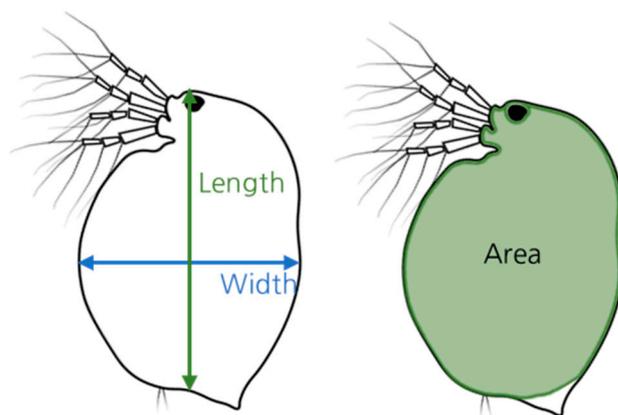


Figure 1. Measurements of *Daphnia magna* individuals' body specifications. Length: from the head to the end of the body, excluding the apical spines; width: carapace width; area: lateral area, excluding the area of the apical spines.

Individuals for which the length, width and area measurements were taken ($n = 90$) were placed in a preweighed aluminum tin capsule (a) and dried in an oven at $60\text{ }^{\circ}\text{C}$. After 24 h, the weight of the dried tin capsule was measured (b) using a microbalance (MYA 2.4Y; RADWAG, Poland). Weight measurements (a) and (b) were taken three times each to minimize the effect of errors during measurement, and the measured biomass of *D. magna* was calculated as follows:

$$\text{Measured biomass (dry weight, mg)} = (b)_{\text{avg}} - (a)_{\text{avg}}. \quad (1)$$

2.3. Statistical Analyses

Statistical analyses were conducted in R [37] to verify the validity of the established length–weight regression equation applied to estimate *D. magna* biomass and derive a more suitable regression equation for estimating the biomass of this species using directly measured biomass and body size. We compared the average biomass values estimated using the length–weight regression equations of Dumont et al. [17] and measured directly with a microbalance through ANOVA with the Kruskal–Wallis test since our data are not normally distributed (Shapiro–Wilk test, $p < 0.05$). The post hoc test was conducted with Bonferroni–Dunn correction, and we interpret that there is no statistically significant difference because the null hypothesis (H_0) that two groups we compared had the same size rejects when the p -value is larger than the $\alpha/2$ ($\alpha = 0.05$). Additionally, we attempted to identify the optimal equation for estimating *D. magna* biomass using regression analysis that included the measured biomass and individual body specifications (i.e., length, width and area). To visualize the regression analysis results, we used the R packages “ggplot2” and “hrbrthemes” [38,39]. Because reliability is maximized when the multiple R^2 value is close to 1, the suitability of the biomass estimation equation derived from the model fitting analysis was determined using the multiple R^2 values.

3. Results

3.1. Comparison of *D. Magna* Biomass Estimates and Measurements

Figure 2 shows the results of comparisons among the dry weight (biomass) values estimated using the *D. magna* biomass calculation formulas presented in Table 1 and the values obtained by directly measuring the dry weight using a microbalance. Differences in the biomass estimates obtained according to the equations of Dumont et al. [17] were detected (Table 2). Particularly, significant differences were found between the values calculated using Equation (1), derived from *D. magna* collected from a lake, and Equation (3), derived from pooled *Daphnia* spp. ($p < 0.025$; Figure 2, Table 2). The biomass estimates calculated using Equations (1)–(3) of Dumont et al. [17] differed from the direct biomass measurements (Table 2). Specifically, the biomass measurements differed significantly

from the value estimated using Equation (3), derived from pooled *Daphnia* spp. ($p < 0.025$; Figure 2, Table 2). Although not statistically significant, the estimates calculated from Equation (2), derived from *D. magna* collected in a river, showed some difference with the measurements ($p = 0.0263$). The direct biomass measurements did not differ significantly from the value estimated using Equation (1), based on *D. magna* from a lake (Figure 2).

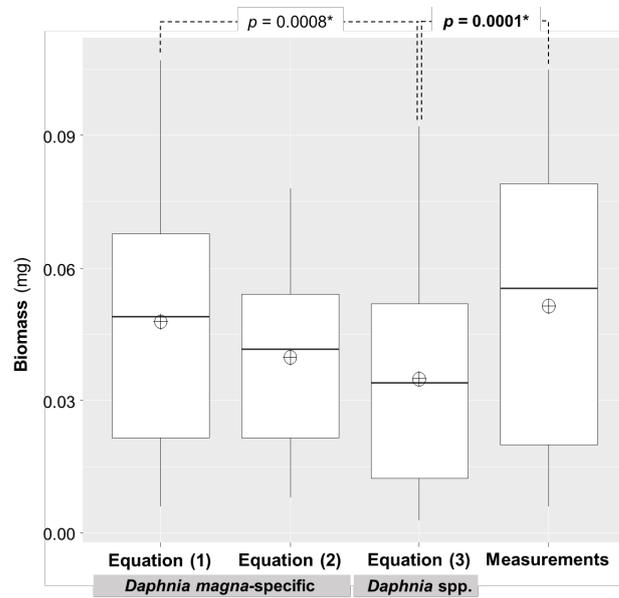


Figure 2. Comparison of the average ($n = 90$) *Daphnia magna* biomass estimates and biomass measurements. Estimates were calculated using Equations (1)–(3) of Dumont et al. [17] Equation (1) is based on *D. magna* from a lake, Equation (2) is based on *D. magna* from a river and Equation (3) is based on pooled *Daphnia* spp. Biomass measurements are shown as \oplus (mean values). p values of < 0.025 were considered statistically significant, and * shows significant differences between biomass values.

Table 2. Mean \pm standard deviation ($n = 90$) of biomass estimates and measurements taken from *Daphnia magna* individuals. Biomass estimates were calculated using length–weight regression equations, i.e., Equations (1)–(3) from Dumont et al. [17].

Biomass (dry weight, mg)	Equation (1) <i>D. magna</i> in Donk Lake	Equation (2) <i>D. magna</i> in Sambre River	Equation (3) ¹ Pooled <i>Daphnia</i> spp.	Measurements
Mean \pm Standard deviation	0.048 \pm 0.026	0.040 \pm 0.018	0.035 \pm 0.022	0.051 \pm 0.030

¹ Equation (3) is the length–weight regression equation proposed by the EPA manual [36] for calculating *D. magna* biomass.

3.2. Derivation of Regression Equations Considering Body Specifications and Biomass Measurements of *D. Magna*, and a Comparison of the Suitability of the Derived Equations

To derive the most suitable regression equation for estimating *D. magna* biomass (dry weight, mg), we used width and area as well as length, having measured the relevant body specifications of each individual (Table 3).

Table 3. Mean \pm standard deviation (with minimum–maximum range) of the body specifications of *Daphnia magna* individuals ($n = 90$). Length, width and area were measured in each individual.

Body Specifications	Length (mm)	Width (mm)	Area (mm ²)
Mean \pm standard deviation (Minimum–Maximum)	1.866 \pm 0.519 (0.796–2.791)	1.176 \pm 0.372 (0.469–1.948)	1.872 \pm 0.962 (0.314–4.073)

Following the regression analysis that included each body specification and biomass measurement of *D. magna*, the equations shown in Figure 3 were derived for each regression type: linear ($y = a \cdot x + b$), logarithmic ($y = a \cdot \ln x + b$) and exponential ($y = a \cdot e^x + b$) functions. The derived *D. magna* length–biomass regression equations were analyzed using the multiple R^2 values 0.904, 0.8689 and 0.8584 for the linear, logarithmic and exponential functions, respectively (Figure 3A–C). For the width–biomass regression equation, the multiple R^2 values were 0.8952, 0.8799 and 0.8383 for the linear, logarithmic and exponential functions, respectively (Figure 3D–F). In the derived area–biomass regression equation, the multiple R^2 values were 0.8892, 0.8774 and 0.5036 for the linear, logarithmic and exponential functions, respectively (Figure 3G–I). Regardless of the body specifications of *D. magna*, high multiple R^2 values were obtained in the following order: linear functions > logarithmic functions > exponential functions. Thus, the linear model was found to be best fitted when body specification–weight regression analyses were conducted (Figure 3). Based on the linear function, the length (prososome length) of *D. magna* is most suitable for estimating their biomass (Figure 3A,D,G).

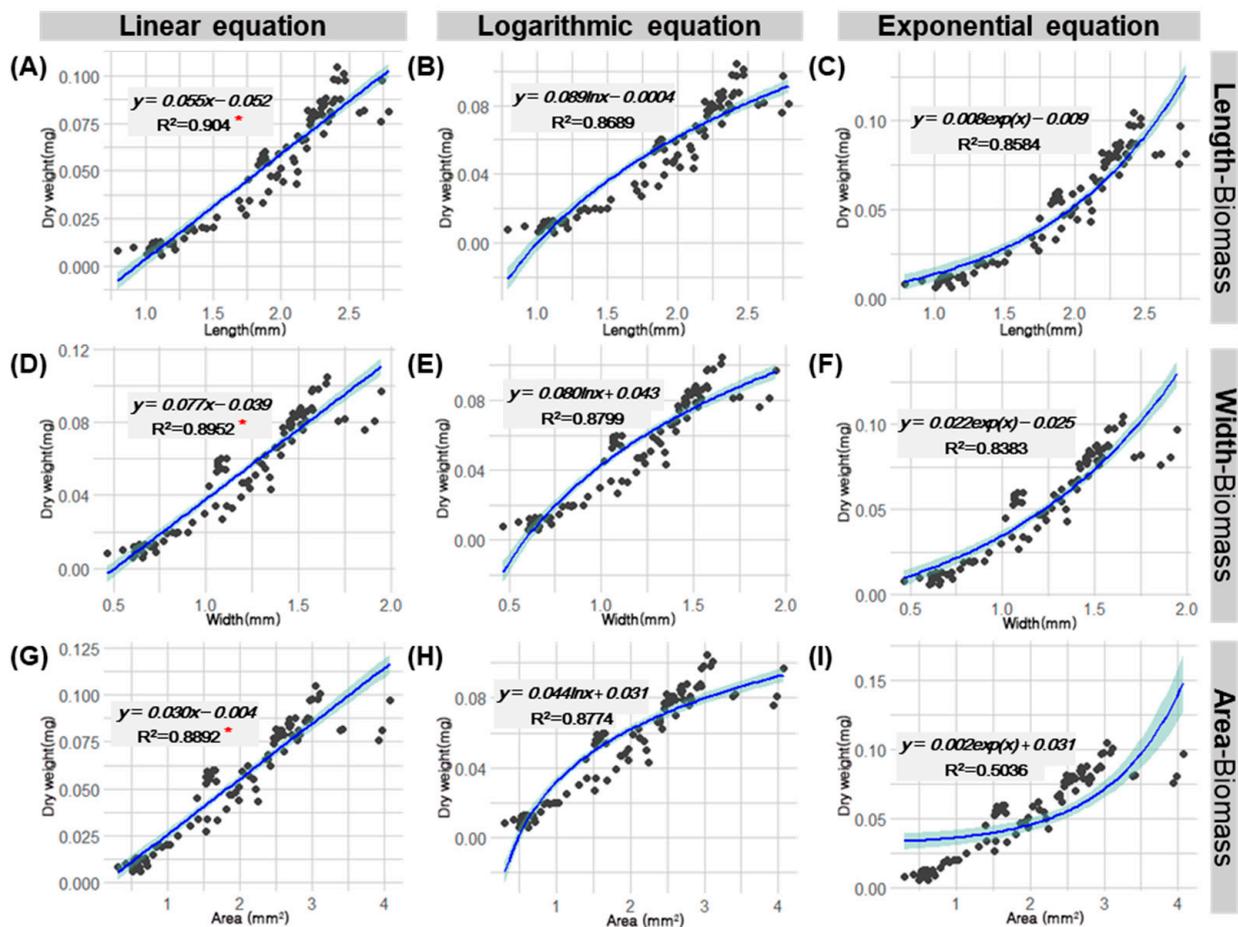


Figure 3. Regression analysis and fitting results between the body specifications of *Daphnia magna* individuals (length, (A–C); width, (D–F); area, (G–I)) and their biomass measurements. *, regression equation with the highest multiple R^2 value among the linear, logarithmic and exponential functions according to body specification factor.

When the residuals of the measurements were visualized for the fitted values (estimates) of the regression equations derived between each body size specification and biomass, in the linear functions, the residuals were relatively uniformly distributed, and the increasing trend of residuals according to those of fitted values was less noticeable compared with the logarithmic and exponential functions (Figure 4). Since negative residuals

occurred in the section where the fitted values are large, the regression equations could lead to overestimation when estimating the biomass of larger individuals (Figure 4A,D,G). However, deviation of the residuals was smaller than other regression functions. Finally, it was shown that the biomass of *D. magna* can be most effectively estimated using the length–weight linear regression equation; $y = 0.055 \cdot x - 0.052$ (Figures 3A and 4A).

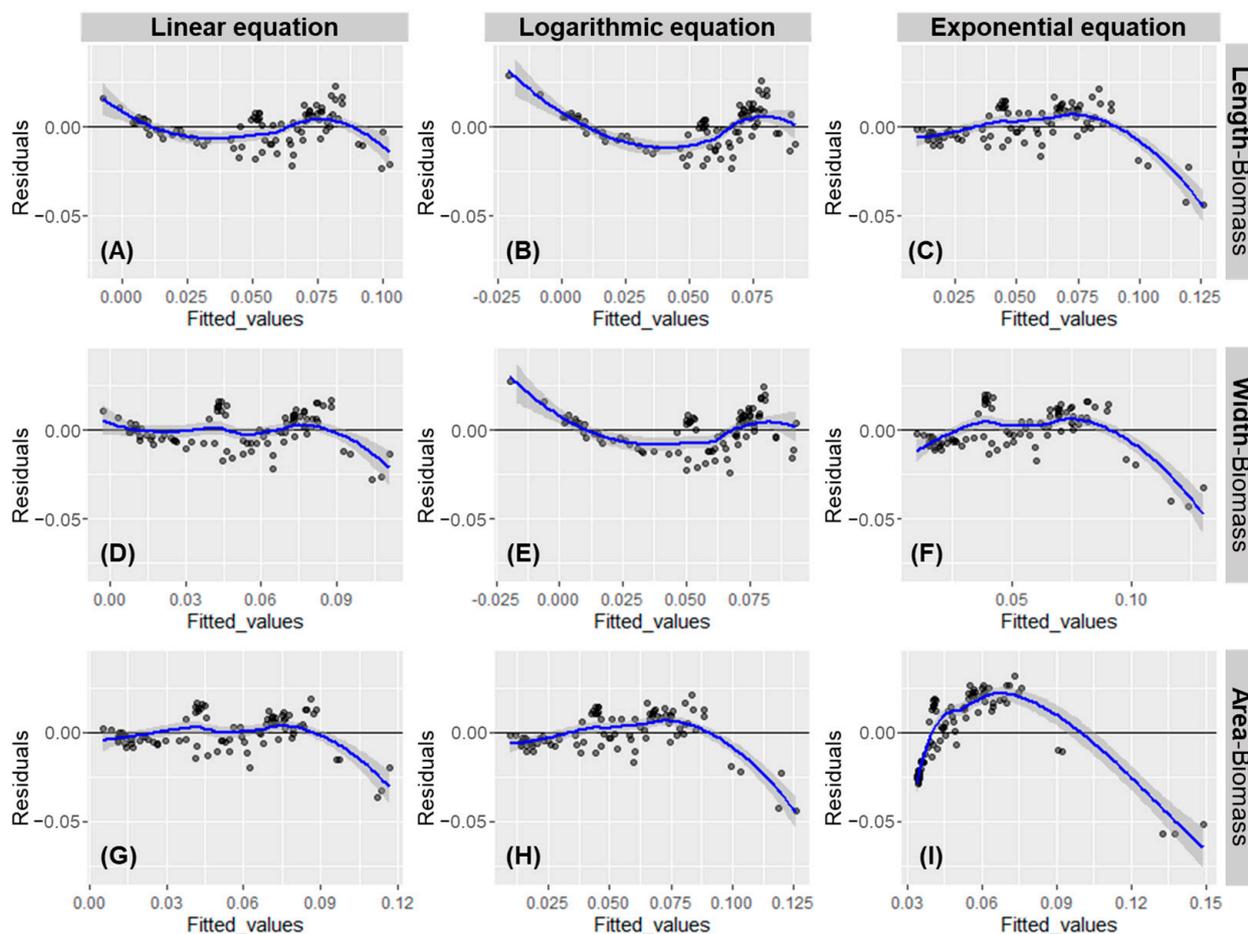


Figure 4. Fitted versus residual value plot of each regression equation derived from the body specifications of *Daphnia magna* individuals (length, (A–C); width, (D–F); area, (G–I)) and their biomass measurements.

4. Discussion

In this study, to develop a more suitable method for estimating the biomass of *D. magna*, the existing length–weight regression equations were evaluated [17], and new estimation equations were derived using regression analyses with the dry weight of *D. magna* according to their body specifications.

The biomass of *D. magna* estimated using the length–weight regression equation proposed by Dumont et al. [17] differed depending on the sampling environment of the individuals used to derive the equation and whether the *Daphnia* species were pooled. Moreover, a certain equation gave values that differed significantly from the biomass measured directly using a microbalance. The biomass of *D. magna* was lower on average in estimates compared with direct measurements. Especially, the estimated biomass was lowest when using the equation derived from pooled *Daphnia* spp., which is currently suggested by the EPA [36] as the equation with which to estimate the biomass of *D. magna*. Accordingly, there is a concern that this equation might underestimate the actual biomass. Additionally, from the slight difference between biomass values estimated from equations derived from the lake- and river-collected *D. magna*, it was judged that the effect of the

sampling environment of *D. magna* individuals used to derive the length–weight regression equations must be considered. Therefore, individual collection in various environments will be important in deriving equations that estimate the biomass of target zooplankton species accurately.

Although new regression equations were derived using the lateral area (containing both width and length information) and width (based on research results indicating that body width is a more stable factor in estimating the biomass of *Daphnia* species than length [25]) of *D. magna*, the existing approach used to estimate length-based biomass was found to be the most appropriate method. Compared with logarithmic and exponential functions, the highest multiple R^2 value was shown in the length–weight linear regression equation, and the residuals between the estimates calculated from the equation and the actually measured biomass using microbalance appeared in a relatively evenly stable distribution. Therefore, the biomass of *D. magna* is considered to be estimated most accurately by the length–weight linear regression equation using prosome length. This may be because of the relatively simple body morphology of *D. magna* compared with that of other *Daphnia* species. Additionally, most of the species in *Daphnia* develop appendages, such as helmets and apical spines, for defensive functions according to predation pressure [40,41], whereas the *D. magna* individuals used in the present study showed little appendage development, resulting in a higher correlation between their body length and dry weight (biomass). The degree of appendage development can affect the accuracy of length-based biomass estimation formulas for *Daphnia* species. For instance, in the case of *Daphnia galeata*, which varies greatly in its appendage development according to predation pressure and season, biomass tends to decrease as the length of the helmet and apical spines decreases [42]. Additionally, our regression analysis including the weight and length of *D. galeata* with its apical spines had a better fit compared with that of a similar analysis including the weight and length without the apical spine [unpublished data]. Given the increased length of appendages due to the morphological changes of cladoceran species, including *Daphnia*, the lateral area, considering both length and width, should be used as a reasonable parameter for estimating biomass [43].

We found that body specification, which is closely related to *Daphnia* biomass, seemed to differ among species (e.g., *Daphnia similoides*, width; *D. galeata* and *Daphnia pulex*, area), and the tendency for biomass to increase was seemingly different as body specifications increased [unpublished data]. This is likely because of differences in body morphology and the degree to which each body specification develops during the individual growth of *Daphnia* species [25]. Regression equations for estimating *Daphnia* biomass derived by pooling various *Daphnia* species without considering the species-specific relationship between body specifications and biomass may lead to errors when estimating the biomass of particular *Daphnia* species. Indeed, to develop more suitable biomass calculation methods, the existing species-specific biomass estimation equations must be reviewed, including those for *D. magna* assessed in this study.

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