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Short Communication

Micro-computed tomography (μCT) as a novel method in ecotoxicology — determination of morphometric and somatic data in rainbow trout (*Oncorhynchus mykiss*)

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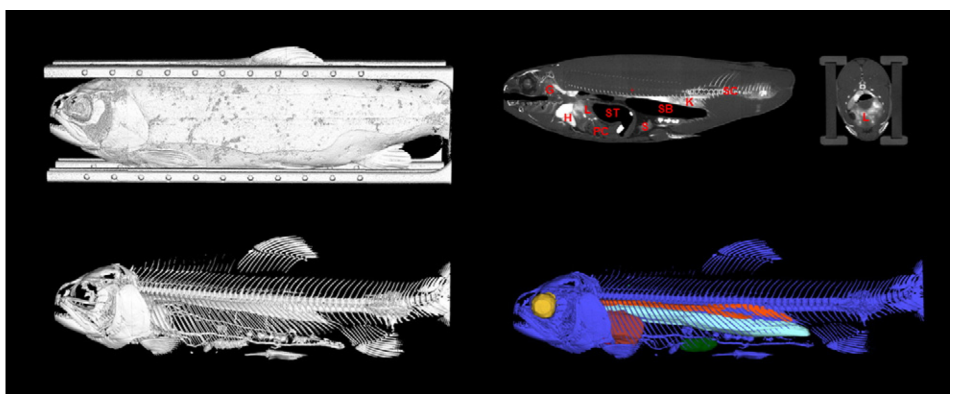
HIGHLIGHTS

- μCT was used for volumetric imaging sediment-exposed and unexposed rainbow trout
- Liver volumes determined by μCT were highly correlated with liver weights.
- The perfusion of organs in fish could also be studied by means of μCT.
- It was shown that μCT is a useful tool in context of ecotoxicological research.

GRAPHICAL ABSTRACT



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ABSTRACT

Fish are important sentinel organisms for the assessment of water quality and play a central role in ecotoxicological research. Of particular importance to the assessment of health and fitness of fish stocks in response to environmental conditions or pollution are morphometric (e.g. Fulton's condition index) and somatic indices (e.g. hepatosomatic, and gonadosomatic index). Standard measurements of somatic indices are invasive and require, by definition, the sacrifice of examined animals, thus prohibiting longitudinal studies and relocation of animals captured in the field. As a potential solution, in the present study, we propose the use of micro-computed tomography (μCT) as imaging modality to non-invasively tomographically image rainbow trout (*Oncorhynchus mykiss*) exposed to different sediment suspensions. We here demonstrate that μCT can be used as a tool to reliably measure the volumes of different organs, which could then be applied as a substitute of their weights in calculation of somatic indices. To the best of our knowledge, this study is the first to report the results of μCT analyses in the

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Liver somatic index
Micro-computed tomography

context of ecotoxicological research in rainbow trout. It has the potential to greatly increase the information value of experiments conducted with fish and also to potentially reduce the number of animals required for studying temporal effects through facilitating longitudinal studies within the same individuals.

1. Introduction

Fish are widely distributed throughout aquatic systems and represent important sentinel organisms for the assessment of water quality (Hallare et al., 2011). Apart from the numerous important ecological functions they fulfill, fish are of great economic importance and represent the main source of animal protein in many regions of the world (Brunner et al., 2009). Aquatic pollution thus not only poses a severe risk to wildlife, but also to human health, which is the reason why fish have always played a central role in ecotoxicological research (van der Oost et al., 2003).

Despite the rapidly growing number of methods to assess mechanistic effects of chemicals (Brinkmann et al., 2010, Wernersson et al., 2015), ecotoxicological research still relies on apical endpoints, i.e. empirically verifiable evidences of exposure, such as effects on development, reproduction, or survival (Villeneuve and Garcia-Reyero, 2011). Of particular importance to the assessment of the fitness of fish stocks are morphometric indices. Such indices enable relatively easy comparisons of length–weight relationships (e.g. Fulton's condition index) or the relative contribution of different organs to the total body weight (somatic indices, e.g. the hepatosomatic index, HSI) between different populations of fishes or treatment groups within an experiment (Bolger and Connolly, 1989). While the application of somatic indices is a reliable means of assessing general environmental quality and the effects of aquatic pollution in fishes, measurement of these parameters is by definition invasive and requires the sacrifice of studied animals, thus prohibiting longitudinal studies and relocation of animals captured in the field.

To overcome these limitations, we investigated the use of micro-computed tomography (μCT) as a non-invasive imaging modality to assess ecotoxicological parameters in studies involving rainbow trout. This technology allows accurate quantitative volumetric imaging of biological specimens, with precise acquisition of three-dimensional data of different organs based on their inherent differences in contrast, especially of bone tissue (Gremse et al., 2014). For being a very suitable technique in longitudinal studies, with highly valuable and reliable anatomical information it has been the main method of choice in several small rodent models, i.e. mice (Ehling et al., 2015, Schürmann et al., 2015), as well as other species and small-bodied fishes such as the zebrafish (Martinez et al., 2014, Martinez et al., 2015, Schulz-Mirbach et al., 2013, Seo et al., 2015). Native μCT scans, i.e. without use of contrast agents, can be applied to differentiate between bones, fat, air, and other soft tissues, while distinction between the latter (e.g. muscle, liver, kidney), as well as better defined visualization of vessels and perfusion parameters may require the use of contrast agents (Gremse et al., 2011). μCT is a powerful, rapid and inexpensive imaging method for tomographic analysis, providing excellent spatial resolution, as well as morphological and structural information (Ehling et al., 2013, Ehling et al., 2014), therefore holding interesting potential in longitudinal assessment of ecotoxicological effects in fish populations. The evaluated fish originated from the exposure experiments of a recently published study on the effects of sediment-borne pollutants in rainbow trout (Brinkmann et al., 2015) and were subjected to non-invasive contrast-enhanced μCT scans. Somatic information from segmented μCT images was subsequently compared with gravimetric data, and we discuss the potential use of μCT to study the perfusion of organs in fish.

2. Methods

2.1. Fish

Rainbow trout were purchased from a commercial hatchery (Mohnen Aquaculture, Stolberg, Germany) and allowed to acclimatize to laboratory conditions for one month prior to use in the experiments. Fish were reared in groups of 100–150 individuals in 1500 L glass fiber-reinforced plastic tanks. In a recirculating system with a 400 L biofilter and UVC-sterilizer, water was continuously exchanged at a rate of approx. 0.5 full replacements per day with municipal tap water. Light and dark phases were 12 h each. Fish were fed with commercial trout pellets (Biomar, Brande, Denmark) at a rate of 1% bodyweight per day. All experiments were conducted in accordance with the Animal Welfare Act and with permission of the federal authorities (Landesamt für Natur,

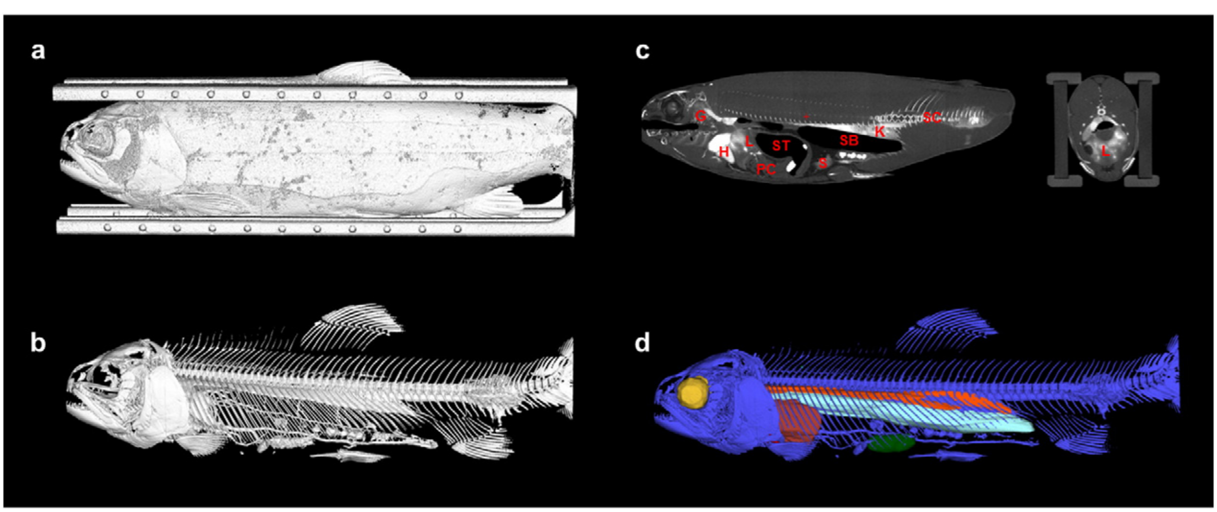

Fig. 1. CT-based reconstruction of a non-invasive in vivo μCT scan of a juvenile rainbow trout with Imeron-enhanced contrast. Contrast-based volume rendering of the entire fish within the scanning bed (a), and bone tissues and blood vessels (b). The following organs/tissues can be identified: G, gills; H, heart; L, liver; ST, stomach; S, spleen; SB, swim-bladder; K, kidney; SC, spinal cord (c). Segmentation of soft tissues after reconstruction of μCT scans (d): dark blue, bony tissues; yellow, eyes; orange, kidney; light blue, swim bladder; light red, heart, dark red, liver; green, spleen. Such images could be used to (non-invasively) determine morphometric indices, such as the hepatosomatic index (HSI), based on the volumes of the respective organ in comparison to the total weight/volume of fish.

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2.2. Experimental design and exposure conditions

As described in Brinkmann et al. (2015), fish were exposed to suspensions of two different sediments, Prossen (PR, Germany, close to the Czech border) and Zollelbe (ZE, Magdeburg, Germany), from the river Elbe over a period of 90 days. Both sediments were diluted 2-fold with sediment from the Rhine harbor Ehrenbreitstein (EBR) in the vicinity of Koblenz, Germany. Sediments were chosen to represent different contamination levels with organic contaminants; these were lowest in EBR, higher in PR and highest in ZE sediment (Appendix A). Experiments were conducted in 550 to 750 L glass fiber-reinforced plastic containers purchased from AGK Kronawitter (Wallersdorf, Germany) as previously described in Brinkmann et al. (2013). Submersible pumps were used to constantly suspend the sediments at a nominal concentration of 5 g L^{-1}. Pumps were separated from the main volume of the tanks by use of perforated stainless steel sheets. Tanks were additionally aerated and tempered through stainless steel heat exchangers. Sediment suspensions were replaced semi-statically with fresh suspensions in intervals of 10 days, and fish were fed commercial trout pellets (Biomar) at a rate of 1% bodyweight per day.

In untreated control animals, as well as 90 days after exposure to the two sediments, $n = 3$ fish per group were randomly removed from the tanks.

2.3. Anesthesia and μCT imaging

Fish were individually anesthetized in a solution of benzocaine in tap water (>250 mg L^{-1}; Sigma-Aldrich, Deisenhofen, Germany). Dose was chosen so that specimens would remain still during the scanning time and avoid motion artifacts; length and body weight were determined. Subsequently, fish were injected into the caudal vein with the contrast agent Imeron 400® (Bracco Imaging, Konstanz, Germany) at a dose of 4 ml kg^{-1} to increase the contrast of richly perfused organs and more accurately allow their segmentation (Hallouard et al., 2010). The most appropriate contrast-enhanced timing was defined in a preliminary longitudinal scan ($n = 1$) immediately post-injection, at 7, 15, 30 and 60 min post-injection (data not shown). Contrast-enhanced μCT scans were performed in all animals 1 h post-injection in a TomoScope 30s Duo (CT Imaging, Erlangen, Germany) with the sequence SQD-6565-360-29, with 2–5 subscans per animal (dependent on the size, 29 s per subscan), and acquisition of a topogram prior to scanning. Obtained datasets were reconstructed in house and further segmented based on anatomical identification of the organs and their contrast-enhanced densities, using the Imalytics Preclinical software (Gremse et al., 2015a). Bone structures were segmented based on their high radiodensity, while other organs (eye, heart, liver, spleen, kidney and swim bladder) where interactively segmented by interactively delineating their boundaries (Gremse et al., 2015b), resulting in a voxel-wise binary mask, providing accurate organ volumes non-invasively. Hepatic blood vessels were manually segmented, following their branching patterns on all transversal, coronal and sagittal views, and allowing visualization and comparison of the hepatic vascularization. Subsequent to the imaging procedure, fish were killed by exsanguination and the livers were carefully excised and weighed.

2.4. Data analysis

All calculations were performed in spreadsheets using Microsoft Excel™ 2013. Graphs were plotted and statistically analyzed by use of the software GraphPad Prism 6 (GraphPad, San Diego, USA). All datasets were analyzed by means of One-way ANOVA ($p \leq 0.05$) with Dunnett's post-hoc test ($p \leq 0.05$). The probability of Type I error (α) was set to

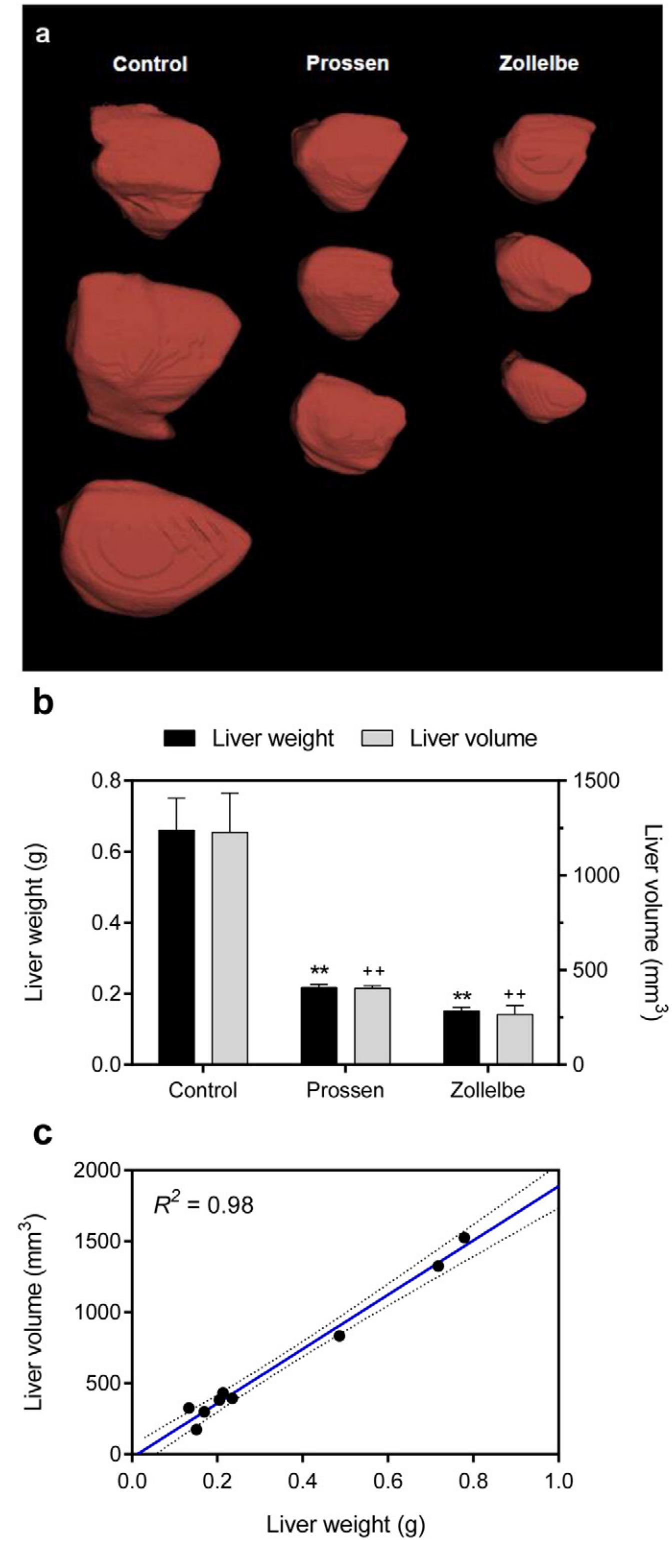

Fig. 2. Non-invasive in vivo segmentation of the liver volumes of $n = 3$ fish per group (a), comparison of gravimetrically determined weight (g, left y-axis) of excised livers with volumes (mm^3, right y-axis) determined by means of μCT analysis (b), as well as correlation analysis of weights and volumes (Pearson's correlation, $R^2 = 0.98$) (c). Livers of control animals were significantly larger than the other groups. Asterisks indicate statistically significant differences of liver weight, while crosses depict statistically significant differences of liver volume compared to unexposed control animals (One-way ANOVA with Dunnett's Post-hoc test, $p \leq 0.01$).

$p \leq 0.05$. Unless indicated, all values are expressed as mean value ± standard deviation.

3. Results and discussion

We here demonstrate that μCT can be efficiently used for segmentation of bone structures, which can be performed automated because of the high radiodensity and thus provides a rapid and reliable means of detecting effects of environmental or anthropogenic stressors on skeletal development in fish (Fig. 1). Using this data, μCT images could be used to indubitably determine meristic formulas of fishes, i.e. the number of fins and scales that can help to identify fish species. Classically, such analyses are performed microscopically through time-consuming staining of calcified structures using alizarin red (Song and Parenti, 1995) or calcein (Parichy et al., 2009), or by maceration of tissues followed by macroscopic inspection (Cowx, 1983). The advantages of μCT can be seen in the non-invasive aspect of the scans, the potential for simplifying the identification of skeletal abnormalities, as well as improved throughput and conservation of the general tissue structure. Application of contrast agents is not even necessary if only the skeletal structures are of interest.

Furthermore, we were able to demonstrate that μCT can be efficiently used for semi-automated and/or manual segmentation of different organs, including the eyes, kidney, swim bladder, heart, liver, and spleen (Fig. 1c and d). The resulting information can be used to identify aberrations and to quantify the volumes of individual organs for non-invasive determination of morphometric indices, such as the HSI, and for comparison among different treatment groups to address potential effects of environmental or chemical stressors.

Although the randomly sampled animals from exposure experiments with the two suspended sediments provided only a small sample size for statistical comparisons, we were able to depict significantly greater liver volumes of untreated control animals compared to sediment-exposed fish by means of μCT imaging (Fig. 2). No differences were observed between the two different sediment treatments. Comparison with the conventional assessment based on the liver weights revealed a high degree of correlation between liver weights and volumes (Fig. 2c; Pearson's correlation, $R^2 = 0.98$, $p < 0.0001$), supporting our data from non-invasive imaging. This finding is particularly promising in context of the potential application of non-invasive μCT in longitudinal studies. This would allow for temporally scanning the same individuals repeatedly without the need to sacrifice groups of fish for each time point, resulting in reduction of the fish number in ecotoxicological experiments, and significantly increasing the statistical power. Furthermore, we were able to demonstrate that functional information, e.g. on the perfusion of organs such as the liver can be derived from manual segmentation (Fig. 3), thereby adding further information depth to ecotoxicological studies with potential use in toxicokinetic modeling (Pöschinger et al., 2014).

In our study, we applied contrast agent to enable better discrimination between different organs and soft tissue types, which would be difficult with native μCT scans, i.e. without injection of contrast agent. It remains to be shown, however, how much contrast agent is needed for sufficient image analysis and how much the fish are affected by possible side effects, particularly in longitudinal contrast agent applications. The advent of phase-contrast μCT devices bears potential to completely avoid the application of contrast agents, however. This technology is based on the phase shift of X-rays in biological tissues and provides higher soft tissue contrast compared to the standard absorption-based contrast. Current implementations are prone to high X-ray doses, long scanning durations, and may require expensive synchrotron facilities, however (Hoshino et al., 2012). Magnetic resonance imaging (MRI)

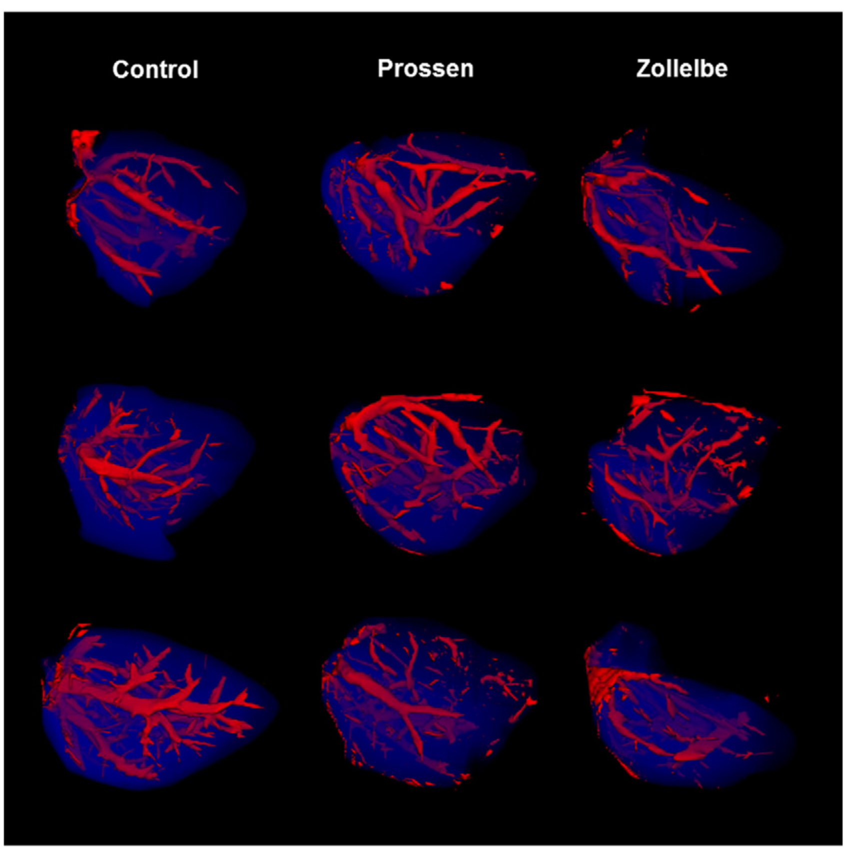

Fig. 3. Non-invasive in vivo segmentation of the total liver (blue) with hepatic blood vessels (red) of $n = 3$ fish per group. Such images could be used to derive functional information on the perfusion of organs and tissues in response to environmental or anthropogenic stressors. Vessels were interactively segmented following the branching patters of the vessels in all axial, coronal and sagittal views. Please note that images of the livers were magnified to similar size.

could be used as alternative technology for anatomical and functional imaging, excelling over µCT in terms of the native soft tissue contrast (Chang et al., 1987). These devices are more expensive, however, and therefore less often available. Another disadvantage is that the skeletal structure is more difficult to assess with MRI (Berker et al., 2012). In summary, we were able to demonstrate that µCT is a useful tool also in context of ecotoxicological research. µCT has the potential to greatly increase the information value of experiments conducted with fish and also to reduce the number of animals required for studying temporal effects through facilitating longitudinal studies within the same individuals, in which each animal serves as its own control. In this respect, our findings strongly endorse the "3R Principle" in Animal Welfare, which advocates for a reduction, replacement and refinement of animals in animal research (Russell and Burch, 1959), holding important potential for non-invasive longitudinal assessment of environmental pollution and its impacts on different fish species.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.11.020.

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