DAIMON Toolbox Fact Sheets:

Methods to Study the Impact of Dumped Munitions on Marine Biota

Assessment category 3: Biological Effects

Toolbox component: Immunotoxicity

Fact Sheet 3.21: Hematology - erythrocytes, hemoglobin, hematocrit and leucocrit

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What is it?

The hematology parameters **erythrocytes** (Ery) and **hematocrit** (Hct) give an overview of the abundance of red blood cells in the blood. Erythrocytes (red blood cells) contain hemoglobin, the iron-containing biomolecule that binds oxygen and is responsible for the red colour of the blood. The hematocrit is the percentage of all red blood cells in the blood. The **leucocrit** (Lct) is the percentage of all white blood cells. **Hemoglobin** (Hb) reversibly stores oxygen, thereby enabling oxygen transport to tissues. The hemoglobin concentration varies depending on fish species. A marked reduction indicates anemia, which may lead to an undersupply of oxygen in the tissues. The four parameters can be used as an indicator of developing or already established disease and of physical stress. Changes may have an infectious (e.g., viral, bacterial, parasitic) or non-infectious (various causes, including exposure to hazardous substances) aetiology.

What does it tell you?

The results of red blood cell parameter are considered as a generic non-specific indicator of habitat quality and environmental health, reflecting the well-being of fish and the status of their specific and non-specific immune system (Silveira-Coffigny et al., 2004).

Changes in the percentage of red blood as well as white blood parameters may be caused by a variety of natural and anthropogenic stressors, including exposure to hazardous substances (Atamanalp et al., 2003; Härdig et al., 1988).

Type of Indicator (tick box)

☒non-specific stress indicator

☐specific for groups of contaminants incl. CWA or explosives

☐CWA-specific indicator

☐specific for substances related to explosives (e.g. TNT)

How to measure it?

Blood collection is best and most easily performed by a puncture of the caudal vein. The puncture site must be wiped clean and dry to avoid possible contamination of the blood sample. The blood

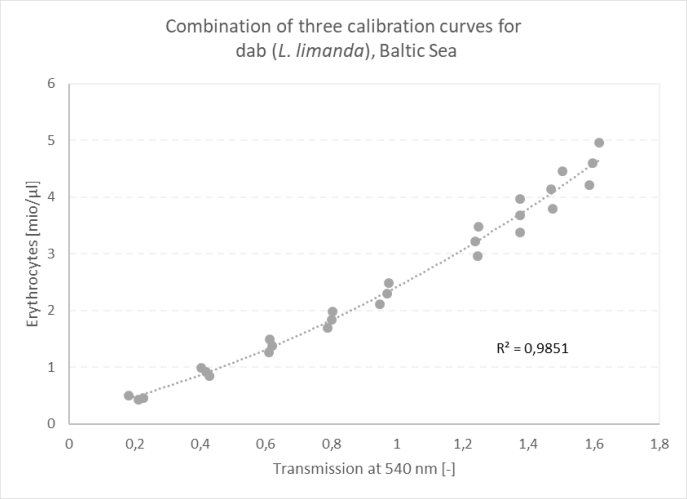
has to be mixed in a tube with an anticoagulant (e.g. 500 µl Microvette®, Sarstedt). It is crucial that for all examinations the same anticoagulant is used as it can have a strong influence on the results (Tavares-Dias & Sandrim 1998). The most widely used and validated anticoagulant is lithium heparin (e.g. 500 µl Microvette®, Sarstedt).

Erythrocytes (Ery): The photometric measurement of erythrocytes takes place at 540 nm. Using a commercially available kit (Ery 142, Diaglobal, Germany), the blood sample (10 µl) is transferred from the tube into a cuvette with 2.5 ml Gower's solution with a pipette. After a given time, the absorbance of the sample is measured at 540 nm. The concentration of erythrocytes is to be calculated by using a species-specific 2nd degree polynominial regression line, calculated based on a calibration curve.

For creation a calibrating curve, the concentration of erythrocytes is to be quantified for each fish species separately by counting cells in a diluted blood sample using a haemocytometer chamber (e.g., Neubauer improved counting chamber) (Bastidas 2017). The dilution of the blood sample is, e.g., 1:200 in a red blood cell pipette. For dilution, the Natt-Hericks solution is used, which stabilizes the cells and stains for better differentiation. After counting the cells, a calibration series is created. Using a commercially available reagent kit (Ery 142, Diaglobal, Germany), the erythrocyte concentration is determined by means of a photometric measurement.

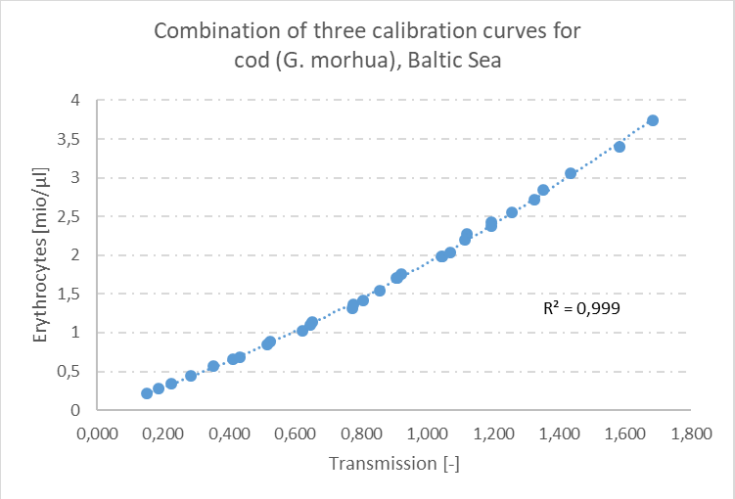
For the calibration curve, 10-12 different blood concentrations (range: 2-24 µl blood in 2.5 ml Gower's solution) of one blood sample are measured. Three different blood samples should be examined for creating one calibration curve each. The three curves are averaged and all further red blood cell concentrations of the samples can be calculated with the help of the regression line (2nd degree polynomial) afterwards. The y-intercept represents the erythrocyte concentration and the x-intercept the measured absorbance.

For dab (*L. limanda*) of the Baltic Sea, the following calibration curve and formula emerged from studies in the DAIMON project:



The 2nd degree polynominial function for dab is: $y = 0{,}8565x^2 + 1{,}3931x + 0{,}1681$

For cod (*G. morhua*) of the Baltic Sea, the following calibration curve and formula emerged from studies in the DAIMON project:



The 2nd degree polynominial function for cod is: $y = 0{,}4811x^2 + 1{,}4303x - 0{,}0125$

Hematocrit (Hct): The blood cells have a higher specific gravity than the surrounding plasma. Therefore, they can be centrifuged off. Whole blood is sampled by using a specific capillary glass tube for haematocrit measurement. One end is closed with plasticine or wax and capillaries are centrifuged in a haematocrit centrifuge (5 min at 10,000 x g; repeat duplication). After centrifugation, red blood cells, white blood cells and plasma in the tube form sharply separated layers (columns). The haematocrit value results from the height of the red cell column as a percentage of the total column. The hematocrit can be read using a corresponding scale and the value is given in % of the red column. (Thrall et al. 2012)

Leucocrit (Lct): The leucocrit reflects the concentration of white blood cells in the blood. It is measured in the same capillary glas tube used for measuring haematocrit on a scale in µm, using a magnifying glass. The leucocytes will deposit in the capillary glass tube on top of the red blood cells (hematocrit).

First, three blood samples with different height (µm) of leucocrit, have to be counted in a heaemocytometer chamber (e.g., Neubauer improved counting chamber). Similar to counting red blood cells, the dilution of a blood sample performed in a white cell pipette diluted with Natt-Hericks solution is needed. The counting result has to be put in correlation with µm of leucocytes and can be calculated easily (rule of three). The result is given in absolute number of leukocytes.

Hemoglobin (Hb): Measuring total hemoglobin is accomplished by applying the cyanmethemoglobin method, commonly by using a commercially available reagent kit (e.g. HB-142 Diaglobal, Germany). A sample of 10 µl of whole blood is added to the test cuvette containing 2 ml test solution, mixed thoroughly and allowed to incubate for at least 2 min. The samples are then measured at 540 nm in a photometer (e.g., Duo Photometer DP200, Diaglobal, Germany). Each sample is to be measured in repeat duplication. The concentration is given as mg/l.

In the test solution, the blood is hemolyzed, the Hb is converted into the stable cyanmethemoglobin and its concentration is determined photometrically. During hemolysis, the nuclei of the erythrocytes form sludge in the solution. This sludge should be removed by a stick or cotton bud. All chemical forms of hemoglobin that can occur in the blood (deoxyhemoglobin, oxyhemoglobin, carboxyhemoglobin, methemoglobin) are thereby converted into

cyanmethemoglobin. This method is independent of whether the individual Hb forms can bind oxygen.

How to analyse and assess the data?

For the measurements of the blood parameters (Ery, Hb, Hct and Lct), different issues have to be taken into account when interpreting the data. The different blood parameters must be considered separately for each fish species. The blood parameters are subject to the natural influences of season, physical influences such as water temperature, water depth (pressure) and salinity as well as chemical inserts in relation to the anticoagulant used and the measurement method (Houston 1996). After trawling and before sampling the fish, there may be changes in blood parameters. The causes are complex. In addition to the changed physical parameters of the holding water compared to the original environment, an adaptation of the fish to the new conditions or a stress response are possible. It is important that the trawling time as well as the sampling time of the caught fish should be kept as short as possible in order to keep an influence on the blood parameters as low as possible. From experience made during studies in the DAIMON project, there is indication that dab showed faster changes in blood parameters after the catch than cod.

From the individual blood parameters (Ery, Hb, Hct, Lct) values, mean values per sample and sampling site can be calculated; e.g., arithmetic means and standard deviation or arithmetic means and 95 % confidence intervals. Depending on the distribution of the data and the form of the mathematical relationship, medians with percentiles are also applicable.

For the assessment of effects on the blood parameters, two commonly applied approaches can be used:

(1) Statistical comparison of mean blood parameter values obtained from impacted areas (e.g. a munitions dumpsite) and from un-impacted reference areas,

(2) The use of assessment criteria (BAC: background assessment criteria; EAC: environmental assessment criteria) reflecting a good, medium or bad fitness status.

So far, no generally applicable assessment criteria for blood parameters in fish have been established. One reason is that each criteria has to be species-specific, because the mean of each blood parameter values and the range of such values occurring in a population differ by species.

In the data analysis and assessment for the DAIMON project, BAC and EAC values for red blood cell parameter (Hct, Hb, Ery) were defined on the basis of the highest 20 % percentile (BAC) and the lowest 5 % percentile (EAC) of all red blood cell values recorded in fish from reference areas, an approach that may also be used in further studies. The following assessment criteria used in DAIMON were (assessment criteria for Lct have not yet been established):

Erythrocytes:

- Dab (*Limanda limanda*): BAC: Ery ≥1,57; EAC: Ery <1,24
- Cod (*Gadus morhua*):BAC: Ery ≥1,4994; EAC: Ery <1,3008

Hematocrit:

- Dab (*Limanda limanda*): BAC: Hct ≥22,4; EAC: Hct <15,6
- Cod (*Gadus morhua*): BAC: Hct ≥33,6; EAC: Hct <29,0

Hemoglobin:

- Dab (*Limanda limanda*): BAC: Hb ≥4,82; EAC: Hb <4,02
- Cod (*Gadus morhua*): BAC: Hb ≥5,18; EAC: Hb <5,06

References

Atamanalp, Muhammed, and Telat Yanik. 2003. 'Alterations in hematological parameters of rainbow trout (Oncorhynchus mykiss) exposed to mancozeb', *Turkish Journal of Veterinary and Animal Sciences*, 27: 1213-17.

Bastidas, Oscar. 2017. 'Cell Counting with Neubauer Chamber - Basic Hemocyter Usage', *Technical Note*, celromics.

Härdig, Jan, Tommy Andersson, Bengt-Erik Bengtsson, Lars Förlin, and Åke Larsson. 1988. 'Long-term effects of bleached kraft mill effluents on red and white blood cell status, ion balance, and vertebral structure in fish', *Ecotoxicology and environmental safety*, 15: 96-106.

Houston, AH, N Dobric, and R Kahurananga. 1996. 'The nature of hematological response in fish', *Fish Physiology and Biochemistry*, 15: 339-47.

Silveira-Coffigny, R, A Prieto-Trujillo, and F Ascencio-Valle. 2004. 'Effects of different stressors in haematological variables in cultured Oreochromis aureus S', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 139: 245-50.

Tavares-Dias, Marcos, and Elziane F Silva Sandrim. 1998. 'Influence of anticoagulants and blood storage on hematological values in tambaqui, Colossoma macropomum', *Acta Scientiarum. Biological Sciences*, 20: 151-55.

Thrall, Mary Anna, Glade Weiser, Robin Allison, and Terry Campbell. 2012. *Veterinary hematology and clinical chemistry* (John Wiley & Sons).