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Biomass measurement of living *Lumbriculus variegatus* with impedance spectroscopy

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Abstract
Impedance spectroscopy is a useful tool for non-invasive and real time measurements of cell suspensions and a variety of biological tissues. The objective of this study was the investigation of the dielectric properties of living aquatic worms (*Lumbriculus variegatus*) using impedance spectroscopy in a frequency range between 100 Hz and 10 MHz. We demonstrate a linear relation between the worm biomass and the phase response of the signal thereby providing a quick and precise method to determine the biomass of aquatic worms *in situ*. Possible applications for non-destructive online biomass monitoring of aquatic worms and other aqueous organisms are discussed. Furthermore, we show that groups of worms fed different diets can be distinguished by the method presented. These results reveal a close relationship between the nutritional composition of the worms and the measured phase response. We also demonstrate that the phase response at 90 kHz does not depend on the worm size. In contrast, the response function for the signal at 440 Hz reveals a linear correlation of average individual worm size and phase. Therefore, we conclude that the measured phase response at 90 kHz qualifies as a measure of the total amount of worm biomass present in the measuring cell, whereas the phase measurement at 440 Hz can be used to estimate the average individual worm size.

Keywords: impedance spectroscopy, *Lumbriculus variegatus*, worm biomass, in situ measurement

Introduction

Electrical properties of biological tissues have been studied for over a century [1]. A large variety of different biological tissues have been investigated with the help of impedance spectroscopy, a detailed review on various human and animal tissue and blood samples can be found, e.g., in the review by Gabriel et al. [2]. Fricke and Curtis [3] investigated the impedance of yeast cell suspensions in the early 1930´s. They concluded that the impedance at the surface of the yeast cell is derived from a poorly conducting membrane which acts as a static capacitor. The dielectric properties of biological cells and their different components (cell wall, membranes and cytoplasm) were summarized by Markx and Davey [4]. Over the last couple of decades a lot of research was done on the impedance of cell suspensions, in which a relationship between capacitance and viable cell number was reported [5-8]. Fehrenbach et al. [5] used on-line capacitive measurements for biomass estimation of *Saccaromyces cerevisiae*, *Pitchia pastoris* and *Streptomyces virginiae* in suspension culture. In their experiments they could directly relate biomass concentration to the capacitance of the suspensions. Some of these techniques can be applied in an on-line measurement, e.g. for monitoring mycelial growth under industrial conditions, and their results are closer to physiological reality than traditional off-line methods. Nowadays, impedance spectroscopy is a well-established technique applied for both monitoring and controlling viable yeast cell concentrations in breweries and in biopharmaceutical industry for monitoring viable cell mass during fermentation processes [9]. Impedance spectroscopy is easy to use compared to traditional methods like cell count or spin down of the yeast slurry since it does not require accurate dilutions or addition of dyes. Yang et al. [10] used impedance measurements to detect viable *S. typhimurium*. They investigated both changes in double-layer capacitance at low frequencies (< 10 kHz) and medium impedance at high frequencies (> 10 kHz). Apart from measurements on cell suspensions, Kim et al. [11] investigated the influence of attached bacteria and biofilms on double-layer capacitance during biofilm formation. They measured a reduction in the double-layer capacitance due to bacterial adhesion and biofilm maturation. A recent review by Heileman et al. [12] covers different methods of interpreting dielectric spectra and summarizes applications of impedance spectroscopy for observations of cell suspension. In this work we show that the method can also be used for the investigation of larger organisms like, in our case, the aquatic worms *Lumbriculus variegatus*.

In general, the use of impedance spectroscopy has two main advantages:

1. It is fast, and can therefore be applied for online-monitoring;
2. It is a non-destructive method and hence can be used for measuring living organisms.

The objectives of this work are to measure mass, size and diet-based distinguishability (differences, variations) of the aquatic worms *Lumbriculus variegatus* using impedance spectroscopy.

Materials and methods

Impedance Analysis

All measurements were performed using an Impedance/Gain Phase Analyzer HP 4194A (Hewlett-Packard, California, U.S.A) which was connected via four BNC cables to a BDS 1200 connection head containing a BDS 1309 measurement cell (NOVOCONTROL Technologies, Germany) thereby applying voltage and measuring current separately in a bipolar electrode configuration. The BDS 1309 consists of two gold plated electrodes with a Teflon® isolation ring in between, the diameter of the electrodes was 11 mm and the distance between the electrodes was 6.1 mm. This sample cell is especially designed for high permittivity liquids. The software WinDETA (NOVOCONTROL Technologies, Germany) was used to calibrate the system using the stray capacity of the cell (1.2 pF) and to perform the measurements. We measured the absolute impedance and phase of different worm biomass quantities in a frequency range from 100 Hz to 10 MHz.

All measurements were performed at room temperature. The analyzer settings were chosen to provide 37 data points on a logarithmic scale from 100 Hz to 10 MHz with threefold averaging. The obtained impedance and phase spectra were fitted with "EIS Spectrum Analyser" software [18] using the Powell algorithm [19] to fit the spectra. Fig 1(a) depicts an equivalent circuit for the system simulating two parallel ways for the current to pass through the cell: electrode – water – electrode (R, W and CPE, C_{aq} and R_{aq}) and electrode – water – worm – water – electrode (R, W and CPE, $C_{aq,w}$ and $R_{aq,w}$, C_w and R_w). The double layer capacitance caused by electrode polarization (EP) is represented by a constant phase element CPE [20] with a Warburg impedance in parallel to account for ion migration. R and W impedance represent bulk properties of the electrolyte solution and diffusion features of the probe in the solution [21]. The electrical properties of water are represented by a resistance R_{aq} and capacitance C_{aq} in parallel. We modelled the aquatic worms as additional capacitance C_w and resistance R_w in parallel, in conjunction with the water between electrode and worms which is represented by a capacitance and resistance in parallel, $C_{aq,w}$ and $R_{aq,w}$. The contribution of EP is shown by simulating curves using the equivalent circuit (Fig. 1 a) without both Warburg impedance and the constant phase element. These simulations are shown as dotted curves in Fig.1 a and b. The contributions of the electrode polarisation are highlighted as blue areas. It should be noted that in Fig 1, the crosses (x) and large dots (●) represent measured values of buffered water and 323 mg worms in water, respectively, whereas the lines were calculated using the model depicted.

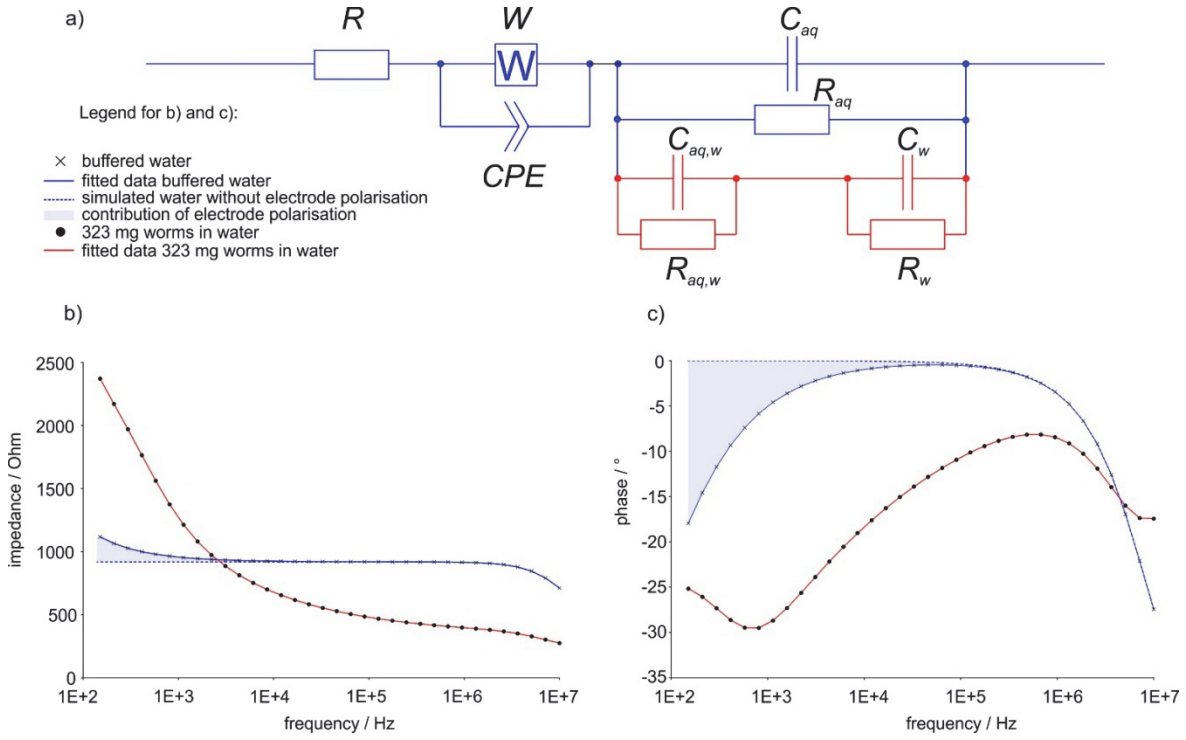

Fig. 1: Equivalent circuit (a), exemplary impedance (b) and phase spectra (c) of tap water and worm biomass in tap water, incl. fitting results. R, W and CPE are resistance, Warburg impedance and constant phase element simulating electrode polarization and ion migration; Caq and Raq the capacitance and resistance of buffered tap water when no current runs through the worms; Cw and Rw are capacitance and resistance of the worms, and Caq,w and Raq,w represent capacitance and resistance of the water between worms and electrodes.

Worms

The aquatic worms investigated in this work are called *Lumbriculus variegatus* (Oligochaeta; Lumbriculidae) (see Fig. 2). When cultivated, these aquatic worms are mainly used as fish feed for ornamental fish. But there is some on-going research on using them to reduce sludge in wastewater treatment plants or food waste streams in order to compact the waste stream thereby creating protein rich worm biomass as a nutrition source for animal feed [22]. Furthermore these worms have been used in toxicology studies and for monitoring water quality [23-27].

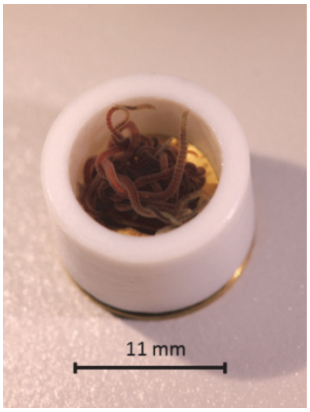

Fig. 2: Lumbriculus variegatus in the open measuring cell

The worms which we used for our experiments were cultured under the same experimental conditions but on different feeds, mainly TetraMin® fish feed and secondary potato sludge. TetraMin® is a commercial tropical fish feed which provides a complete diet for ornamental fish. Secondary potato sludge was collected from the process water of a potato starch factory.

In order to represent the environment of a real reactor (and thus the applicability of this method) as closely as possible, tap water was used as basis for the growth medium. Because living aquatic worms are constantly exchanging substances via their permeable skin (uptake of nutrients and exclusion of catabolic substances), this tap water was pH buffered. A controlled temperature and water flow system with 12 plastic flow-through beakers was utilized to test the different worm foods. Each beaker had a working volume of 770±8 mL and a surface area of 57 cm^2. Water was continuously discharged with a drain pipe positioned approximately 2 cm above the artificial sediment (at the 150 mL mark). The beakers were submerged in a temperature controlled bath at 19±1 °C and retained in dark conditions until the food containing sediment was replaced and/or sampling occurred. Water was supplemented at a flow rate of 4.34 mL min^{-1}, resulting in refreshment rates of 4 and 8 day^{-1}, respectively. The water (pH 7.2, hardness 77 mg $CaCO_3$) was a mixture of 70% tap water (Leeuwarden, the Netherlands) and 30% softened water. The water was disinfected with UV-C light to prevent inoculation of the beakers with microorganisms and was aerated to keep oxygen on a saturated level, pH was controlled by dosing HCl (37%) with the use of pH controlled dose pump (StepDos O8S). It was then supplied on the surface of each beaker by a needle dispenser, creating a constant flow of small droplets.

The total wet weight of the aquatic worms was determined with the help of a fine mesh on a laboratory balance. Prior to weighing, worms were kept in fresh buffered tap water without any food for 2 hours in order to give them time to purge their guts. Afterwards the worms were cleaned with fresh buffered tap water. The clean worms were collected on top of the mesh and paper towels were gently pressed against the back of the mesh for 10 seconds to drain the remaining water. Average individual weight was calculated by dividing worm biomass by worm number.

As a concluding statement, we would like to point out that *Lumbriculus variegatus* are able to perform osmoregulation. This ability makes them extremely adaptive and enables them to live in environments with fluctuating ionic contents, like for example ditches or tarns. The measurements presented in this work were performed on worms in the same environment as the reactor in which they were grown. This environment will differ largely in various applications such as food waste streams or wastewater treatment plants. In principle, the evaluation presented focusses on two distinct frequencies: 90 kHz and 440 Hz. At 90 kHz the contribution of electrode polarisation is very small, and it is safe to assume that this evaluation will work mostly independent from the worm environment. However, at 440 Hz electrode polarisation is large, and although it can be taken into account for the data presented, such a procedure might not be applicable at higher ion concentrations.

Results and discussion

Fig. 3a shows the measured impedance spectra for 6 different amounts of biomass of living *Lumbriculus variegatus* in buffered tap water. Taking the electrode polarisation of the buffered tap water into account (see Figs. 1c and 3b) one can immediately identify the α- and β-dispersion regions of the spectra of the samples containing worms: α-dispersion up until 1 kHz, and β-dispersion starting from that value.

At frequencies above 1 kHz the impedance decreases with worm biomass due to higher conductivity of the worm's intra- and extracellular body fluids compared to the surrounding tap water. At frequencies below 1 kHz an increase of the impedance occurs due to additional capacitive effects due to polarisation of the worm membranes, represented by C_w. The influence of the worm biomass on the overall impedance is smallest at 1 kHz. At that frequency the conduction mainly occurs through the water surrounding the worms and due to larger amounts of worms inside the measurement cell the amount of charge carriers is reduced, therefore the impedance is higher. Fig. 3b shows the measured phase response of these samples.

The relationship between the observed phase response and the amount of worm mass seems inversely proportional up to an isophasic point at approximately 3 MHz and directly proportional afterwards.

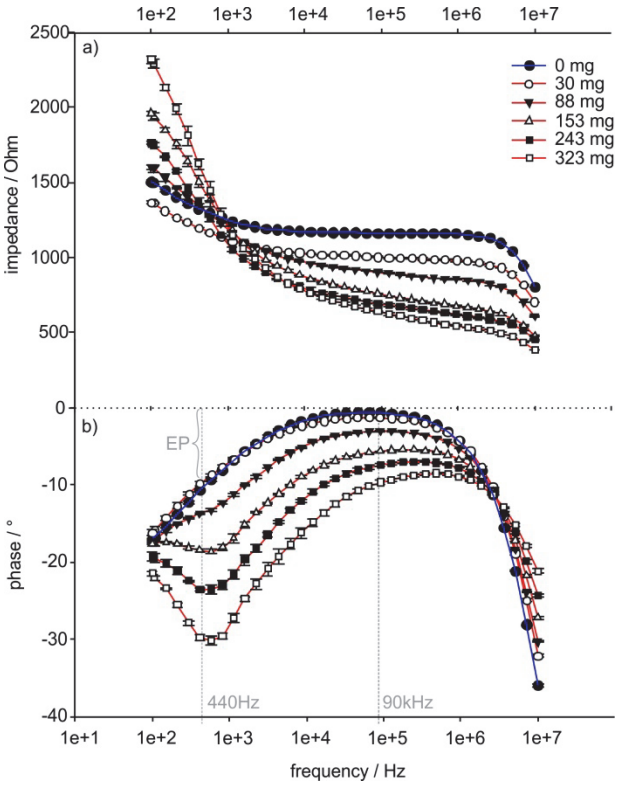

Fig. 3: Impedance (a) and phase (b) against frequency plotted for 6 different amounts of living *Lumbriculus variegatus* fed with TetraMin® measured in buffered tap water. The two evaluation frequencies and the contribution of electrode polarization to the phase shift are marked grey.

The largest phase differences were found at 570 Hz, but the correlation between biomass and phase response is not linear at that frequency. Up until approximately 2 kHz, the phase response of the smallest amount of worms measured (30 mg) is slightly higher than that of tap water. In order to investigate this feature, the influence of the worms on the buffered tap water was investigated. The buffer was measured before and after 60 min (the typical time of a threefold measurement) exposure to living aquatic worms. The result is given in Fig. 4 showing the same features as the 0 mg and 30 mg curves in Fig. 3: A decrease of the impedance at all frequencies accompanied by a small increase of the phase in the low frequency range only. This effect can be explained by the fact that the skin of the worms may leak small amounts of proteins or salts, thereby reducing impedance and increasing electrode polarisation. If there is sufficient water left in the cell, its impedance is lower at low frequencies where ion movement plays a major role. This effect disappears as soon as the biomass reaches a certain volume. In order to avoid this effect we chose 90 kHz as measurement frequency, which allows good resolution and small deviations in between single measurements (small error bars), and the results (Fig. 5) are not influenced by small composition changes of the buffered tap water (see Fig. 4).

It is important to point out that the general contribution of electrode polarisation at this frequency is very small (see Fig 1c). Since it is a feature of the liquid, not of the worms, it can be assumed constant for all worm concentrations. Because the evaluation is based upon phase differences, electrode polarisation contributions cancel each other out. In Fig. 5 we present the aforementioned linear relation between worm biomass and phase response.

Apart from the worm mass investigation, as feasibility study two groups of worms feeding on different diets were compared in order to see whether such a differentiation is possible. Fig. 5 shows that this might indeed be the case, since the slopes for worms with different diets are different. A covariance analysis [28] was performed to determine whether this difference is significant or not. In detail, this covariance analysis was executed using an independent variable m (mass) as covariate and a dependent variable ϕ (phase) as outcome in a two group analysis of covariance. Two procedures were carried out to do the comparison: Firstly, two regression lines $\phi_1 = k_1 m + c_1$ and $\phi_2 = k_2 m + c_2$ were computed, then the two regression coefficients were compared in order to check whether they are significantly different. This method is equivalent to evaluating whether interaction between groups and covariates exists. Secondly, we assumed that the two regression coefficients are not significantly different and calculated a common regression slope for the whole set of data. We then compared the mean dependent ϕ values (adjusted for the common regression slope). This second part is a covariance analysis where the dependent variable is ϕ and the covariate is m. The first procedure is equivalent to evaluating the presence of interaction between the covariates and the groups, and the second procedure is a simple analysis of covariance for two groups with one covariate. The result of this analysis is given in Tab. 1.

Tab.1: Covariance analysis of the regression slopes of mass (m) against phase (ϕ) at 90 kHz of worms fed with either TetraMin® (T) or secondary potato sludge (S) as shown in Fig. 5. SD means standard deviation.

	TetraMin® (T)	**Sec. potato sludge (S)**
n	18.00	30.00
$mean_m$ / mg	140.00	142.00
SD_m / mg	118.27	110.09
$mean_\phi$ / °	-4.11	-4.50
SD_ϕ / °	3.31	3.63
r	-1.00	-1.00
t	-46.18	-65.32
p	< 0.0001	< 0.0001
Slope *k* / (°/mg)	-0.0278	-0.0328
Const *c* / °	-0.2227	0.163
Com. Slope / (°/mg)	-0.0308	
Com. const. / °	-0.534	

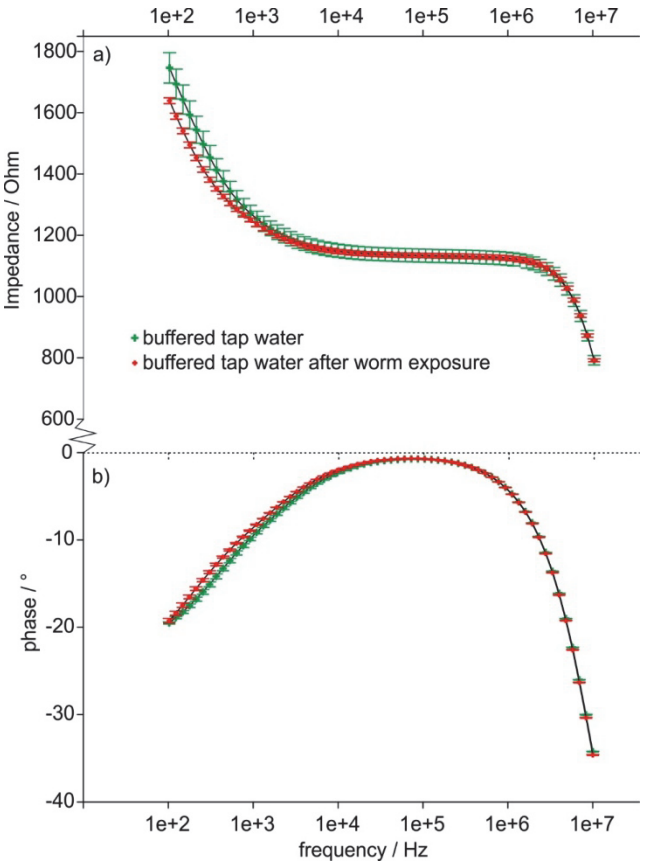

Fig. 4: Comparison of impedance (a) and phase (b) of buffered tap water before and after 60 minutes exposure to living aquatic worms.

The covariance analysis shows that the difference between the two slopes is highly significant ($P<0.0001$); so the slope of the regression lines depends on the diet of the worms. As can be seen in Fig. 4, changes in the composition of the buffered tap water due to the metabolism of the worms are not visible at this frequency (90 kHz). Instead this effect is caused by small changes in the composition of the worms which can be associated with the composition of the feed. A steeper decline equals a stronger phase response dependence which in turn signifies a higher capacitance of the sample. Such an increased capacitance of the worms fed with potato sludge results either from more worms with a high permittivity, or fewer worms with a lower permittivity than the worms fed with TetraMin®. Whereas the exact composition of aquatic worms is complex and cannot be simplified easily, it can be expected that the substance with the lowest permittivity accumulated in a worm is fat, and the highest permittivity is found in ionic solutions within the worms. A possible, reasonable consequence from this train of thought would be that the worms fed with potato sludge either contain less fat or more ionic solutions than the ones fed with TetraMin®. A validation of this assumption which will include an actual fat measurement and the analysis of the admittance spectra is planned and will be subject of a work subsequent to this one.

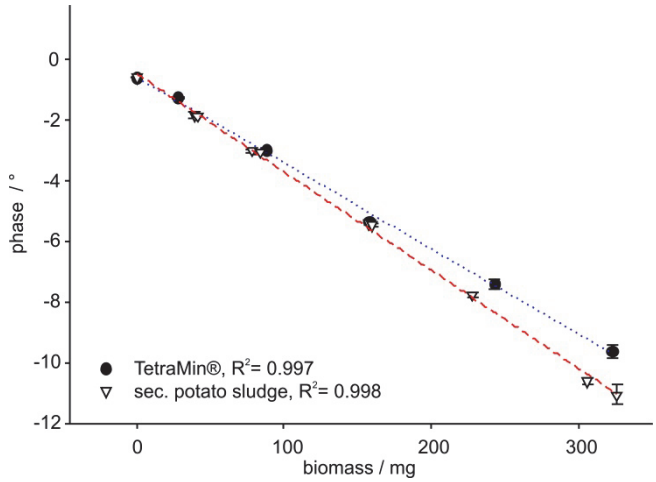

Fig. 5: Phase response at 90 kHz against worm biomass of two different kinds of *Lumbriculus variegatus* measured in tap water. The two groups of worms were fed with TetraMin® and secondary potato sludge, respectively.

Because individual worms always differ in size from each other, the ratio of worm surface to worm mass and thus the amount of membrane (skin) per worm is different at each measurement. This difference is visible in the region where membrane polarization is predominant, but does not influence measurements at higher frequencies, as illustrated in Figs 6 and 7: Fig. 6 shows the dependence of average worm size on the phase response at 440 Hz. At this frequency, the phase shift consists of electrode polarization (see also Fig. 1b) and α-dispersion. Although clearly larger than at 90 kHz the electrode polarization contributions can again be assumed to be constant for different worm concentrations, thereby cancelling each other out when phase shifts are compared. The resultant slope is thus a measure for α-dispersion. Whereas this evaluation worked well for the data presented, we would like to emphasize that it should be applied with cation since electrode polarization may vary with time or other factors that cannot be controlled. Fig. 7 shows the independence of the average worm size (no significant phase difference) in the β-region at 90 kHz for the same group of worms.

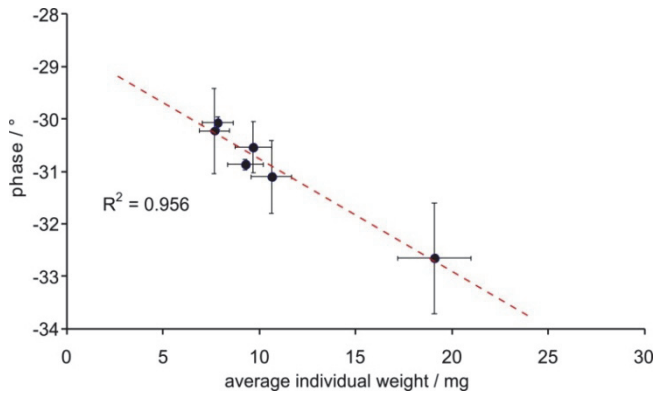

Fig. 6: Phase response per mg of worm for 6 groups of differently sized groups of worms from different diets at 440 Hz. Diets from left to right: TetraMin® / starch mix COD/N=37; TetraMin® / starch mix COD/N=56; TetraMin® pure; Okara; soy meal; chlorella. Error bars are due to worm weight variation (abscissa) and measurement error (ordinate), respectively.

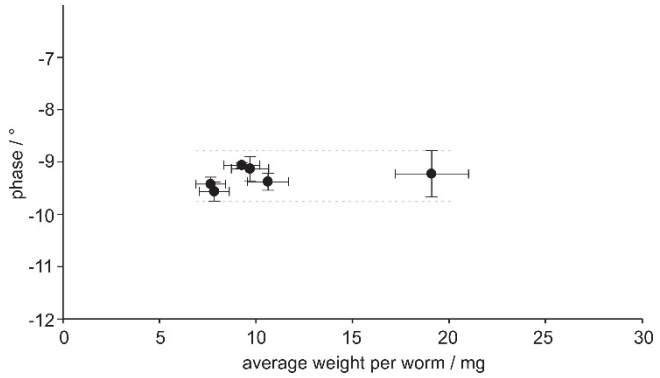

Fig. 7: Phase response per mg of worm for 6 groups of differently sized groups of worms at 90 kHz. Diets from left to right: TetraMin® / starch mix COD/N=37; TetraMin® / starch mix COD/N=56; TetraMin® pure; Okara; soy meal; chlorella. Error bars are due to worm weight variation (abscissa) and measurement error (ordinate), respectively. The dotted grey lines are a visual aid showing the upper and the lower end of the measurement error.

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Conclusions

Measurement of impedance and phase shift in the frequency range from 100 Hz to 10 MHz provides information about the biomass of living aquatic worms. We have shown that the biomass of the aquatic worm *Lumbriculus variegatus* can be measured *in situ* by impedance spectroscopy using information from both α- and β-dispersion. There is a linear relation between the phase shift at 90 kHz (β-dispersion) and the total amount of worm biomass which is independent from the average individual worm weight. In contrast to this, a linear dependence of the phase shift on the average individual worm weight was observed at 440 Hz (α-dispersion). Moreover it is possible to distinguish between different groups of worms based on their nutritional composition by measuring changes in the phase response at 90 kHz. The authors are planning to further investigate this relationship in subsequent work. Worm behaviour was monitored before and after measurements and no abnormalities were observed, thus it can be concluded that the worms are not negatively affected by the applied measurement signal. Therefore, these measurements are a suitable basis for future online impedance biomass monitoring of *Lumbriculus variegatus* and other aquatic invertebrates for instance in flow through bioreactors for food industry waste streams.

Acknowledgements

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