Fish and Shellfish Immunology 88 (2019) 179–188

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Characterisation of Chinook salmon (*Oncorhynchus tshawytscha*) blood and validation of flow cytometry cell count and viability assay kit

Ronald Lulijwa[a,d], Andrea C. Alfaro[a,*], Fabrice Merien[a,c], Mark Burdass[b], Tim Young[a], Jill Meyer[a], Thao V. Nguyen[a], Caroline Trembath[b]

[a] Aquaculture Biotechnology Research Group, School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Private Bag 92006, Auckland, 1142, New Zealand
[b] Nelson Marlborough Institute of Technology (NMIT), H-Block, 322 Hardy Street, Private Bag 19, Nelson, 7042, New Zealand
[c] AUT-Roche Diagnostics Laboratory, School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Private Bag 92006, Auckland, 1142, New Zealand
[d] National Agricultural Research Organisation (NARO), Rwebitaba Zonal Agricultural Research and Development Institute (Rwebitaba-ZARDI), P. O. Box 96, Fort Portal, Uganda

ARTICLE INFO

Keywords:
Fish peripheral blood mononuclear cells
Immunology
Muse® Cell analyser

ABSTRACT

New Zealand Chinook salmon (*Oncorhynchus tshawytscha*) industry has great potential for growth and expansion. While production is relatively free of health problems, there is limited literature on haematology, and immunological tools to safeguard against possible future health threats. The current study aim was to characterise New Zealand farmed *O. tshawytscha* peripheral blood cellular composition, develop a micro-volume method to isolate peripheral blood mononuclear cells (PBMCs) and validate a microcapillary flow cytometry assay kit for PBMC cell count and viability assessment. We used light microscopy to characterise peripheral blood and PBMC cellular composition in combination with a flow cytometer Sysmex XT 2000i Haematology Analyser. ImageJ version 1.52 was used for cell size characterisation of freshly stained blood. The stability of PBMCs stained with the Muse® Cell Count and Viability Assay Kit and the Trypan blue assay stains were studied at 4 °C and 21 °C for 60 min; while the Muse® Cell Count and Viability Assay Kit was validated against the Trypan blue assay haemocytometer chamber to assess PBMC count and viability. Findings showed that *O. tshawytscha* smolt yearlings had total blood cell counts in the range of 1.9–2.7 × 10^6 μL^{-1}. Differential cell counts revealed five cell types, comprising 97.18% erythrocytes, 2.03% lymphocytes, 0.67% thrombocytes, 0.09% monocytes, and unquantifiable neutrophils. Using micro-volumes of blood and Lymphoprep™, we successfully isolated fish PBMCs. Significantly, stained PBMCs remained stable for up to 45 min at 4 °C and 21 °C; while validation of the Muse® protocol showed that this microfluidic instrument delivered more accurate and precise viability results than the haemocytometer. The Muse® protocol is rapid, easy to use, has quick calibration steps, and is suitable for field use to facilitate onsite sample processing. These findings pave the way for future assessments of fish health and *in vitro* immunological studies in *O. tshawytscha*.

1. Introduction

Immunity is an integrated physiological and barrier system that protects animals from threats posed by pathogens [1,2]. The vertebrate immune machinery consists of the innate and adaptive immune systems; and teleosts are the only vertebrate ancestral group that possess both systems [3]. The innate immune response consists of physical barriers (e.g. the skin, scales, gut mucosa and gill epithelia), humoral and cellular components. This system acts as the first line of defence against pathogens. The humoral component includes complement proteins, lysozymes, proteases, esterases, antimicrobial peptides (AMPs) and immunoglobulins, like IgM, IgD and IgT or IgZ [4,5]. The cellular immune constituents include leucocytes, such as T and B lymphocytes, neutrophils, eosinophils, basophils, thrombocytes, cytotoxic cells (natural killer cells), mast cells (MCs), dendritic cells (DCs), macrophages and their precursor monocytes [6,7]. Neutrophils, eosinophils and basophils have a granulated cytoplasm, thus called granulocytes [8]. Macrophages and neutrophils are professional phagocytes [9,10] with a high phagocytic ability and capacity [10]. These cells produce antimicrobial nitrogen and oxygen products, cytokines, and

* Corresponding author.
E-mail address: andrea.alfaro@aut.ac.nz (A.C. Alfaro).

https://doi.org/10.1016/j.fsi.2019.02.059
Received 12 December 2018; Received in revised form 22 February 2019; Accepted 25 February 2019
Available online 26 February 2019

ingest a wide range of particle sizes [11].

When the physical barriers fail to prevent pathogen attacks and entry, other innate immune components become involved through pattern recognition receptors (PRRs) on cell surfaces, which recognise common conserved pathogen-associated molecular patterns (PAMPs) not expressed in multicellular organisms [6,12]. These PAMPs include viral RNA and bacterial DNA, fungal β1,3-glucans, bacterial cell wall peptidoglycans, polysaccharides and lipopolysaccharides (LPS) [12]. The recognition of PAMPs initiates cellular responses designed to kill and eliminate microbial pathogens [13] via phagocytosis and exocytosis [14]. Phagocytosis initiates release of cytokines, followed by antigen presentation through the major histocompatibility complex (MHC), resulting in the development of adaptive immunity [15]. Overall, the adaptive immune component relies on the humoral (antibody) and cellular responses, and is characterised by specific antigen recognition, which invokes a quick and strong secondary pathogen-specific response [15].

In aquaculture, disease outbreaks caused by viruses, bacteria, and parasites, routinely lead to heavy industry losses [reviewed in Ref. [16]]. Amidst these challenges, aquaculture has gained significant progress, and is presently recognised as one of the fastest growing food production sectors worldwide [17]. To keep up with demand, aquaculture farms aim to produce high quantity, of healthy and fast-growing fish, under optimal husbandry and management practices. Combined with other diagnostic tools, fish haematology can reveal important information on fish physiology and health, with high application potential to investigate and monitor stress responses, diagnose diseases and identify nutritional problems [reviewed in Ref. [16]]. For instance, haematology combined with humoral, cellular immune parameters and gene expression have been employed in several fish species to demonstrate immunomodulatory effects of: dietary supplements [18,19]; immunostimulants and pathogen challenge [20–23]; temperature perturbations [24–26]; and hypoxia [27]. Furthermore, salinity stress [28]; crowding stress [29]; heavy metals [30,31]; hormonal components [32]; and melatonin [33] have been shown to induce dramatic changes in leucocyte counts.

However, most fish immunology studies are conducted *in vivo*, which can be time consuming, costly, and often involve fish euthanasia. Thus, there is a need for alternative models that are less invasive, can be performed under controlled *in vitro* environments, with nominal biofluid or tissue requirements, and which are less time consuming and more affordable. To perform *in vitro* immunological studies, fish immune cells are sourced from the thymus, kidney, spleen, or peripheral blood [34]. These cells can be isolated from lymphoid tissues and blood by either hypotonic lysis or density gradient centrifugation [35,36]. Indeed, isolated peripheral blood mononuclear cells (PBMCs) composed of suspended lymphocytes and adherent monocytes have been previously used to investigate immunomodulatory effects of: dietary supplements and disease resistance [18,37]; pathogen challenge [22,38], and vaccine efficacy [23,39,40], among others. Thus, fish PBMCs present an important opportunity to model several biological variables via integrated techniques, including microscopy, flow cytometry, RT-qPCR, spectrophotometry, western blotting, among others.

Specifically, flow cytometry has been used to characterise fish PBMCs in rainbow trout (*Oncorhynchus mykiss*) [35], sea bass (*Dicentrarchus labrax*) [41], striped catfish (*Pangasius hypothalamus*), and European eel (*Anguilla anguilla*) [36]. Flow cytometry has also been used to assess PBMCs for: production of reactive oxygen species (ROS) [22,42]; antibody detection [43,44]; phagocytosis [26,42,45,46] and natural cytotoxicity [47]. Consequently, flow cytometry tools such as the Muse® Cell Analyser, offer accurate and fast quantitative assessment of single cells compared to traditional methods. Fortunately, the Muse® Cell Analyser has been successfully used in our laboratory to characterise haemocytes in black-footed abalone (*Haliotis iris*) [48,49]; to study immune responses of shellfish to pathogen infections [50]; T. V. [51]; T. V [50–52]. and stressors [53]; T. [53,54]; and to assess the immune status of spiny lobster (*Jasus edwardsii*) with tail fan necrosis [55].

While the Muse® Cell Analyser has been shown to work well with shellfish haemolymph, the platform has not yet been used on fish blood, which first requires isolation of PBMCs to exclude nucleated erythrocytes. Fish PBMCs have previously been isolated for immunological investigations in Atlantic salmon (*Salmo salar*) [43,45], and in Chinook salmon (*Oncorhynchus tshawytscha*) in Oregon state USA [56], but this information is still lacking for cultured *O. tshawytscha* in New Zealand. This presents an opportunity to incorporate the Muse® Cell Analyser as a fish health assessment tool for the aquaculture industry and other research-based applications.

The New Zealand aquaculture sector was valued at over US$ 800 million in 2016 of which *O. tshawytscha* contributed 22% and 12% by value and production volume respectively [57]. The species is the second most important aquaculture product after the Greenshell™ mussel (*Perna canaliculus*), making New Zealand, a global leader in production and supply. Despite the economic importance of *O. tshawytscha*, there is a lack of literature on haematological and immunological aspects of this species. Continued growth of the salmon industry will necessitate species-specific immunological information and development of tools to monitor and assess fish health. Thus, the present study characterised the cellular composition of *O. tshawytscha* peripheral blood, and developed a micro-volume method to isolate fish PBMCs. We also characterised isolated PBMCs and used them to validate a commercial cell count and viability assay kit using the Muse® Cell Analyser.

2. Materials and methods

2.1. Fish samples

Nineteen yearling smolts (weight = 317.2–544.5 g; total length = 23.7–28.0 cm) were obtained from the Nelson Marlborough Institute of Technology (NMIT) aquaculture facility (Glenduan, Nelson, New Zealand). Fish had been maintained on a saltwater recirculating system (temp = 14.26 ± 0.35 °C; DO = 7.30 ± 0.11 mgL^{-1}; pH = 8.12 ± 0.21; NH_4^- = 0.14 ± 0.13 mgL^{-1}; NO_2^- = 0.28 ± 1.12 mgL^{-1}), and they were fed to satiation daily with EWOS Microboost 2.2 mm commercial diet (Cargill) with 50% crude protein and 22% lipid. Ethical approval was obtained from the NMIT Animal Ethics Committee (NMIT-AEC-2017-NMIT-01).

2.2. Fish blood sampling

Fish were captured by scoop net, individually anaesthetised with AQUI-S® (25 mgL^{-1} of culture water for 2–3 min), and length/weight measurements quickly recorded. Approximately 1.4 mL of peripheral blood was withdrawn via caudal vein puncture using a non-heparinised 3.0 mL syringe attached to a 20 gauge Terumo needle. Each sample was quickly distributed into three 400 μL BD Microtainer® Lithium Heparin tubes (Becton Dickinson, USA). Blood was mixed by inverting the tube 10 times, as per the manufacturer's recommendations, kept chilled at 4 °C, and processed within 5–10 min for PBMC isolation.

2.2.1. Peripheral blood total cell count

Serial dilutions of 1 in 20 were made by adding 10 μL of peripheral blood into 190 μL of diluting fluid in an Eppendorf tube to a final dilution of 8000. At each dilution, the suspension was mixed by vortexing for 20 s. Subsequently, a haemocytometer chamber was filled with 10 μL using a micropipette and cells counted at 400x magnification, and recorded. Two replicate counts were conducted for each blood sample, and total cell counts were estimated for 10 individuals according to the formula:

$$Count\ per\ Litre = [(Number\ counted)/(Volume\ (\mu L)]\ x\ Dilution\ Factor\ x\ 1{,}000{,}000$$

2.2.2. Isolation of fish PBMCs

Fish PBMCs were isolated using a modified protocol by Ref. [36] with minor modifications. Briefly, all buffers and solutions were kept chilled at 4 °C during isolations. A micro-heparinised blood volume of 284 μL was quickly diluted 1:1 with sterile filtered (40 μm) phosphate-buffered saline (PBS), pH 7.4. PBMCs were obtained by centrifuging (Heraeus Labofuge 200; Thermo Scientific) the mixture at 3000 rpm (971 g) for 20 min over a layer of 682 μL Lymphoprep™ (1.077 g mL^{-1}) (Stemcell Technologies, Australia) in a 1.5 mL Eppendorf tube. Cells at the interface were aspirated with a pipette and washed twice in 500 μL of sterile filtered PBS by centrifuging at 2500 rpm (674 g) for 7 min. The resulting cell pellet was re-suspended to a final cell concentration of 10^5 - 10^6 cells mL^{-1} in sterile filtered PBS supplemented with 2% fetal calf serum (FCS) and kept at 4 °C for further analysis.

2.2.3. Peripheral blood and PBMC slide smear preparation

To prepare fresh peripheral blood smears, approximately 10 μL of fresh non-heparinised sample were transferred to glass slides immediately after withdrawal; smears were made, and quickly stained with modified Giemsa Differential Quick Stain (Polysciences, Inc.). Briefly, slides were fixed by dipping in methanol for 10 s, quickly dipped five times in stain 1 (orange) and stain 2 (blue), immediately washed with distilled water and mounted with coverslips. Images were taken with a Nikon Digital Sight DS-Fi1camera (Nikon Corporation, Japan) attached to an inverted Nikon Eclipse TS100 microscope using NIS-Elements software at 400x magnification. Cell and nuclear size parameters (μm) were measured using an ImageJ version 1.52a Java application for cell size characterisation [58]. For differential cell counts, 10 μL of fresh heparinised blood was used to prepare slide smears, while isolated PBMC slides were prepared by adhering a monolayer of 10 μL cell suspension on glass slides. Slides were left to air dry overnight, fixed with methanol, and were subsequently stained with Giemsa. Peripheral blood stained slides were observed under a microscope at1000x magnification with oil immersion for white blood and red blood cell counts. Peripheral blood and PBMC slides were also observed, and targeted photographs taken under a 1000x magnification with oil immersion using an optical microscope (Leica ICC50 HD). In addition, the cellular composition of isolated PBMCs was compared with peripheral blood haematograms, using the Sysmex XT 2000i haematology analyser following the manufacturer's protocol.

2.3. Stability of stained PBMCs

The stability of isolated PBMCs stained with the Muse® Count and Viability Assay Kit stain (Merck KGaA, Darmstadt, Germany) and 0.4% Trypan blue (TB) assay stain (Sigma-Aldrich, New Zealand) [59] was assessed over 1-h at room temperature (21 °C). Fish PBMC suspensions at 10^6 cells mL^{-1} in sterile filtered PBS with 2% FCS from five biological replicates were separated into paired samples, and repeatedly measured for viability using the Muse® Cell Analyser and the haemocytometer chamber at 5, 15, 30, 45 and 60 min. Duplicate measurements (technical replicates) for each sample were taken. This protocol was repeated to determine the stability of fish PBMCs kept at 4 °C using the Muse® Count and Viability Assay Kit only, with five additional PMBC samples. Repeated-measures ANOVA was performed for each time-series dataset to determine approximate limits for PBMC stability using Minitab 16 statistical software at $\alpha = 0.05$.

2.4. Positive control for setting Muse® gate parameters

2.4.1. Procedure for dead/live cell positive controls

To establish correct Muse® gating parameters, fish PBMCs at 1×10^6 cells mL^{-1} from one fish were used. Dead cells were prepared via thermal stress induction at 100 °C for 5 min, to obtain assay cell suspensions at high and low viability. The high and low viability cell samples were used to set the viability profile and gates for the Muse® Cell Analyser, Cell Count and Viability assay Kit. The haemocytometer chamber was used to verify the cell counts and viability profile of the two sample preparations at high and low viability.

2.4.1.1. Estimating method accuracies. Cell viability and cell count data obtained using the Muse® Cell Analyser and haemocytometer chamber were compared to determine congruence. A series of 57 PBMC samples at low, medium and high viability were prepared from 19 fish using proportions (100%, 50% and 0%) of heat-killed cells at 100 °C for 5 min from each individual. Duplicate measurements were taken for each method and data for mean cell viability and cell count for the haemocytometer chamber was plotted against data for the Muse® Cell Analyser. A scatter plot was generated with a line of best fit, and the R^2 value was determined; while regression equations were established, and regression were tested for significance at $\alpha = 0.01$.

2.4.1.2. Method repeatability (assessing intra-assay precisions). Cell count and viability data obtained from 57 PBMC suspensions at low, medium and high viability from 19 fish were measured in duplicates using the Muse® Cell Analyser and the Trypan blue haemocytometer chamber technique. To assess intra-assay precisions, scatter plots of individual duplicate measurements for cell viability and live cell counts obtained at low, medium and high viability were produced separately for the two methods. A line of best fit was plotted, linear equations and R^2 values were determined. The Muse® instrument specificity was tested by running blank sterile filtered PBS with 2% FCS. In addition, method repeatability was assessed by determining the percentage coefficients of variation using the mean cell count and viability data at low, medium, and high viability profiles for both methods.

3. Results

3.1. Peripheral blood cellular characterisation

Five types of cells in Giemsa-stained peripheral blood were identified, comprising erythrocytes, lymphocytes, thrombocytes, monocytes and neutrophils. Neutrophils were observed, but not sufficiently quantifiable. Generally, erythrocytes dominated cell composition (Fig. 1). The high dominance of erythrocytes was also confirmed via differential cell counts where total leucocytes accounted for less than 3%, and majority were lymphocytes (Table 1).

Erythrocytes were the largest cells with ellipsoid, round to oval shape, and had the lowest nuclear to cytoplasmic (N: C) ratio (Table 1; Figs. 1–2). Immature erythrocytes were more rounded with a larger circular nucleus, while mature cells were more ellipsoidal, with a centrally located round to ellipsoid nucleus that stained deep blue (basophilic) against a pinkish (eosinophilic) cytoplasm. Ellipsoid erythrocytes were the most common cell type in salmon peripheral blood.

Thrombocytes were the second largest cells. They were ellipsoidal, spindle to oval, usually with a spine-like structure at one end of the cell. Ellipsoid and spike shaped thrombocytes were the most common in salmon blood. They had the second lowest N: C ratio after erythrocytes (Table 1). Like erythrocytes, the nucleus varied in shape from ellipsoid to oval, and stained basophilic, while the cytoplasm stained eosinophilic.

Monocytes were the third largest blood cells and possessed an irregular macrophage-like structure and a kidney shaped nucleus that sits on one side of the cell. The nucleus stained basophilic with a violent blue cytoplasm and had a moderate to high N: C ratio after thrombocytes. Small and large monocytes were observed, with cell surfaces exhibiting cytoplasmic extensions or pseudopodia-like structures.

Although lymphocytes were the most numerous of the leucocytes

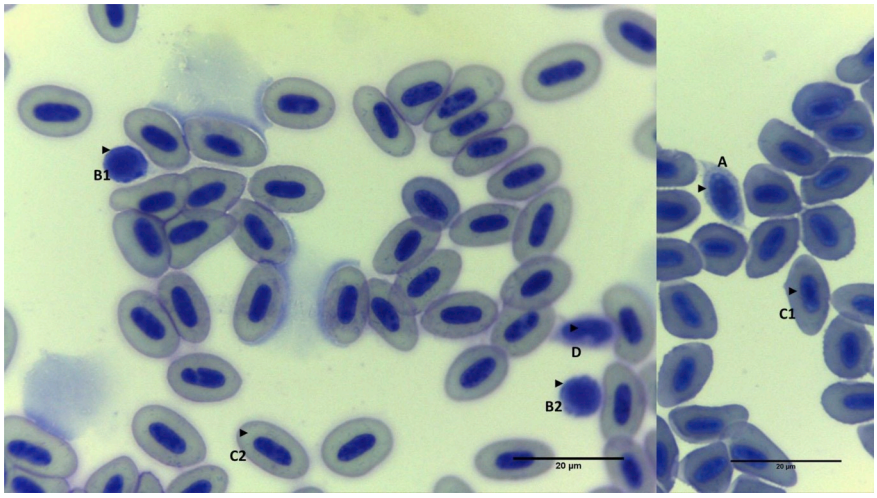

Fig. 1. Giemsa stained *O. tshawytscha* yearling smolt heparinised peripheral blood cells taken with a Leica ICC50 HD camera attached to a Leica binocular at 1000x magnification. (A) thrombocyte, (B1-B2) lymphocytes, (C1-C2) mature nucleated erythrocytes, and (D) monocyte. [1.5].

observed in peripheral blood, they were the smallest cells, had a circular or bell shaped structure, with a very thin cytoplasmic rim. Lymphocytes stained deeply basophilic and presented with the highest N: C ratio in most of the cells and commonly stained basophilic with a thin violent blue cytoplasm (Figs. 1–2). By size, small and large cells were clearly visible in peripheral blood.

Isolated PBMCs were predominantly composed of lymphocytes and monocytes (Fig. 3), with the composition being confirmed with a Sysmex XT 2000i haematology analyser scattergrams (Fig. 4). Specifically, nucleated fish peripheral blood cells (Fig. 4A) presented as a cluster of varying cellular complexity, while isolated PBMCs appeared in scattergrams as two populations composed of smaller and less complex lymphocytes (pink) and more complex monocytes (green) as indicated by side scatter (SSC) (internal organelles and granularity) on the x-axis vs. side fluorescence (SFL) (DNA/RNA) on y-axis (Fig. 4B). Blue dots on the PBMC scattergram represent cellular debris or ghost cells.

3.2. Stability of stained PBMCs

Regarding the stability of stained PBMCs, repeated measures ANOVA revealed approximate limits for PBMC stability at 45 min with the Muse® Count and Viability Assay Kit stain at 21 °C and 4 °C; while the Trypan blue assay stain showed stability limit up to 30 min of incubation at 21 °C (Table 2).

3.3. Estimating method accuracies

The accuracy of the Muse® Count and Viability Assay Kit was benchmarked against the traditional haemocytometer chamber with the Trypan blue exclusion assay. A linear regression model across a wide range of measurements, revealed that comparative method accuracies

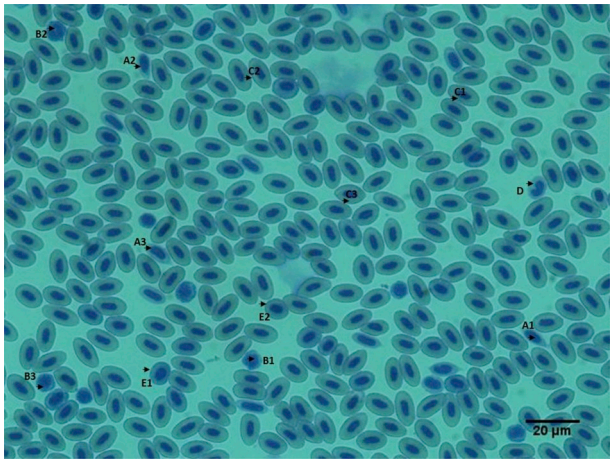

Fig. 2. Modified Giemsa differential quick stained *O. tshawytscha* yearling smolt fresh un-heparinised blood cells, taken with a Nikon Digital Sight DS-Fi1camera attached to an inverted Nikon Eclipse TS100 microscope at 400x magnification. (A1-A3) thrombocytes, (B1-B3) lymphocytes, (C1-C3) mature erythrocytes, (D) monocyte, and (E1-E2) immature erythrocytes. This picture was specifically used to determine cell size. [Single].

were good for cell viability ($R^2 = 0.87$; $p < 0.01$), and reasonable for cell count ($R^2 = 0.74$; $p < 0.01$) (Fig. 5).

3.4. Method precision using intra-assay plots

The ability of the Muse® Cell Analyser to consistently produce results was also assessed against the haemocytometer chamber over a wide range of viability profiles. Results of a linear intra assay regression

Table 1
Cellular profile of healthy *O. tshawytscha* smolt peripheral blood (mean ± SD; n = 10).

Parameters	Erythrocytes	Thrombocytes	Lymphocytes	Monocytes
Composition (%)	97.18 ± 1.67	0.67 ± 0.92	2.03 ± 1.36	0.09 ± 0.29
Cells μL^{-1}	2,254,132 ± 6,785	15,463 ± 3,762	47,093 ± 5,526	2,109 ± 1,188
Total cell count μL^{-1}	2,319,500 ± 407,041			
Cell length (µm)	11.45 ± 1.01	11.74 ± 0.95	6.48 ± 0.76	7.07 ± 0.50
Cell width (µm)	6.44 ± 0.51	4.39 ± 0.49	6.12 ± 0.86	5.51 ± 0.79
Nuclear length (µm)	5.92 ± 0.67	7.08 ± 0.74	5.01 ± 0.83	5.96 ± 0.60
Nuclear width (µm)	2.80 ± 0.34	2.97 ± 0.36	4.36 ± 0.85	3.50 ± 0.90
Nuclear: Cytoplasm (N:C) ratio	0.21–0.24	0.39–0.42	0.49–0.60	0.45–0.61

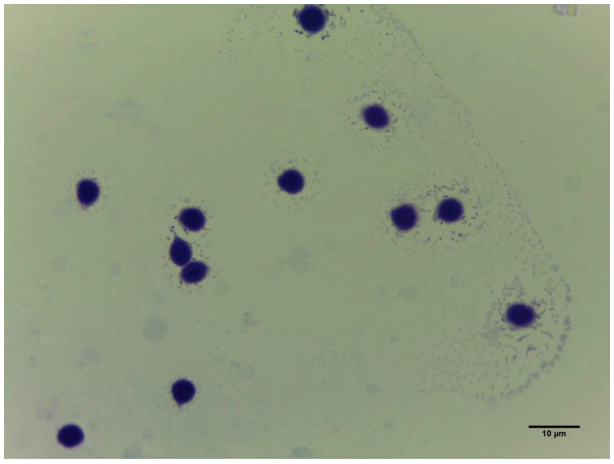

Fig. 3. Giemsa stained *O. tshawytscha* smolt isolated PBMCs taken with a Leica ICC50 HD camera attached to a Leica binocular microscope at1000x magnification. [Single].

revealed that the Muse® protocol performed with slightly better precision than the haemocytometer method in giving consistent results, as indicated by the higher R^2 range of 0.85–0.92 compared to 0.71–0.85, respectively (Fig. 6).

The slightly better precision of the Muse® Cell Analyser in estimating cell viability in comparison to the haemocytometer chamber is further revealed by low % CV among results obtained with the Muse® Cell Analyser in comparison to the haemocytometer chamber (Table 3).

On the other hand, observations of cell counts at different viability profiles revealed that the Muse® Cell Analyser is comparable to the haemocytometer chamber in consistency as revealed with R^2 range of 0.84–0.99 for the Muse® and to 0.88–0.98 for the haemocytometer chamber (Fig. 7).

Comparing method repeatability in estimating live cell counts, results obtained with the Muse® Cell Analyser showed closer grand mean % CV to that obtained with the haemocytometer chamber results (Table 4).

4. Discussion

In this study, we characterised peripheral blood cells in *O. tshawytscha* yearling smolts using light microscopy and flow cytometry, we successfully established a micro blood volume (< 300 µL) density gradient centrifugation method to isolate and purify PBMCs, and validated an efficient assay kit to assess PBMC count and viability using a portable flow cytometry platform, the Muse® Cell Analyser. Differential cell count revealed five types of peripheral blood cells, consisting of erythrocytes, lymphocytes, thrombocytes, monocytes and neutrophils, which is consistent with previous reports in juvenile *O. tshawytscha* from British Columbia, Canada [60]. Since healthy fish were used in our study, neutrophils were observed, but were not quantifiable in peripheral blood as their presence in circulation has been associated with immunological response [10]. Overall, erythrocytes were the most abundant cell types in peripheral blood, similar to previous reports in juvenile *O. tshawytscha* [60] and *S. salar* [2,61]. The observed erythrocyte population in this study is within the range of 10^6 cells μL^{-1} for healthy *S. salar* [2,62] and *O. mykiss* [63,64]; although lower numbers of erythrocytes were observed in *O. tshawytscha* [60]. Scientific evidence shows that fish erythrocyte counts can be influenced by several extrinsic factors (e.g., temperature, oxygen, salinity, pH, season, culture system, diet) and intrinsic ones like age, sex, maturity, ploidy and metabolic activity [reviewed in Ref. [65]].

On the other hand, total leucocytes comprised around 3% and mostly consisted of lymphocytes, as previously reported in *O. mykiss* [35] and other teleosts [22,36]. Salmonid peripheral blood cellular profile has been reported to vary with species, age, season, photoperiod, sexual maturity and diet [44,66–72]; Jiří. Overall, lower leucocyte counts are an excellent indicator of depressed immunity, while variations in circulating leucocyte to erythrocyte levels have been used to assess stress and disease susceptibility [60]. For example, total leucocyte counts have been used to assess immune effects caused by physical [24–29]; and chemical [30–33,73] stressors. Furthermore, haematological parameters combined with humoral, cellular immune responses and molecular techniques are routinely used to assess immunomodulatory effects of dietary supplements and immunostimulants

Table 2
Percent viability of stained PBMCs over time at 21 °C and 4 °C (data points are shown as mean ± SD, n = 5 fish). Repeated measures ANOVA tests were performed at α = 0.05. Values with the same superscript in a column are not significantly different. Values are an average of duplicate readings for both viability assessment techniques.

Time (min)	Muse® Count and Viability stain 21 °C	Trypan blue stain 21 °C	Muse® Count and Viability stain 4 °C
5	92.07 ± 4.16[a]	92.59 ± 3.65[a]	88.40 ± 5.44[a]
15	90.66 ± 5.58[a]	87.16 ± 8.30[ab]	86.86 ± 5.72[a]
30	88.99 ± 5.27[a]	85.54 ± 7.18[ab]	85.15 ± 4.86[a]
45	86.50 ± 4.20[ab]	82.41 ± 7.43[b]	82.00 ± 4.99[ab]
60	82.50 ± 5.30[b]	81.44 ± 8.77[b]	77.57 ± 6.19[b]
F-value	5.85	3.69	6.22
p-value	0.001	0.011	0.000

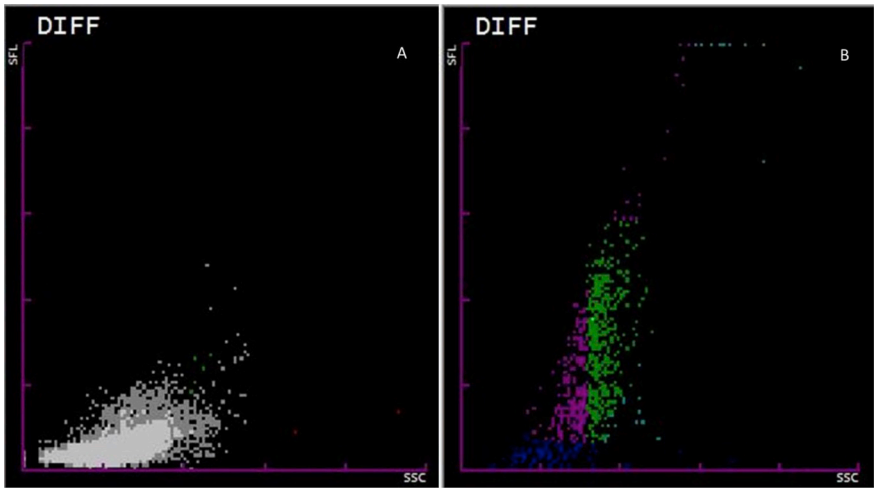

Fig. 4. Sysmex XT 2000i differential scattergrams of fish peripheral blood, showing whole peripheral blood nucleated cells (A) and differential scattergrams of isolated fish PBMCs (B), lymphocytes are orange, monocytes are green, and blue are cellular debris or ghost cells. SFL: side fluorescence; SSC: side scatter; and DIFF: differential. [1.5]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

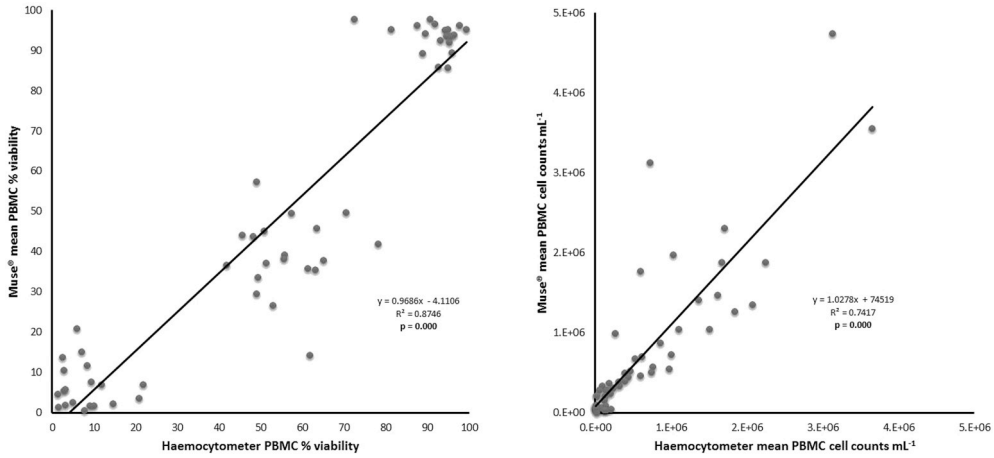

Fig. 5. Linear regression of mean duplicate cell % viability (left) and viable cell count (right) profiles obtained via the haemocytometer chamber and Muse® Cell Analyser at low, medium and high viability cell suspensions (data comes from 57 samples and n = 19 fish). [2.0].

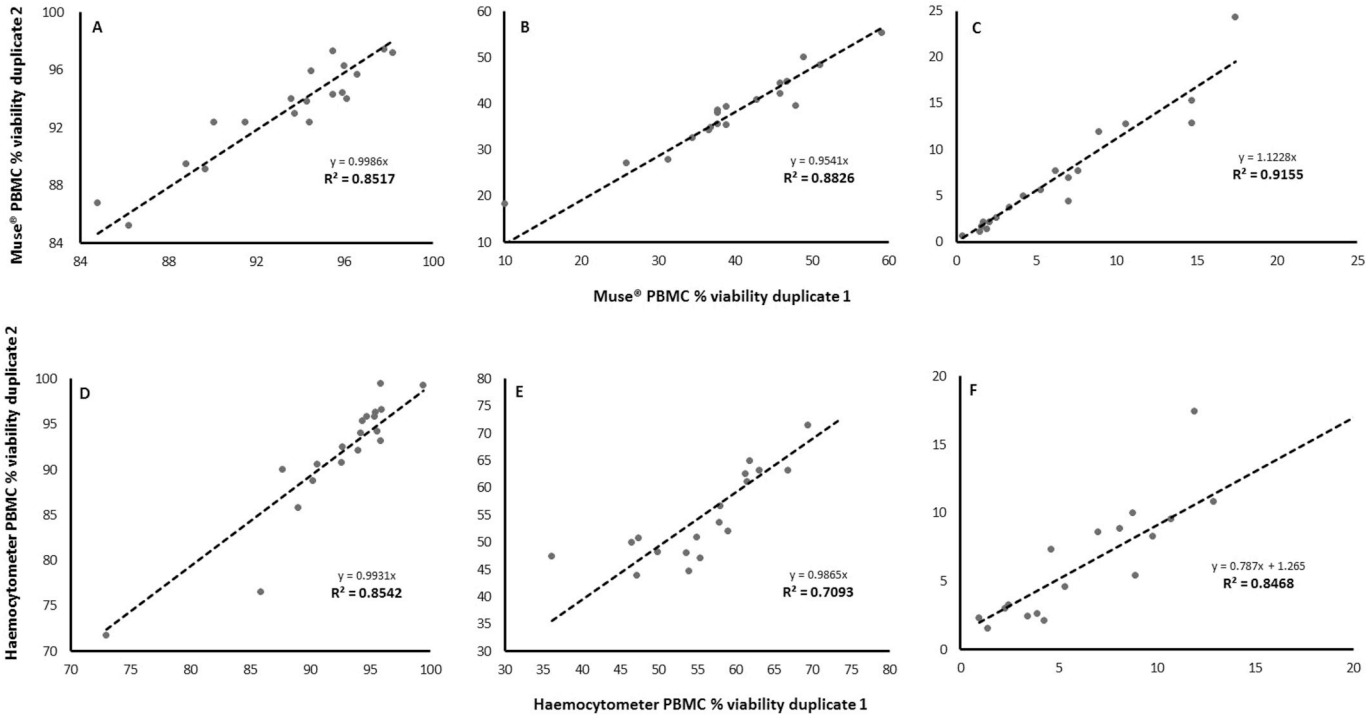

Fig. 6. Regression profiles of duplicate cell viability assessed by the haemocytometer chamber and Muse® Cell Analyser, at three target viabilities (high, medium and low, n = 19). Panel A–C are viability results with the Muse® Cell Analyser at (A) high viability, (B) medium viability, (C) low viability. Panel D–F are viability results with the haemocytometer chamber at (D) high viability, (E) medium viability, and (F) low viability. [2.0].

Table 3
Precision of haemocytometer chamber and Muse® Cell Analyser in assessing PBMC viability at low, medium and high viability samples (data are grand mean from duplicate readings from 19 fish).

Method	Target Viability (%)	Grand mean measured viability (%)	Standard deviation	% CV/ RSD
Haemocytometer	100.00	91.87	1.25	1.41
	50.00	56.31	3.09	5.82
	0.00	7.90	1.43	21.24
Average				9.49
Muse®	100.00	93.27	0.81	0.87
	50.00	38.99	1.90	6.14
	0.00	6.53	0.82	12.60
Average				6.53

in farmed fish [18,19,21,37,74,75]. This integrated approach has also been applied to investigate immunological effects of pathogen challenges and vaccine efficacy in aquaculture [22,23,39,40].

For erythrocytes, mature and immature (erythroblasts) cells are observed with varied shapes, lowest N: C ratios, deeply basophilic nuclei and eosinophilic cytoplasm. A deeply basophilic nucleus is suggestive of chromatin richness, while an eosinophilic cytoplasm has been associated with haemoglobin richness in fish, and variations in morphology are associated with extrinsic and intrinsic factors [reviewed in Ref. [76]]. Although erythrocytes are respiratory cells with a primary gas-transport role, evidence of immunological function is accumulating. For example, expression of immune genes for toll-like receptors and/or interferon regulatory factors have been reported in Nile tilapia (*Oreochromis niloticus*) [23], *S. salar* [77] and *O. mykiss* [78]. In addition, fish erythrocytes appear to contain high numbers of lysosomes and

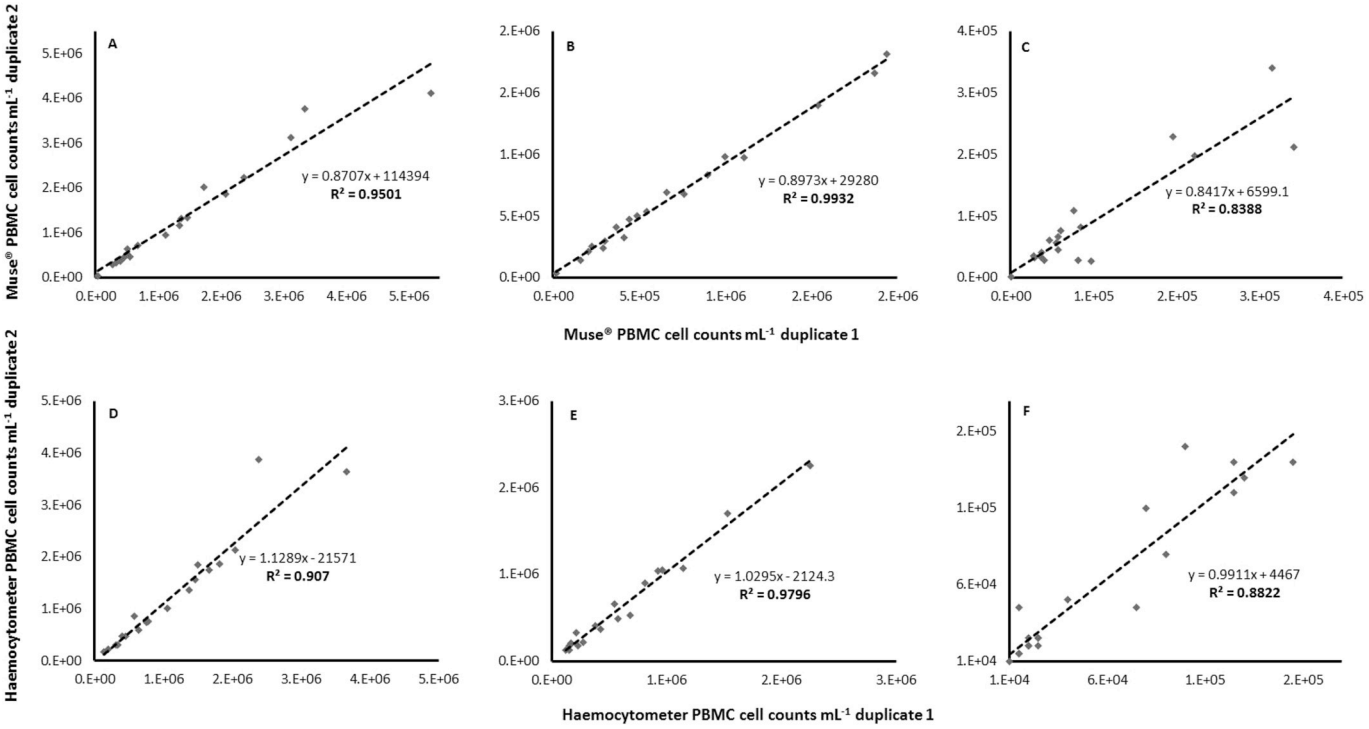

Fig. 7. Regression profiles of duplicate viable cell counts assessed by haemocytometer chamber and Muse® Cell Analyser, at three target viabilities (high, medium and low, n = 19). Panel A–C are live cell count results with the Muse® at (A) high viability, (B) medium viability, (C) low viability. Panel D–F are count results with the haemocytometer at (D) high viability, (E) medium viability, (F) low viability. [2.0].

Table 4
Repeatability of haemocytometer chamber and Muse® Cell Analyser in assessing PBMC cell counts (n = 19).

Method	Cells mL^{-1}	Mean cells mL^{-1}	Standard deviation	% CV/ RSD
Haemocytometer	1,200,000	1,199,605	104,019	7.69
	600,000	624,868	48,939	10.85
	72,000	60,394	7,629	12.12
Average				10.22
Muse®	1,200,000	1,389,038	116,941	7.69
	600,000	676,804	41,624	7.59
	72,000	93,782	16,845	19.34
Average				11.40

mitochondria [23], which further supports evidence of immunological function.

Thrombocytes facilitate fast blood clotting after injury and control fluid loss [76]. Elevated thrombocyte counts in fish have been associated with high clotting capacity and chronic stress [79]. Their observed cellular and nuclear shapes have been previously reported in several teleosts [reviewed in Ref. [76]] and *D. labrax* [41,80–82]. Similar to the current study, thrombocyte cell widths of 3.0–4.5 μm have been reported in goldfish (*Carassius auratus*) [83]. Furthermore, spindle and spike shaped cells were the most frequent thrombocytes in the current study as previously reported in *D. labrax* [41], while spherical cells were the most common in *C. auratus* [83]. The observed thrombocyte shapes in *O.tshawytscha* suggest possession of marginal microtubules and a surface canalicular system previously reported in other fish species [41,84,85], and has been associated with cell shape maintenance [84].

Morphological characteristics of monocytes or macrophages in our study have previously been noted in a review for teleost fishes by Ref. [76]; irregular cell surfaces with protrusions or ridges reported in *D. labrax* [41] and *C. auratus* [83]. Macrophages are a central component of the innate and adaptive immune responses in teleosts [86]. Macrophages are professional phagocytes, and have the potential to kill bacteria and protozoans via production of toxic reactive oxygen species and nitrogen intermediaries, lytic enzymes, and antimicrobial peptides [10]. The production of cytotoxic compounds is facilitated via respiratory bursts [22,87] and phagocytosis [37,75,88].

The dominance of the leucocyte population by lymphocytes, and their small size range was previously reported in coho salmon (*Oncorhynchus kisutch*) [89]. A large nucleus that stains basophilic, with a thin cytoplasmic rim and an amoeboid cell shape have also been reported in several fish species [41,80]. In the current study, we also observed small and large lymphocytes in *O. tshawytscha*, indicative of T and B cells, respectively. While B lymphocytes with surface immunoglobulins have previously been reported in salmonids [43,56]; non-immunoglobulin producing T lymphocytes were reported in *S. salar* [90]. Additionally, some lymphocytes exhibit exterior cytoplasmic protrusions, an indicator of phagocytic capability, while their basophilic cytoplasm and nucleus stain uptake suggests acidic content. Generally, lymphocytes serve immunological functions and can triple their size (plasma cells) following activation in response to antigens [76,91].

Slide smears of purified PBMCs in *O. tshawytscha* confirmed that cells were dominated by lymphocytes, which is consistent with PBMC composition previously reported in several salmonids and other fish species [89,92–94]. Isolated PBMC composition was also confirmed by peripheral blood and PBMC scattergram comparisons for which SSC, clearly illustrated two populations, consistent with results reported in several studies [35,36,41]. PBMC haematograms suggested that monocytes are more complex in structure, possibly due to higher nuclear protein content and presence of cytoplasmic organelles, as they emitted more fluorescence compared to the less complex lymphocytes.

In Giemsa-stained PBMCs, monocyte and lymphocyte nuclei stained deeply basophilic, probably due to chromatin richness, which has been associated with innate immune responses in previous studies among salmonids [89,95–98] and other fish species [22,36,40,99]. To the best of our knowledge, the present contribution is the first to report on the

isolation of *O. tshawytscha* PBMCs for characterisation, which is the first step towards effective *in vitro* immunological and physiological studies.

Despite the successful isolation of PBMCs, we experienced challenges, during preliminary laboratory work (results not shown) on isolating fish PBMCs using anticoagulated blood couriered to our lab and used 24 h after sampling. The aim was to isolate fish PBMCs by density gradient centrifugation following the protocol by Ref. [36] and the SepMate™ tubes (Stemcell Technologies, Australia). Overall, we noted that anticoagulant-treated blood processed 24 h after sampling failed to separate into PBMCs. Furthermore, anticoagulant treated fish peripheral blood stored for an hour and samples with coagulation particles also failed to isolate into PBMCs. Only freshly taken and quickly heparinised or EDTA preserved blood successfully separated into PBMCs without delay, hence the need to run samples as quickly as possible following draw from fish. Using less than 300 µL of fish blood, the technique enables collection of 1.0–1.5 mL of PBMCs at 10^5-10^6 cells mL^{-1}, sufficient to conduct common immunological assays, e.g. phagocytosis, ROS and nitric oxide (NO) production. This is a huge sampling advantage as it does not require fish euthanisation, and presents benefits for on-farm individual fish sampling for longitudinal experiments.

With respect to PBMC stability, cells stained with the Muse® Count and Viability Assay Kit stain remained significantly stable within 45 min at 21 °C and 4 °C; while Trypan blue assay stained cells were significantly stable within 30 min at 21 °C. These findings provide important insights into practical handling of fish PBMCs during laboratory assays, and reveal an important time range within which results can be reliably obtained. Overall, reliable measurements of cell viability can be obtained when working with fish PBMCs within the first 45 min following staining, irrespective of storage conditions. However, it is highly recommended that cell viability assessments are made on fresh samples that have been stained for 10 min when possible to minimise the potential for compromising results.

Meanwhile, validation findings showed that the accuracy and precision of the semi-automated Muse® Count and Viability assay Kit was similar or better than the manual Trypan blue and the haemocytometer chamber method for fish PBMC viability and cell count analysis. These results align with other validation studies using this platform and kit for different cell types from humans, hamsters, insects, and microalgae [100,101]. In the current study, lower grand mean % CV values for viability results were obtained with the Muse® Cell Analyser in comparison to the improved haemocytometer, while cell count overall mean % CV values were close for both methods. However, the Muse® Cell Analyser is a portable and cost-effective bench top flow cytometer engineered with a precise microcapillary system that uses fluorescence to accurately, and precisely quantify multiple cell parameters at once, helping to save time. The Muse® Cell Analyser also uses micro sample volumes, requires minimal sample preparation, and works with adherent and non-adherent cells over a wide size range from 2 to 60 µm diameter. The instrument is very robust and has higher sample throughput than using a traditional manual haemocytometer chamber since it provides results and produces cell population profile plots in only 1–2 min, compared to 5–7 min per sample for haemocytometer. These findings underscore the strength of using the semi-automated Muse® Cell Analyser to quickly process fish PBMC samples using only 20 µL of biofluid.

The use of fish PBMCs and the Muse® Cell Analyser provides a highly improved method to elucidate immune responses in fish, and can be easily deployed to the field for monitoring and performing health assessments in farmed fish without killing stocks. The Muse® Cell Analyser can be used with a range of cell health assessment kits, including the Muse® Oxidative Stress Kit for quantification of ROS, the Muse® Nitric Oxide Kit for intracellular NO activity, the Muse® MultiCaspase Assay Kit for rapid and quantitative measurements of cell death, and the Muse® MitoPotential Assay Kit for simultaneous measurement of changes in mitochondrial potential and cell death. Other kits include the Muse® Caspase-3/7 Kit to quantify cellular apoptosis and cellular plasma membrane permeabilization, and the Muse® Annexin V and Dead Cell Assay to quantify live cells, early and late apoptosis and cell death. Some of these kits have been earmarked for further immunological studies with *O. tshawytscha*.

4.1. Conclusions and recommendations

In this study, we demonstrated that *O. tshawytscha* yearling smolts peripheral blood is dominated by erythrocytes, while lymphocytes were the most common leucocytes. In addition, differential cell count revealed five cell types including erythrocytes, thrombocytes, lymphocytes, monocytes and unquantifiable neutrophils. The study also provides a micro-volume method to successfully isolate fish PBMCs through density gradient centrifugation. Using the isolated PBMCs, we validated a microcapillary flow cytometry Muse® Cell Count & Viability Assay Kit against the traditional manual haemocytometer chamber Trypan blue assay method for cell viability. We conclude that we successfully characterised fish peripheral blood cellular composition; and that the portable Muse® Cell Analyser can accurately and precisely be used to assess *O. tshawytscha* PBMC health in field or laboratory conditions. The protocol is easy-to-use, fast, cheap, and maybe used to monitor fish health or assess physiological responses *in vitro* using a high throughput Muse® Cell Analyser.

Acknowledgements

The authors would like to thank Leanne D Jones of the Nelson Marlborough Institute of Technology (NMIT) for her generous support in maintaining the study animals during laboratory sessions at the Glenduan Aquaculture Park in Nelson, New Zealand. We are also thankful to the technical support at the Auckland University of Technology, particularly Meena Patel, Kathryn Hattersley at NMIT medical laboratory and the Cawthron Institute during the course of this study. This work was greatly improved by discussions with the Aquaculture Biotechnology Research Group at AUT and Drs. Jane Symonds and Seumas Walker at Cawthron Institute. This project was supported by a New Zealand AID doctoral scholarships to Ronald Lulijwa under the supervision of Andrea C. Alfaro and Fabrice Merien and the AUT doctoral student research budget.

References

[1] G.J. Lieschke, N.S. Trede, Fish immunology, Curr. Biol. 19 (16) (2009) R678–R682.
[2] K. Sandnes, Ø. Lie, R. Waagbø, Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon, Salmo salar, J. Fish. Biol. 32 (1) (1988) 129–136.
[3] J.H.W.M. Rombout, H.B.T. Huttenhuis, S. Picchietti, G. Scapigliati, Phylogeny and ontogeny of fish leucocytes, Fish Shellfish Immunol. 19 (5) (2005) 441–455.
[4] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol. 35 (6) (2013) 1729–1739 https://doi.org/10.1016/j.fsi.2013.09.032.
[5] B. Magnadóttir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2) (2006) 137–151 https://doi.org/https://doi.org/10.1016/j.fsi.2004.09.006.
[6] R. Castro, C. Tafalla, Overview of fish immunity, Mucosal Health in Aquaculture, 2015, pp. 3–54 Retrieved from https://www.sciencedirect.com/science/article/pii/B9780124171862000029 https://doi.org/10.1016/B978-0-12-417186-2.00002-9.
[7] F. Merien, A journey with elie Metchnikoff: from innate cell mechanisms in infectious diseases to quantum biology, Front. Public Health 4 (2016) 125.
[8] T.J. Bruce, M.L. Brown, A review of immune system components, cytokines, and immunostimulants in cultured finfish species, Open J. Anim. Sci. 7 (03) (2017) 267.
[9] J.R. Mathias, M.E. Dodd, K.B. Walters, S.K. Yoo, E.A. Ranheim, A. Huttenlocher, Characterization of zebrafish larval inflammatory macrophages, Dev. Comp. Immunol. 33 (11) (2009) 1212–1217.
[10] N.F. Neumann, J.L. Stafford, D. Barreda, A.J. Ainsworth, M. Belosevic, Antimicrobial mechanisms of fish phagocytes and their role in host defense, Dev. Comp. Immunol. 25 (8) (2001) 807–825 https://doi.org/https://doi.org/10.1016/S0145-305X(01)00037-4.
[11] M. Rabinovitch, Professional and non-professional phagocytes: an introduction, Trends Cell Biol. 5 (3) (1995) 85–87.

[12] K. Elward, P. Gasque, “Eat me” and “don't eat me” signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system, Mol. Immunol. 40 (2–4) (2003) 85–94.
[13] A.M. Rieger, D.R. Barreda, Antimicrobial mechanisms of fish leukocytes, Dev. Comp. Immunol. 35 (12) (2011) 1238–1245.
[14] S.K. Whyte, The innate immune response of finfish–a review of current knowledge, Fish Shellfish Immunol. 23 (6) (2007) 1127–1151.
[15] S.A. Martin, E. Król, Nutrigenomics and immune function in fish: new insights from omics technologies, Dev. Comp. Immunol. 75 (2017) 86–98.
[16] F. Fazio, Fish hematology analysis as an important tool of aquaculture: a review, Aquaculture 500 (2019) 237–242 https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.10.030.
[17] FAO, The State of World Fisheries and Aquaculture 2014. Opportunities and Challenges, Food and Agriculture Organisation of the United Nations, Rome, 2014.
[18] A. Brum, S.A. Pereira, M.S. Owatari, E.C. Chagas, F.C.M. Chaves, J.L.P. Mouriño, M.L. Martins, Effect of dietary essential oils of clove basil and ginger on Nile tilapia (Oreochromis niloticus) following challenge with Streptococcus agalactiae, Aquaculture 468 (2017) 235–243 https://doi.org/https://doi.org/10.1016/j.aquaculture.2016.10.020.
[19] A.T. Mansour, M.Á. Esteban, Effects of carbon sources and plant protein levels in a biofloc system on growth performance, and the immune and antioxidant status of Nile tilapia (Oreochromis niloticus), Fish Shellfish Immunol. 64 (2017) 202–209 https://doi.org/https://doi.org/10.1016/j.fsi.2017.03.025.
[20] N. Abu-Elala, M. Marzouk, M. Moustafa, Use of different Saccharomyces cerevisiae biotic forms as immune-modulator and growth promoter for Oreochromis niloticus challenged with some fish pathogens, Int. J. Vet. Sci. Med. 1 (1) (2013) 21–29 https://doi.org/https://doi.org/10.1016/j.ijvsm.2013.05.001.
[21] J.D. Biller-Takahashi, L.S. Takahashi, C.M. Marzocchi-Machado, F.S. Zanuzzo, E.C. Urbinati, Disease resistance of pacu Piaractus mesopotamicus (Holmberg, 1887) fed with β-glucan, Braz. J. Biol. 74 (3) (2014) 698–703.
[22] Z. Sha, L. Wang, L. Sun, Y. Chen, Y. Zheng, M. Xin, ... S. Chen, Isolation and characterization of monocyte/macrophage from peripheral blood of half smooth tongue sole (Cynoglossus semilaevis), Fish Shellfish Immunol. 65 (Supplement C) (2017) 256–266 https://doi.org/https://doi.org/10.1016/j.fsi.2017.04.015.
[23] Y. Shen, D. Wang, J. Zhao, X. Chen, Fish red blood cells express immune genes and responses, Aquacult. Fish. 3 (1) (2018) 14–21 https://doi.org/https://doi.org/10.1016/j.aaf.2018.01.001.
[24] A.-C. Cheng, S.-A. Cheng, Y.-Y. Chen, J.-C. Chen, Effects of temperature change on the innate cellular and humoral immune responses of orange-spotted grouper Epinephelus coioides and its susceptibility to Vibrio alginolyticus, Fish Shellfish Immunol. 26 (5) (2009) 768–772 https://doi.org/https://doi.org/10.1016/j.fsi.2009.03.011.
[25] D. Ndong, Y.-Y. Chen, Y.-H. Lin, B. Vaseeharan, J.-C. Chen, The immune response of tilapia Oreochromis mossambicus and its susceptibility to Streptococcus iniae under stress in low and high temperatures, Fish Shellfish Immunol. 22 (6) (2007) 686–694.
[26] J. Wang, D.-Q. Lu, B. Jiang, H.-L. Luo, G.-L. Lu, A.-X. Li, The effect of intermittent hypoxia under different temperature on the immunomodulation in Streptococcus agalactiae vaccinated Nile tilapia (Oreochromis niloticus), Fish Shellfish Immunol. 79 (2018) 181–192 https://doi.org/https://doi.org/10.1016/j.fsi.2018.04.040.
[27] S. Gallage, T. Katagiri, M. Endo, M. Maita, Comprehensive evaluation of immunomodulation by moderate hypoxia in S. agalactiae vaccinated Nile tilapia, Fish Shellfish Immunol. 66 (2017) 445–454 https://doi.org/https://doi.org/10.1016/j.fsi.2017.05.041.
[28] J. Taylor, M. Needham, B.P. North, A. Morgan, K. Thompson, H. Migaud, The influence of ploidy on saltwater adaptation, acute stress response and immune function following seawater transfer in non-smolting rainbow trout, Gen. Comp. Endocrinol. 152 (2–3) (2007) 314–325.
[29] N. Sadhu, S.K. Sharma, S. Joseph, P. Dube, K. Philipose, Chronic stress due to high stocking density in open sea cage farming induces variation in biochemical and immunological functions in Asian seabass (Lates calcarifer, Bloch), Fish Physiol. Biochem. 40 (4) (2014) 1105–1113.
[30] M. El-Boshy, A. El-Ashram, E. Risha, F. Abdelhamid, E. Zahran, A. Gab-Alla, Dietary fucoidan enhance the non-specific immune response and disease resistance in African catfish, Clarias gariepinus, immunosuppressed by cadmium chloride, Vet. Immunol. Immunopathol. 162 (3–4) (2014) 168–173 https://doi.org/10.1016/j.vetimm.2014.10.001.
[31] M. Radhakrishnan, Immunological effect of cadmium in Heteropneustes fossilis Bloch, Glob. Vet. 4 (6) (2010) 544–547.
[32] H. Xu, X. Zhang, H. Li, C. Li, X.-J. Huo, L.-P. Hou, Z. Gong, Immune response induced by major environmental pollutants through altering neutrophils in zebrafish larvae, Aquat. Toxicol. 201 (2018) 99–108 https://doi.org/https://doi.org/10.1016/j.aquatox.2018.06.002.
[33] M. Kepka, E. Szwejser, L. Pijanowski, B.M.L. Verburg-van Kemenade, M. Chadzinska, A role for melatonin in maintaining the pro- and anti-inflammatory balance by influencing leukocyte migration and apoptosis in carp, Dev. Comp. Immunol. 53 (1) (2015) 179–190 https://doi.org/https://doi.org/10.1016/j.dci.2015.07.011.
[34] N.S. Trede, L.I. Zon, Development of T-cells during fish embryogenesis, Dev. Comp. Immunol. 22 (3) (1998) 253–263.
[35] T.L. Crippen, L.M. Bootland, J.-A.C. Leong, M.S. Fitzpatrick, C.B. Schreck, A.T. Vella, Analysis of salmonid leukocytes purified by hypotonic lysis of erythrocytes, J. Aquat. Anim. Health 13 (3) (2001) 234–245.
[36] M.-A. Pierrard, K. Roland, P. Kestemont, M. Dieu, M. Raes, F. Silvestre, Fish peripheral blood mononuclear cells preparation for future monitoring applications, Anal. Biochem. 426 (2) (2012) 153–165.
[37] H. Van Doan, W. Tapingkae, T. Moonmanee, A. Seepai, Effects of low molecular weight sodium alginate on growth performance, immunity, and disease resistance of tilapia, Oreochromis niloticus, Fish Shellfish Immunol. 55 (2016) 186–194 https://doi.org/10.1016/j.fsi.2016.05.034.
[38] C. Pietsch, R. Vogt, N.F. Neumann, W. Kloas, Production of nitric oxide by carp (Cyprinus carpio L.) kidney leukocytes is regulated by cyclic 3′,5′-adenosine monophosphate, Comp. Biochem. Physiol. Mol. Integr. Physiol. 150 (1) (2008) 58–65 https://doi.org/10.1016/j.cbpa.2008.03.003.
[39] M. Galeottia, N. Romano, D. Volpatti, C. Bulfon, A. Brunetti, P.G. Tiscar, ... L. Abelli, Innovative vaccination protocol against vibriosis in Dicentrarchus labrax (L.) juveniles: improvement of immune parameters and protection to challenge, Vaccine 31 (8) (2013) 1224–1230 https://doi.org/10.1016/j.vaccine.2012.12.041.
[40] H.Y. Huang, Y.C. Chen, P.C. Wang, M.A. Tsai, S.C. Yeh, H.J. Liang, S.C. Chen, Efficacy of a formalin-inactivated vaccine against Streptococcus iniae infection in the farmed grouper Epinephelus coioides by intraperitoneal immunization, Vaccine 32 (51) (2014) 7014–7020 https://doi.org/10.1016/j.vaccine.2014.08.039.
[41] M. Esteban, J. Muñoz, J. Meseguer, Blood cells of sea bass (Dicentrarchus labrax L.). Flow cytometric and microscopic studies, Anat. Rec. 258 (1) (2000) 80–89.
[42] H.C. Samaï, D. Rioult, A. Bado-Nilles, L. Delahaut, J. Jubréaux, A. Geffard, ... S. Betoulle, Procedures for leukocytes isolation from lymphoid tissues and consequences on immune endpoints used to evaluate fish immune status: a case study on roach (Rutilus rutilus), Fish Shellfish Immunol. 74 (2017) 190–204.
[43] I.A. Hedfors, H. Bakke, K. Skjodt, U. Grimholt, Antibodies recognizing both IgM isotypes in Atlantic salmon, Fish Shellfish Immunol. 33 (5) (2012) 1199–1206 https://doi.org/10.1016/j.fsi.2012.09.009.
[44] F.E. Pettersen, M. Ulvenes, G.O. Melingen, H.I. Wergeland, Peripheral blood and head kidney leucocyte populations during out-of-season (0+) parr-smolt transformation and seawater transfer of Atlantic salmon (Salmo salar L.), Fish Shellfish Immunol. 15 (5) (2003) 373–385.
[45] G.T. Haugland, A.-E.O. Jordal, H.I. Wergeland, Characterization of small, mononuclear blood cells from salmon having high phagocytic capacity and ability to differentiate into dendritic like cells, PLoS One 7 (11) (2012) e49260.
[46] G.T. Haugland, A. Rønneseth, H.I. Wergeland, Flow cytometry analyses of phagocytic and respiratory burst activities and cytochemical characterization of leucocytes isolated from wrasse (Labrus bergylta A.), Fish Shellfish Immunol. 39 (1) (2014) 51–60.
[47] S. Chilmonczyk, D. Monge, Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens, Fish Shellfish Immunol. 9 (4) (1999) 319–333 https://doi.org/https://doi.org/10.1006/fsim.1998.0188.
[48] R. Grandiosa, M.-L. Bouwman, T. Young, F. Mérien, A.C. Alfaro, Effect of anti-aggregants on the in vitro viability, cell count and stability of abalone (Haliotis iris) haemocytes, Fish Shellfish Immunol. 78 (2018) 131–139 https://doi.org/https://doi.org/10.1016/j.fsi.2018.04.038.
[49] R. Grandiosa, M. Fabrice, P. Krish, A. Andrea, Innovative application of classic and newer techniques for the characterization of haemocytes in the New Zealand black-footed abalone (Haliotis iris), Fish Shellfish Immunol. 48 (2016) 175–184 https://doi.org/10.1016/j.fsi.2015.11.039.
[50] R. Grandiosa, F. Mérien, T. Young, T. Van Nguyen, N. Gutierrez, E. Kitundu, A.C. Alfaro, Multi-strain probiotics enhance immune responsiveness and alters metabolic profiles in the New Zealand black-footed abalone (Haliotis iris), Fish Shellfish Immunol. 82 (2018) 330–338.
[51] T.V. Nguyen, A.C. Alfaro, T. Young, F. Merien, Tissue-specific immune responses to Vibrio sp. infection in mussels (Perna canaliculus): a metabolomics approach, Aquaculture 500 (2019) 118–125 https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.09.061.
[52] T. Nguyen, A.C. Alfaro, F. Merien, R. Lulijwa, T. Young, Copper-induced immunomodulation in mussel (Perna canaliculus) haemocytes, Metall 10 (2018) 965–978.
[53] A.C. Alfaro, T.V. Nguyen, D. Mellow, A metabolomics approach to assess the effect of storage conditions on metabolic processes of New Zealand surf clam (Crassula aequilatera), Aquaculture 498 (2019) 315–321 https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.08.065.
[54] T.V. Nguyen, A.C. Alfaro, T. Young, S. Ravi, F. Merien, Metabolomics study of immune responses of New Zealand Greenshell™ mussels (Perna canaliculus) infected with pathogenic Vibrio sp, Mar. Biotechnol. 20 (3) (2018) 396–409 Nguyen2018 https://doi.org/10.1007/s10126-018-9804-x.
[55] H. Zha, G. Lewis, A. Alfaro, S. Wang, Y. Dong, R. Grandiosa, A. Jeffs, Immune status of the spiny lobster Jasus edwardsii with tail fan necrosis, Dis. Aquat. Org. 126 (3) (2017) 229–238.
[56] R.H. Milston, A.T. Vella, T.L. Crippen, M.S. Fitzpatrick, J.A.C. Leong, C.B. Schreck, In vitro detection of functional humoral immunocompetence in juvenile chinook salmon (Oncorhynchus tshawytscha) using flow cytometry, Fish Shellfish Immunol. 15 (2) (2003) 145–158 https://doi.org/10.1016/s1050-4648(02)00151-1.
[57] FAO, Fishery and Aquaculture Statistics. Global Aquaculture Production 1950-2016 (FishstatJ), FAO Fisheries and Aquaculture Department, 2018 Retrieved 10/11/2018, from www.fao.org/fishery/statistics/software/fishstatj/en.
[58] R. Wayne, ImageJ Java Application, National Institutes of Health, USA, 2018 Retrieved from https://imagej.nih.gov/ij/.
[59] W. Strober, Trypan blue exclusion test of cell viability, Current Protocols in Immunology, John Wiley & Sons, Inc., 2001Retrieved from https://doi.org/10.1002/0471142735.ima03bs111 https://doi.org/10.1002/0471142735.ima03bs111.
[60] P.A. Ackerman, B.J. Wicks, G.K. Iwama, D.J. Randall, Low levels of environmental

ammonia increase susceptibility to disease in Chinook salmon smolts, Physiol. Biochem. Zool. 79 (4) (2006) 695–707.
[61] T. Benfey, A. Sutterlin, The haematology of triploid landlocked Atlantic salmon, Salmo solar L, J. Fish. Biol. 24 (3) (1984) 333–338.
[62] V. Vikeså, L. Nankervis, E. Hevrøy, High dietary energy level stimulates growth hormone receptor and feed utilization in large Atlantic salmon (Salmo salar L.) under hypoxic conditions, Aquacult. Nutr. 23 (6) (2017) 1193–1203.
[63] A.L. Morgan, K.D. Thompson, N.A. Auchinachie, H. Migaud, The effect of seasonality on normal haematological and innate immune parameters of rainbow trout Oncorhynchus mykiss L, Fish Shellfish Immunol. 25 (6) (2008) 791–799 https://doi.org/https://doi.org/10.1016/j.fsi.2008.05.011.
[64] J. Řehulka, B. Minařík, E. Řehulková, Red blood cell indices of rainbow trout Oncorhynchus mykiss (Walbaum) in aquaculture, Aquacult. Res. 35 (6) (2004) 529–546.
[65] M. Witeska, Erythrocytes in teleost fishes: a review, Zool. Ecol. 23 (4) (2013) 275–281.
[66] R.A. Barnhart, Effects of certain variables on hematological characteristics of rainbow trout, Trans. Am. Fish. Soc. 98 (3) (1969) 411–418.
[67] D. Conroy, Studies on the haematology of the Atlantic salmon (Salmo salar L.) Symposium conducted at the meeting of the Symp, Zool. Soc. Lond. 30 (1971) 101–127.
[68] J. Härdig, L.B. Höglund, On accuracy in estimating fish blood variables, Comp. Biochem. Physiol. Physiol. 75 (1) (1983) 35–40.
[69] H.R. Jamalzadeh, M.R. Ghomi, Hematological parameters of Caspian salmon Salmo trutta caspius associated with age and season, Mar. Freshw. Behav. Physiol. 42 (1) (2009) 81–87.
[70] H. Lane, Progressive changes in haematology and tissue water of sexually mature trout, Salmo gairdneri Richardson during the autumn and winter, J. Fish. Biol. 15 (4) (1979) 425–436.
[71] M. Leonardi, A. Klempau, Artificial photoperiod influence on the immune system of juvenile rainbow trout (Oncorhynchus mykiss) in the Southern Hemisphere, Aquaculture 221 (1–4) (2003) 581–591.
[72] J. Řehulka, Influence of astaxanthin on growth rate, condition, and some blood indices of rainbow trout, Oncorhynchus mykiss, Aquaculture 190 (1) (2000) 27–47 https://doi.org/https://doi.org/10.1016/S0044-8486(00)00383-5.
[73] M.R. Arkoosh, A.L. Van Gaest, S.A. Strickland, G.P. Hutchinson, A.B. Krupkin, M.B.R. Hicks, J.P. Dietrich, Dietary exposure to a binary mixture of polybrominated diphenyl ethers alters innate immunity and disease susceptibility in juvenile Chinook salmon (Oncorhynchus tshawytscha), Ecotoxicol. Environ. Saf. 163 (2018) 96–103.
[74] M. Furne, E. Holen, P. Araujo, K.K. Lie, M. Moren, Cytokine gene expression and prostaglandin production in head kidney leukocytes isolated from Atlantic cod (Gadus morhua) added different levels of arachidonic acid and eicosapentaenoic acid, Fish Shellfish Immunol. 34 (3) (2013) 770–777.
[75] H. Van Doan, S.H. Hoseinifar, W. Tapingkae, S. Tongsiri, P. Khamtavee, Combined administration of low molecular weight sodium alginate boosted immunomodulatory, disease resistance and growth enhancing effects of Lactobacillus plantarum in Nile tilapia (Oreochromis niloticus), Fish Shellfish Immunol. 58 (2016) 678–685 https://doi.org/10.1016/j.fsi.2016.10.013.
[76] T.M. Clauss, A.D. Dove, J.E. Arnold, Hematologic disorders of fish, Vet. Clin. Exot. Anim. Pract. 11 (3) (2008) 445–462.
[77] S.T. Workenhe, M.J. Kibenge, G.M. Wright, D.W. Wadowska, D.B. Groman, F.S. Kibenge, Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes, Virol. J. 5 (1) (2008) 36.
[78] D. Morera, N. Roher, L. Ribas, J.C. Balasch, C. Doñate, A. Callol, F.W. Goetz, RNA-Seq reveals an integrated immune response in nucleated erythrocytes, PLoS One 6 (10) (2011) e26998.
[79] S. Espelid, G.B. Løkken, K. Steiro, J. Bøgwald, Effects of cortisol and stress on the immune system in Atlantic salmon (Salmo salarL.), Fish Shellfish Immunol. 6 (2) (1996) 95–110.
[80] T. Doggett, J. Harris, Ultrastructure of the peripheral blood leucocytes of Oreochromis mossambicus, J. Fish. Biol. 34 (5) (1989) 747–756.
[81] B. Köllner, U. Fischer, J.H.W.M. Rombout, J. Taverne-Thiele, J. Hansen, Potential involvement of rainbow trout thrombocytes in immune functions: a study using a panel of monoclonal antibodies and RT-PCR, Dev. Comp. Immunol. 28 (10) (2004) 1049–1062.
[82] A. López-Ruiz, M.A. Esteban, J. Meseguer, Blood cells of the gilthead seabream (Sparus aurata L.): light and electron microscopic studies, Anat. Rec. 234 (2) (1992) 161–171.
[83] L.J. Watson, I.L. Shechmeister, L.L. Jackson, The hematology of goldfish, Carassius auratus, Cytologia 28 (2) (1963) 118–130.
[84] M.S. Cannon, H.H. Mollenhauer, T.E. Eurell, D.H. Lewis, A.M. Cannon, C. Tompkins, An ultrastructural study of the leukocytes of the channel catfish, Ictalurus punctatus, J. Morphol. 164 (1) (1980) 1–23.
[85] A. Savage, The ultrastructure of the blood cells of the pike Esox lucius L, J. Morphol. 178 (2) (1983) 187–206.
[86] D.K. Chaudhary, N. Sood, P. Pradhan, A. Singh, P. Punia, N. Agarwal, G. Rathore, Establishment of a macrophage cell line from adherent peripheral blood mononuclear cells of Catla catla, Vitro Cellular & Developmental Biology-Animal, vol. 48, 2012, pp. 340–348 (6).
[87] L. Grayfer, J.W. Hodgkinson, M. Belosevic, Antimicrobial responses of teleost phagocytes and innate immune evasion strategies of intracellular bacteria, Dev. Comp. Immunol. 43 (2) (2014) 223–242 https://doi.org/https://doi.org/10.1016/j.dci.2013.08.003.
[88] M.M.A. Mahmoud, M.M.M. El-Lamie, O.E. Kilany, A.A. Dessouki, Spirulina (Arthrospira platensis) supplementation improves growth performance, feed utilization, immune response, and relieves oxidative stress in Nile tilapia (Oreochromis niloticus) challenged with Pseudomonas fluorescens, Fish Shellfish Immunol. 72 (2018) 291–300 https://doi.org/10.1016/j.fsi.2017.11.006.
[89] E. Kawahara, S.-i. Oshima, S. Nomura, Separation of lymphocytes from the peripheral blood of coho salmon Oncorhynchus kisutch, Nippon Suisan Gakkaishi 56 (2) (1990) 363-363 https://doi.org/10.2331/suisan.56.363.
[90] B. Dannevig, B. Brudeseth, T. Gjøen, M. Rode, H. Wergeland, Ø. Evensen, C.M. Press, Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (Salmo salarL.), Fish Shellfish Immunol. 7 (4) (1997) 213–226.
[91] S. Mumford, J. Heidel, C. Smith, J. Morrison, B. MacConnell, V. Blazer, Fish histology and histopathology, US Fish and Wildlife National Conservation Training Center, Amerika Serikat, 2007.
[92] P. Blaxhall, A comparison of methods used for the separation of fish lymphocytes, J. Fish. Biol. 18 (2) (1981) 177–181.
[93] K.S. Daiku, Separation of lymphocytes from the peripheral blood of rainbow trout and goldfish, Bull. Jpn. Soc. Sci. Fish. 47 (10) (1981) 1281–1288.
[94] C.A.K. Kalgraff, H.I. Wergeland, E.F. Pettersen, Flow cytometry assays of respiratory burst in Atlantic salmon (Salmo salar L.) and in Atlantic cod (Gadus morhua L.) leucocytes, Fish Shellfish Immunol. 31 (3) (2011) 381–388.
[95] S.K. Dügenci, N. Arda, A. Candan, Some medicinal plants as immunostimulant for fish, J. Ethnopharmacol. 88 (1) (2003) 99–106.
[96] K.A. Gross, R.N. Morrison, R. Butler, B.F. Nowak, Atlantic salmon, Salmo salar L., previously infected with Neoparamoeba sp are not resistant to re-infection and have suppressed phagocyte function, J. Fish. Dis. 27 (1) (2004) 47–56 https://doi.org/10.1046/j.1365-2761.2003.00514.x.
[97] T. Korytář, H.D. Thi, F. Takizawa, B. Köllner, A multicolour flow cytometry identifying defined leukocyte subsets of rainbow trout (Oncorhynchus mykiss), Fish Shellfish Immunol. 35 (6) (2013) 2017–2019.
[98] F.E. Pettersen, I. Fyllingen, A. Kavlie, N.P. Maaseide, J. Glette, C. Endresen, H.I. Wergeland, Monoclonal antibodies reactive with serum IgM and leukocytes from Atlantic salmon (Salmo salarL.), Fish Shellfish Immunol. 5 (4) (1995) 275–287 https://doi.org/https://doi.org/10.1006/fsim.1995.0027.
[99] K. Roland, P. Kestemont, L. Hénuset, M.-A. Pierrard, M. Raes, M. Dieu, F. Silvestre, Proteomic responses of peripheral blood mononuclear cells in the European eel (Anguilla anguilla) after perfluorooctane sulfonate exposure, Aquat. Toxicol. 128 (2013) 43–52.
[100] K. Gillis, K. Chowdhury, J. Clor, K. Tyagarajan, Simplified Methods for Algal Characterization Using Microcapillary Flow Cytometry, MilliporeSigma, Hayward, CA, United States:, 2016.
[101] E.M.D. Millipore, Precise and accurate counts and viability measurements across multiple cell lines using the Muse™ cell count & viability assay, Biotechniques 52 (3) (2012) 200–203 https://doi.org/10.2144/000113827.