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REVIEW

REVIEWS IN Aquaculture

New wine in old bottles: Modification of the Na^+/K^+-ATPase enzyme activity assay and its application in salmonid aquaculture

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Abstract

The Na^+, K^+ ATPase (NKA) enzyme is important to generate the transmembrane ion gradient in the gills, intestine, and kidneys, hence, is vital for secondary transport of fluids and different solutes in teleosts. Gill NKA enzyme activity is often used as a proxy for parr–smolt transformation (PST) during which anadromous salmonids prepare for seawater (SW). Increased intensification and production of larger smolts in modern salmonid aquaculture has resulted in reports of gill NKA activity being less reliable as a proxy for smolt quality. Consequently, changes in mRNA *nka-α1b/α1a* ratios in gills are increasingly used as indicators of PST. However, *nka* isoform mRNA abundance may not reflect translation into the functional protein, nor the activity of the mature enzyme. This may limit the predictive power of molecular markers under certain environmental conditions, rearing regimes and biological scenarios. During PST, the osmoregulatory transformations necessary for SW tolerance and survival does not only occur in the gills. Equally important are the changes in ion transporting activities, including NKA activity, in the intestine and kidneys. However, to our knowledge, there are no previous studies addressing the timing and concurrent changes in NKA activity in the three osmoregulatory tissue during PST. Here we present modifications and optimization of the NKA enzyme activity protocols for gill, intestinal and kidney tissue and outline how to best utilize NKA activity measurements as part of a more holistic approach to evaluate overall smolt quality in modern aquaculture.

KEYWORDS
aquaculture, fish, Na^+/K^+-ATPase, osmoregulation, parr–smolt transformation, seawater tolerance

1 | INTRODUCTION

Since J. C. Skou first suggested that transport of Na^+ and K^+ across cell membranes is linked to an active Na^+, K^+ ATPase (NKA) enzyme,[1,2] it is now established that the electrogenic transmembrane P-type NKA pump is present in all animal cells. The NKA enzyme establish a membrane potential by pumping three sodium ions out of the cell and two potassium ions into the cell for every single ATP consumed. Thus, the membrane potential generated by the NKA pump is important for ion regulation and secondary transport of fluids and different solutes.[3] In teleosts, the NKA enzyme is particularly abundant in ion transporting organs, such as the gills, intestine, and kidneys.

The initial protocols measuring NKA activity in teleosts are time consuming as they required prolonged ultracentrifugation to isolate enzyme fractions.[4,5] Studies of NKA enzyme activity during developmental events such as parr–smolt transformation (PST) in salmonids and acclimation to different salinities in euryhaline teleosts often entail large number of samples to be analysed.[6,7] This prompted the development of simplified protocols where NKA activity is measured in crude suspensions of grinded tissues or partially purified membrane preparations.[8,9] The caveat with these protocols is that they require larger amounts of tissue and yield lower specific enzyme activity than those measured in membrane preparations. The next generation micro assay protocol using 96-well microplate readers, developed by McCormick,[10] counteracts draw backs with earlier methods and permits time efficient measurement of NKA enzyme activity in crude homogenate of small tissue gill biopsies, while retaining sufficient specific enzyme activity levels compared with earlier protocols. Hence, it has been one of the most widely applied protocols in fish physiology, particularly in gills, where increasing gill NKA enzyme activity is often used as a proxy for PST.[7] The readers are referred to Zaugg[9] and McCormick[10] for more detailed descriptions of the NKA enzyme activity methods and/or protocols.

In general, teleost fishes are continually exposed to osmotic forces across all epithelial surfaces, and transitions between different environmental salinities requires substantial changes in osmoregulatory capacity to maintain homeostasis.[6] Bigger fish has a larger volume to epithelial surface ratio than smaller fish, which contributes to an inherent higher seawater (SW) tolerance with increasing fish size, and this may occur independent of developmental changes related to PST.[11,12] Anadromous salmonids go through physiological, morphological, and behavioural changes during PST, gradually preparing them for marine life.[13,14] It should be noted, however, that physiological changes during PST may be quite dynamic depending on rearing conditions and strains, both wild and domesticated. For instance, changes in gill NKA activity in different populations of naturally migrating Sockeye salmon (*O. nerka*), smolts are found to vary considerably.[15] While in naturally migrating masu salmon (*O. masou*), smoltification can be induced by both photoperiod and size, indicating that size can be a driver for PST.[16] Moreover, changes in gill NKA activity levels of freshwater (FW) Atlantic salmon smolts do not always correlate well with long-term NKA activity levels and growth performance in SW.[17] Intensification of rearing conditions and production of ever larger smolts in modern salmonid aquaculture has resulted in more frequent reports of morphological and physiological changes associated with PST occurring independent of the PST process. For instance, increased silvering and elevated gill NKA enzyme activity levels normally occurring only in smolts during PST are reported in juvenile parr reared under conditions not indented to stimulate PST (e.g., use of continuous light). Such biological 'artifacts' in fish kept at intensive rearing conditions highlight the challenges with using only one single metric as a proxy for PST. Consequently, the industry questions the application of gill NKA activity as a reliable proxy for smolt quality, and the use of enzyme activity has dropped significantly since it was introduced in the early 2000s. The industry is therefore searching for new tools to better evaluate smolt quality under modern production regimes.

Intensive smolt production and acclimatization of salmonids to SW requires comprehensive knowledge of key biological processes associated with PST, but also a reliable repertoire of methods and knowledge on how to apply them to better evaluate smolt quality. Here we argue that measurements of osmoregulatory transporters in gills as the only reliable proxy is inadequate. New molecular methods are promising but the know-how about these methods under industrial smolt production are still in its infancy and thus have limitations. Instead, we want to revisit the NKA enzyme activity micro assay protocol published by McCormick[10] and argue that it is still a reliable biomarker if applied correctly. We suggest a more holistic approach, which includes all three osmoregulatory organs (gills, intestine, and kidneys) when evaluating overall smolt quality and SW readiness in modern aquaculture production. Thus, this review aims to (1) present modification of protocols for tissue sampling and analysis of NKA enzyme activity in gills, intestine and kidneys and discuss their relevance and application in modern aquaculture, and (2) discuss the application of physiological and molecular biomarkers for assessment of smolt quality in intensive aquaculture.

2 | THE APPLICATION OF NA^+/K^+-ATPase ENZYME ACTIVITY IN FISH PHYSIOLOGY AND AQUACULTURE

Due to its key role in the development of hypo-osmoregulatory ability, gill NKA activity has become a widely used measure of osmoregulatory capacity in teleost fishes.[6] Research outlining commercial out of season production protocols of Atlantic salmon smolts during the 1990s suggested that the increasing gill NKA activity also could be used as a proxy for SW tolerance.[18–21] Once sufficient data on gill NKA activity emerged from industrial rearing conditions, NKA enzyme activity was successfully introduced and a widely used biomarker by the salmon industry from the early 2000s. From a practical point of view, gill tissue was collected by farmers in smolt facilities, frozen and shipped to biotechnology companies for analysis and advice on smolt status. The farmers could then, following consultation, plan transport and SW transfer of smolt groups. Hence, for more than a decade, gill NKA activity was considered a highly predictable biomarker for SW tolerance, and thus smolt quality. However, large smolts (>200 g)

display a less synchronous PST[22] and when reared in flow-through systems (FTS) may display a greater fold increase in gill NKA activity than cohorts reared in recirculating aquaculture systems (RAS).[23] This concurs with recent concerns raised by the industry regarding variations in gill NKA activity under intensive rearing protocols in both FT and RAS, making it difficult to predict smolt development-based measurements of gill NKA activity alone. Large investments in RAS facilities and rapid implementation of new production strategies have resulted in an increased proportion of large smolt (>200 g) produced in Norway,[24] a development expected to continue rapidly worldwide. Thus, there is a need to better understand the physiological responses of smolts and larger post-smolts produced under intensive conditions in RAS. The application and limitations of the methods used to evaluate smolt quality in contexts of new production strategies and technologies have motivated modifications of the NKA enzyme activity assay. Furthermore, attention to the role of the intestine and kidneys in osmoregulation of smolt and post-smolt, reared under industrial conditions, is still limited.

2.1 | The NKA enzyme, its distribution and main components in gills, intestine and kidneys

The NKA enzyme is an oligomeric protein that comprise of α-subunits, β-subunits and FXYD (also named gamma subunit) protein chains.[25] The α-subunit contains binding sites for Na^+, K^+, ATP and the inhibitor, ouabain, and hence provides the major catalytic and ion transporting capacity of the enzyme. The β-subunit is suggested to be involved in the occlusion of K^+ and regulation of Na^+ and K^+ affinity, and it plays a major role in stabilizing the folding of the α-subunit, whilst the FYXD proteins are known to modulate the NKA enzyme by changing its affinity for both Na^+ and K^+.[26] In salmonids, five different α-isoforms (α1a, α1b, α1c, α2 and α3), each having a salmonid specific paralogue, and four β-isoforms (β1a, β1b, β2 and β3b) have been identified.[27–29] Several studies suggest that differential expression of α-subunit isoforms may be an important mechanism for altering NKA enzyme activity as a response to different physiological requirements[30–35] (see Figure 1).

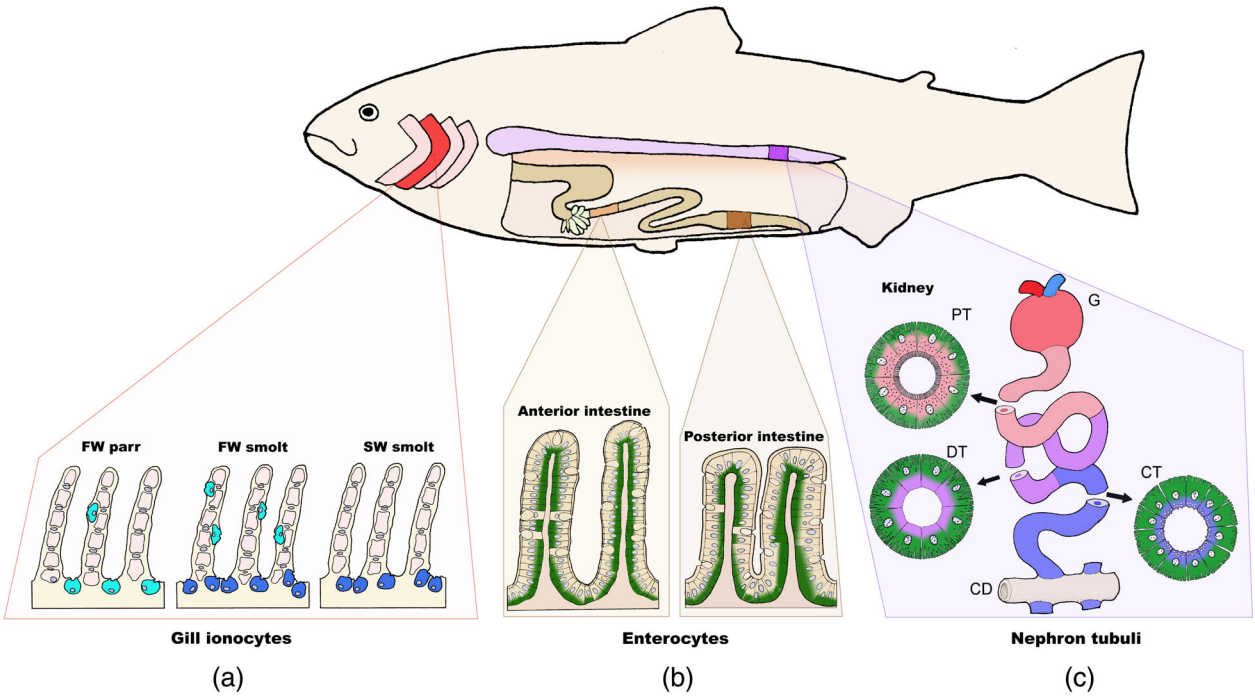

FIGURE 1 Schematic overview showing localization of catalytic alfa (α) Na^+/K^+-ATPase (NKA) subunit in gill ionocytes, intestinal enterocytes and kidney tubule cells. In gills the NKAα subunit are located in specialized cells in the gill termed ionocytes. In salmonids a freshwater (FW) catalytic NKAα subunit (NKAα1a isoform) and seawater (SW) NKAα subunit (NKAα1b isoform) have been discovered in gill ionocytes at different stages of smoltification: (1) FW parr stage: the FW ionocytes type (NKAα1a subunit; turquoise) are well distributed in the gill filament and lamellae; (2) FW smolt stage: the FW ionocytes (NKAα1a; turquoise) are distributed on the lamellae and the SW ionocytes (NKAα1b; dark blue) are distributed on the filament; and (3) SW smolt stage: only SW ionocytes (dark blue) are distributed on the filament. In the gut the NKAα pump (green; general NKAα5 antibody) are equally distributed in the villus both in anterior and posterior intestine (more abundant in anterior intestine). The NKAα subunit are located at the basolateral region of the enterocytes but there are a NKAα1c isoform that appears to be abundant, especially in anterior intestine, during FW smolt stage and SW smolt stage (not shown). In the kidney the NKAα subunit (green; general NKAα5 antibody) are distributed in all parts of the nephron tubule, including proximal tubule (PT; pink), distal tubule (DT; purple) and collecting tubule (CT; dark blue) but are more abundantly located in the last parts of the nephron (DT and CT). Immunolocalization of a specific NKAα isoform in the kidney are yet to be investigated during FW parr, FW smolt and SW smolt. The catalytic part of NKA pump is known to generate a favourable transepithelial membrane potential that can drive water and ion transport in all three osmoregulatory organs in salmonids. Thus, measuring the catalytic NKA activity in gills, gut, and kidney during smoltification and after SW acclimation can be useful to evaluate overall osmoregulatory capacity. The illustrations are based on the papers of McCormick and authors,[32] Sundh and authors[35] and Engelund and Madsen.[55]

NKA enzyme activity in the gills, intestine and kidneys increases during PST in Atlantic salmon as part of the preparation for a marine life.[35–38] Of these three organs, gills are the most widely studied organ with respect to differential regulation of NKA-α-subunits during smoltification, sexual maturation and changes in environmental salinity.[31–33,39,40] Concurrent with the industry's decreasing confidence in the gill NKA enzyme activity as a single reliable proxy for smolt quality several biotechnology companies have introduced quantitative mRNA expression assays based on key components of the NKA enzyme and other ion transporters in gills. Differential NKA-α-subunit isoform expression, or NKA-α1b/α1a ratios, provides a very sensitive indicator of smolt development.[33,41,42] Increased NKA-α1b isoform mRNA and protein levels has largely been linked to elevated enzyme activity in gills of smolts.[31–33] Measures at the transcript level often constitute an earlier indicator of the subsequent protein expression and changes in NKA enzyme activity levels. Similarly, if smolts are not transferred to SW they will start to revert back to a FW state, a process referred to as de-smoltification.[7] De-smoltification is associated with decreasing gill NKA-α1b, concurrent with increasing gill NKA-α1a expression prior to any observable changes in NKA enzyme activity,[33] thereby providing an early warning of de-smoltification and thus give the farmer sufficient time to react. Interestingly, decreasing enzyme activity and upregulation of gill NKA-α1a mRNA levels in maturing salmonids[39,43] suggests that the NKA enzyme and subunit expression may provide useful markers to evaluate osmoregulatory capacity in maturing individuals.

Contrary to the abundant literature of changes in gill NKA enzyme activity and NKA-α-subunit isoform expression at the gene level, less is known about the presence and potential differential expression of NKA-α-subunit isoforms in the intestine and kidneys. In salmonids elevated intestinal NKA enzyme activity appears to be coupled with upregulation of the NKA-α1c isoform mRNA and protein.[35,44] In the kidneys, both the *nka-α1a* and *α1b* mRNA isoforms are present in Atlantic salmon,[30] while in rainbow trout the *nka-α1b*, *α1c* and *α3* subunits are expressed.[29] Despite intestinal and kidney NKA activity increase during PST,[37,38,45] the limited knowledge about expression patterns of NKA-α-subunits, and to some extent NKA enzyme activity in the kidneys, it will require more documentation before introducing NKA subunit expression as smolt markers in intestine and kidneys. Nevertheless, differential expression of NKA-α-subunits or other ion transporters in all three osmoregulatory organs may provide useful biomarkers at the gene or protein level, particularly since the technology has become automated and more cost effective in recent years. However, the caveat with measuring mRNA abundance is that transcripts are often very sensitive and small changes in the environment, particularly ion composition, salinity and other water quality parameters[46–48] may result in changes in NKA-α1b/α1a ratios that not necessarily reflect a true smolt development. Furthermore, differential NKA isoform transcript expression may not reflect translation into protein and the activity of the mature enzyme, which limits the predictive power of mRNA expression as molecular markers. We argue that the NKA enzyme activity may be a better predictor of overall SW readiness under most environmental conditions, rearing regimes and biological scenarios in salmon aquaculture.

2.2 | Optimization of the NKA enzyme activity micro assay method in salmon aquaculture

The micro assay method of McCormick[10] has become one of the most widely used protocols to measure NKA enzyme activity in osmoregulatory tissues. It was also the method of choice when gill NKA enzyme activity was introduced as a biomarker for smolt quality in salmon farming. Briefly, the NKA activity method is enzymatically coupled with pyruvate kinase and lactic dehydrogenase, resulting in the oxidation of nicotinamide adenine dinucleotide, which is directly measured in a temperature-controlled microplate reader. Total protein concentration in the crude tissue homogenate is determined using standard commercial protein kits according to the manufacturer's instructions. The final NKA enzyme activity is reported as μmoles ADP per mg protein per hour. The protocol by McCormick[10] was developed to permit nonlethal gill biopsies using minimal amounts tissue but is also applied to measure activity in the intestine[37] and kidneys,[38,49] both of which play important roles in osmoregulation.[50,51] The industry has reported large variations in gill NKA activity under production of smolts and post-smolts. Hence, this raises the question whether reported variation in NKA activity is a consequence of domestication and/or intensive rearing conditions, or whether the procedures of the NKA protocols itself require modifications when used in larger smolts. The optimized protocols for gills, intestine and kidneys described below are based on several replicated pilot experiments, enabling consistent measurements of NKA activity in gills, intestine and kidneys during PST in Atlantic salmon. The following adjustments and standardizations of the assay is important to yield consistent results when applying the method on multiple tissues (e.g. gills, intestine, kidneys) as well as in larger salmon kept under industrial production environments. The reader is referred to the original method paper for the recipes and in-depth description of the protocol.[10]

2.2.1 | Standardization of the NKA enzyme activity method in gills

The NKA activity assay performance is very consistent when sampling, preservation, tissue storage and assay procedures are standardized. Expression of gill ion transporters can differ significantly between anterior and posterior gills in euryhaline crabs,[52,53] and despite not being able to document significant differences between anterior and posterior gills in Atlantic salmon, it is recommended to always sample the second gill arch from either side, preferentially the left side of the fish. This prevents any potential sampling bias introduces by collecting different gill arches. The gill tissue should be submerged in ice-cold SEI buffer (250 mM sucrose, 10 mM ethylenediaminetetraacetic acid [EDTA], 50 mM imidazole[10]) and immediately snap frozen in liquid nitrogen or on dry ice. Analysing tissue within 1 week is recommended, but if not possible, at least avoid more than 30 days storage, even at −80°C (Table 1). When stored at −20°C, tissue should be analysed within a week to avoid loss of

TABLE 1 Overview of tissue amount used in optimized Na^+, K^+ ATPase (NKA) assay, yielding the preferred protein concentrations (μg/10 μL), recommended centrifugation, storage time and temperature for gill, intestinal and kidney tissues.

Tissue	Tissue amount (mg)	Protein (μg/10 μL)	Centrifugation	Storage time	Temperature (°C)
Gills	1.2–1.6	6–8	6500*g* (2 min)	<7 days (30)	−20 (−80)
Posterior intestine	1–1.4	3–5	8000*g* (4 min)	<3 days	−80
Anterior intestine	1–1.4	3–5	8000*g* (4 min)	<3 days	−80
Kidney	1.2–1.6	6–8	6500*g* (2 min)	<7 days (30)	−20 (−80)

Note: This is based on sampling and analysis of gills, anterior intestine, posterior intestine, and kidney of more than 600 individuals.

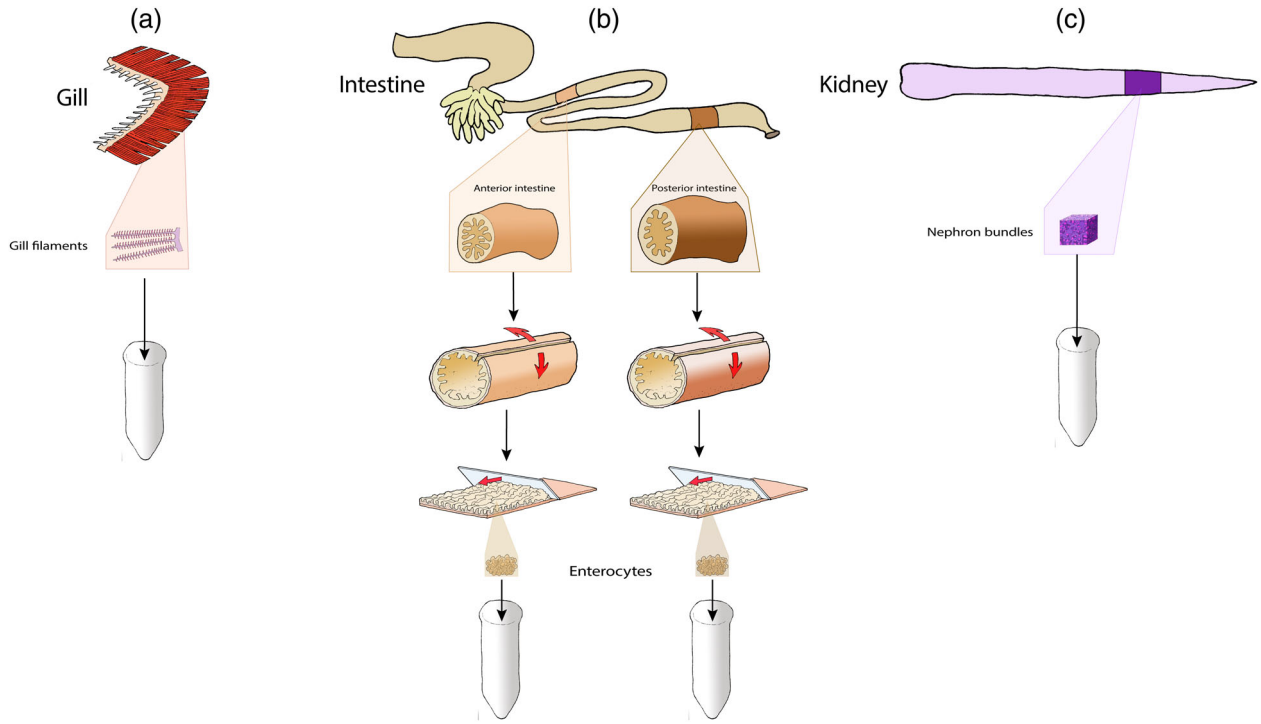

FIGURE 2 General overview of sampling procedure for enzymatic Na^+/K^+-ATPase (NKA) activity measurement in gill, intestine and kidney. All three organs (second gill arch, anterior part of the anterior/posterior intestine and posterior part of the kidney) are collected directly after fish have been euthanized. Gill filaments (approx. 1.2–1.6. mg) are removed from the upper part of the gill arch, an area rich in ionocytes, and transferred to a sampling tube (with ice-cold SEI buffer) for analysis. The intestine is removed from the fish in its entirety and further divided in anterior and posterior intestine. Then a longitudinal incision is made along both sections before its laid out with the serosa side down and the luminal/mucosal side facing up. Hence, gentle scrapings of the mucosal side using a glass microscope slide to maximize abundance of the enterocytes is crucial (approx. 1–1.4 mg) that can be transferred to the sampling tube (with intestinal ice-cold SEIGE buffer) for analysis. The posterior part of the kidney (approx. 1.2–1.6 mg) is removed, an area that are homogenous and rich in nephrons, acquiring the defined nephron tubule cells to be transferred to sampling tube (with ice-cold SEI buffer) for analysis. Modifications and standardization of NKA enzyme activity for gill, gut and kidney are based on the following papers Sundell et al.[37] and Takvam et al.[38]

enzyme activity. Inappropriate storage and repeated freeze–thaw cycles may significantly reduce NKA enzyme activity, which increase the risk of failure to detect peak NKA activity levels in smolts. Although the total abundance of ionocytes remains relatively stable during PST in FW, the number of NKA-α1b ionocytes, primarily found in the filament, increase in number whilst NKA-α1a ionocytes are located on both filaments and lamellae[32,54] (Figure 1a). In SW smolts, the NKA-α1b ionocytes are found on the filament, while NKA-α1a ionocytes disappear[32] (Figure 1a). Location of ionocytes, and thus presence of the NKA enzyme, should be kept in mind when dissecting gill tissue. Several filaments are removed from the mid-section of the second gill arch (Figure 2a), cut in small sections, mixed, and randomly selected and weighed. We recommend using between 1.2 and 1.6 mg of filament tissue (Table 1), ensuring standardization irrespective of gill size. Once filament tissue has been selected and weighted it is homogenized with a motorized pestle in a total volume of 125 μL buffer, that is, 100 μL SEI buffer + 25 μL SEID buffer (0.5% Na deoxycholate acid in SEI buffer) before the crude homogenate are centrifugated at 6500*g* for 2 min (Table 1) to ensure sufficient precipitation of all cell debris. Remaining steps of the analysis are completed in a 96-well plate according to McCormick.[10]

2.2.2 | Standardization of the NKA enzyme activity method in intestinal tissue

The intestinal NKA enzyme is the major driving force of intestinal fluid and secondary ion/nutrient transport,[50] and increased intestinal NKA activity is an important part of developing hypo-osmoregulatory capacity during PST.[37] NKA measurements in intestinal tissues using the micro assay method in research and industrial production of large smolts often led to large variations and/or inconsistent assay performance until introducing the below modifications. Inconsistencies could partially be due to different feeding protocols and/or food deprivation protocols prior to collecting samples, as this may alter epithelial properties[56,57] and thus impact NKA activity. We recommend that fish are fed, not food deprived, prior to collecting tissue as to avoid perturbations of the epithelium. The intestine is cut anterior to the pyloric caeca and anus, then removed from the body cavity and further divided in anterior and posterior intestine (Figure 2b). Nutrients and faeces are pushed out by gently scraping of the intestinal sections. Then a longitudinal incision is made along both sections before its laid out with the serosa side down and the luminal/mucosal side facing up (Figure 2b). The NKA pump is primarily located in the basolateral area of the enterocytes, being more abundant in the mucosal folds of the anterior intestine compared to the posterior intestine[35] (Figure 1b). Hence, the samples are generated by gentle scrapings of the mucosal side using a glass microscope slide to maximize abundance of the enterocytes (Figure 2b). Contamination of connective tissue from the lamina propria layer may follow if too much force is applied. The intestinal scrapings are then thoroughly mixed using small forceps before 1–1.4 mg tissue is weighed and transferred to 0.6-mL tubes containing 100 µL intestinal ice-cold SEIGE buffer (see below) directly during sampling. This ensures appropriate amounts of tissue assayed and minimize errors due to potential variations in activity in anterior and posterior regions of the intestinal tract. The digestive properties of the intestine make it vulnerable to rapid degradation and the intestinal samples should thus be preserved in a modified intestinal ice-cold SEIGE buffer (300 mM sucrose, 45 mM EDTA, 50 mM imidazole, 200 mM glycine, 50 mM ethylene glycol-bis (β-aminoethyl ether)- N,N,N′,N′-tetraacetic acid [EGTA]; including 1 tablet Complete™ protease inhibitor cocktail (04693124001 Roche) for each 10 mL batch of intestinal ice-cold SEIGE buffer) to minimize degradation during lysis and homogenization. All tissues should be snap frozen directly in liquid nitrogen or dry ice and stored at −80°C until analysed no more than 3 days after sampled to avoid loss of NKA enzyme activity (Table 1). At the day of analysis, samples are thawed on ice and homogenized with motorized pestle in a total volume of 125 µL buffer, that is, 100 µL SEIGE buffer + 25 µL SEID buffer, before the crude homogenate are centrifugated at 8000*g* for 4 min (Table 1) to ensure sufficient precipitation of all cell debris. This step is particularly important due to the high content of mucus that can lead to a viscous supernatant making it difficult to pipette. Remaining steps of the analysis are completed in a 96-well plate according to McCormick.[10]

2.2.3 | Standardization of the NKA enzyme activity method in the kidneys

The teleost kidneys are a paired longitudinal structure located dorsally of the abdominal cavity and the nephrons constitute the secretory units, which contains high abundance of the NKA enzyme[51] (Figure 1c). Kidney NKA activity in Atlantic salmon has been reported,[45,49] but to the best of our knowledge, no previous studies has characterized NKA activity from the anterior towards the posterior parts of the kidneys. Five areas (A–E; excluding the head kidney) of the kidneys were characterized and the density of nephrons (histological preparations) and NKA enzyme activity was found to be highest in the posterior D and E areas (see supplementary material from Takvam et al.[38]). The NKA pump are found in all parts of the nephron tubules,[30,55] with higher abundance in the distal and collecting tubule compared to the proximal tubule (Figure 1c). In the proximal tubule, the NKA enzyme is found more basolateral in the cell, while in the distal and collecting tubule both basolateral and lateral location in the cell can be observed (Figure 1c). Sampling of kidney tissue is done by an incision at the posterior D and E area where the nephron densities and thus activity levels are high (Figure 2c; see supplemental data for view of D and E area of kidneys from Takvam et al.[38]). The connective tissue covering the kidneys ventrally should be removed before 1.2–1.6 mg tissue is dissected out and directly snap frozen in ice-cold SEI buffer[10] and stored as described above for the gills (Table 1) to avoid loss of activity. See supplementary material from Takvam et al.[38] for further details regarding the sampling protocol. Once kidney tissue has been selected and weighted its homogenized with motorized pestle in 125 µL buffer, that is, 100 µL SEI buffer + 25 µL SEID buffer, before the crude homogenate is centrifugated at 6500*g* for 2 min (Table 1) to ensure sufficient precipitation of all cell debris. Remaining steps of the analysis are completed in a 96-well plate according to McCormick.[10]

3 | EVALUATION OF SW TOLERANCE AND SMOLT QUALITY IN MODERN AQUACULTURE

Since the mid-2000s, investments in larger and more technologically sophisticated RAS facilities have increased significantly, with the emergence of new production-related challenges and risks.[58,59] Recycling water permits reduced consumption of intake water per produced biomass, constant high temperature, use of continuous light and increased salinity which has resulted in increased proportion of average smolt size, from 50 to 80 g in 2008 to approximately 250 g in 2022 with several companies producing smolts up to 500–600 g in 2023.[24] Despite RAS facilities provide greater control with rearing conditions, the industry experience increased incidents of production disorder such as mineral precipitation in the kidneys,[60–62] haemorrhagic smolt syndrome,[63,64] compromised cardiovascular physiology,[65] multifactorial gill disease[66,67] and other issues related to growth, health and general performance.[68] These disorders may partly be a consequence of rapid changes in technology (from FTS to RAS) and/or smolt protocols (photoperiod,

temperature and salinity) where the physiological requirements of the salmon are challenged.[58,59] While still in FW, changes in photoperiod cues induce a biological cascade of preparative changes that ultimately results in a smolt fully prepared for marine life,[13] while other cues, such as temperature, more govern the rate in which the juvenile salmon develops.[69] Thus, it is imperative that environmental cues are timed and implemented carefully, allowing the juveniles to enter the smolt window (e.g., period), with all physiological systems in synchrony, so they are fully prepared and ready for transfer to the marine environment.[70] However, industry reports several biological traits, such as increased silvering, elevated gill NKA activity and ability to ion regulate when challenged with SW, all associated with PST, to occur independent of applying standard photoperiod protocols, making it increasingly difficult to accurately time the "smolt window" and evaluate quality of large smolts (>250 g). Evidence for a similar PST-independent increase in body silvering, gill NKA activity and SW tolerance in 100–150 g Atlantic salmon kept on continuous light is emerging from the scientific literature.[24,71–73] Arguably more holistic approaches in assessing overall smolt quality and SW readiness is needed to better understand these issues.

3.1 | Whole animal approach in assessment of SW tolerance and smolt development

As part of optimizing the NKA enzyme activity assay we found increased NKA enzyme activity in gills, intestine and kidneys during PST, consistent with the notion of preparatory change to secure sufficient hypo-osmoregulatory capacity when entering the marine environment.[38,74] Gills have been extensively studied and are thus the key organ monitored through smolt development in Atlantic salmon.[13,32,33,75] Similarly, intestinal absorption of water and monovalent ions during PST and SW transfer have been linked to increased NKA enzyme activity.[35,37,50,76] Despite NKA activity in the kidneys has been studied to some extent in euryhaline species,[77–81] only three studies are available in Atlantic salmon.[38,45,49] Our findings have shown a clear difference in the onset and increase in NKA activity where these events during the PST occurs later in the kidneys and anterior/posterior intestine compared to the gills (Figure 3). As a result, caution should be exercised when only using the gills as reporter organ to monitor smolt development. The major benefit of using a more holistic approach is that it gives a more comprehensive evaluation, which arguably provide a more accurate and robust assessment of the overall physiological state of the fish. It is not uncommon for land-based facilities to use different rearing protocols, even within the same company. Such differences may cause different basal levels of NKA activity depending of growth rate, temperature, salinity and light regimes applied. Establishing experience-based enzyme activity levels during production of large smolts and post-smolts in each facility would provide useful baseline data aiding the interpretation of changes and/or potential deviations in NKA activity in modern aquaculture.

3.2 | Variations in the rearing conditions impact NKA enzyme activity

Salmonids display a considerable plasticity in life-history strategies regarding age and size at puberty[82] and maturing salmonids display a decrease in gill NKA activity levels upon coastal arrival, with further reductions in enzyme activity following river entry.[43,83] Rearing of larger smolt and post-smolt in land based facilities under elevated temperature (>13°C) and constant light may lead to increased proportion of early puberty in males,[84,85] which may cause difficulties when interpretating changes in NKA enzyme activity and α-subunit expression, as early puberty results in lower gill NKA enzyme activity and elevated expression of NKA α1a mRNA.[86]

Different salinity in the rearing water may also elicit changes gill NKA activity and/or α-subunit isoform expression. In salmonids,

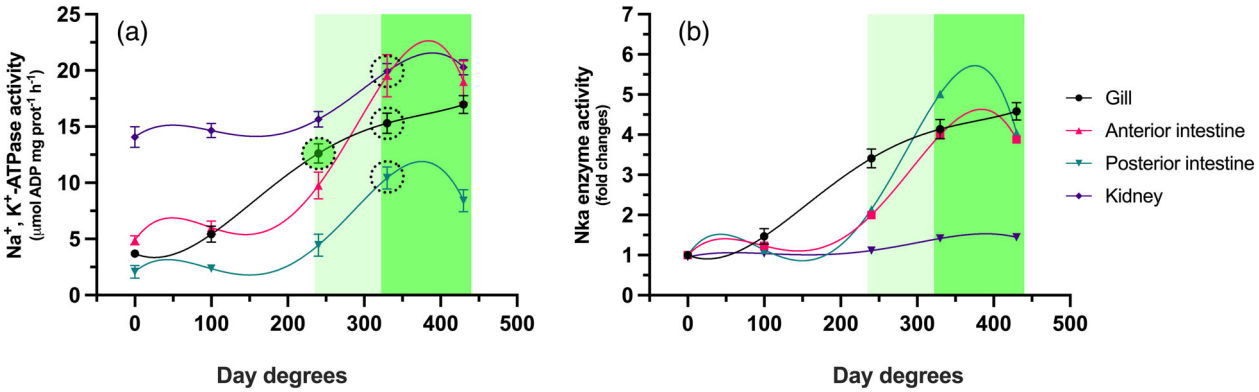

FIGURE 3 Changes in NKA enzyme activity in gills, anterior intestine, posterior intestine and kidney of Atlantic salmon shown as absolute (a: µmol ADP per mg protein per hour) or fold changes (b) during parr-smolt transformation as a function of day degrees (d°C) when reared at 10C. Zero d°C represents when light is switch from a 6 weeks winter signal (12 h light:12 h dark) to spring signal (24 h light) and represents the onset of parr–smolt transformation. Dotted light-green circles indicate significant increase in gill NKA activity at 240 d°C. The dotted shaded light-green areas indicate the period during which NKA activity increased significantly in anterior intestine, posterior intestine and kidney, representing the smolt window. Data are reproduced from Takvam et al.[38] and Takvam.[74]

gill NKA activity and NKA-α1b expression generally increase, while NKA-α1a decrease following exposure to SW.[29,31,54,87] The response may, however, vary depending on the fish size,[12,88] magnitude of salinity exposure and developmental stages. A gradual increase in salinity combined with a stimulatory photoperiod result in an increase of gill NKA activity in juvenile salmon.[89] Still, increases in gill NKA activity appears to require salinity levels higher than 15 ppt when juvenile salmon are exposed to both stimulatory photoperiod and salinity.[90] Larger Atlantic salmon smolts, approximately 100 g, exposed to 12 ppt did not elicit any increase in gill NKA activity but display an increase in NKA-α1b mRNA expression,[24] suggesting that NKA α-subunit expression is more sensitive to salinity changes than the mature enzyme.

The NKA enzyme activity and α-subunit expression may also be sensitive to changes in water qualities. For instance, Atlantic salmon smolts exposed to low pH and elevated levels of aluminium often display reduced gill NKA activity at peak of smoltification, both after chronic[91] and 2–3 days episodic exposure.[47] Interestingly, the decrease in gill NKA α1b-subunit expression in smolts experiencing suboptimal water qualities are replaced by a compensatory increase of α1c isoform expression, suggesting a certain plasticity in recovering from suboptimal water quality exposure.[47,48] Recent findings shows that zebrafish, *Danio reiro*, embryos exposed to cypermethrin display reduced NKA enzyme activity.[92] Similarly, NKA activity decrease in Indian stinging catfish, *Heteropneustes fossilis*, exposed to elevated lead (Pb) levels suggests that NKA enzyme activity or NKA subunit expression may be sensitive biomarkers for a wide range of environmental toxins.[93] The response in NKA activity and subunit expression may either be a direct effect of changes in concentrations of water constituents themselves, or an indirect and/or additive effect of elevated levels of circulating stress hormones, particularly cortisol.[94] In vivo treatment of juvenile salmon with cortisol elicit change in both the gill enzyme activity and expression of NKA-α1b- and α1a-subunit mRNA expression.[95] Hence, suboptimal rearing conditions causing stress-related increase in circulating cortisol levels may provide adverse responses in NKA enzyme activity and α-subunit expression, particularly during the sensitive smolt stage.[96]

In summary, suboptimal rearing conditions clearly elicit adverse responses in NKA enzyme activity either as direct response to changes in the rearing environment (suboptimal water quality, temperature, photoperiod, salinity, etc.) or indirectly through increased stress. We argue that future efforts should be made to include NKA measurements in all three osmoregulatory organs together with monitoring multiple biological traits to provide a better understanding of the physiological changes occurring in a group and/or population of fish.

3.3 | Evaluation of smolt quality should include assessment of multiple biological traits

One of the most used methods for evaluating SW tolerance, and thus smolt status, is subjecting smolts to a standardized SW challenge test.[97] Smolts are transferred directly to full-strength SW for 24 h and plasma ions or osmolality is measured. High-quality smolts will be able to regulate ion levels within 10% of levels normally observed in FW smolts. Poor-quality smolts are often unable to sufficiently regulate ion levels and it is not uncommon to observe up to 30% variations in plasma ion levels. It should be noted, however, that larger fish often display greater SW tolerance as a function by size[11,90] and ability to ion regulate may thus not necessarily be indicative of a true physiological smolt. Hence, SW challenge tests of large smolts may be deceptive and not necessarily reflect later performance in the marine environment. Morphological indicators of PST such as condition factor and silvering should be used with caution during production of large smolt, as increased silvering occurs in larger fish independent of PST,[98] while the traditional decrease in condition factor in smolts[14] may be less pronounced under intensive rearing of larger salmon.[24] We often refer to developmental trajectories or developmental changes related to the number of day degrees (d°C), which basically is the number of days multiplied with the water temperature, for example, 10 days in 10°C water constitute 100 d°C. As mentioned, gill NKA activity is widely used, and increases between 200 and 250 d°C after the spring signal (continuous light) is turned on (Figure 3), thus, the smolt window of approximately 250–350 d°C has been defined before loss of activity, de-smoltification, occur around 500 d°C.[20,99,100] Increases in NKA enzyme activity occur later in both intestine and kidneys, approximately 320–460 d°C (Figure 3). Furthermore, a defined range of d°C when de-smoltification and loss of NKA enzyme activity occurs in intestine and kidneys are, as far as we are aware of, not available. A more in-depth high-resolution sampling and analysis before, during and after PST are required to establish the exact timing of increases (peak smolt) and decreases (de-smoltification) in all three tissues. Nonetheless, our results suggest that consecutive analysis of NKA enzyme activity as a proxy for peak smolt in all three osmoregulatory organs simultaneously would be beneficial and potentially more reliable assessment (in contrast to using gills only) of peak smolt development and readiness for entry to SW. Significant increase of NKA enzyme activity in both intestine and kidneys occurs later in the 'smolt window' than observed for enzyme activity in gills (Figure 3). New recommendations of a peak smolt 'smolt window' needs to encompass all three tissues, and should arguably be 400–460 d°C. However, more data from both small- and large-scale experiments will be required to validate these ranges.

One major advantage of the NKA enzyme activity method is that it is easy, fast, and applicable for both research and industry purposes. With a training period of 1–2 weeks, an experienced technician can learn how to run the method in all three tissues using the modified protocol presented here. Once the method has been established, our experience is that two persons can run up to 50–100 fish per day for all three tissues. It should be noted that the use of different technologies (RAS, FTS, hybrid systems), temperatures, photoperiods (winter signal or continues light), salinities in the water (5–20 ppt), transition feeds and smolts sizes may affect the physiological responses.[22–24,101–103] Thus, acquiring sufficient reference data of concurrent NKA activity in all three organs in natural smolting salmon as well as in large smolts reared under industrial conditions will be important to gage the

robustness of using the NKA enzyme activity method as a proxy for smoltification. One may also consider applying molecular markers, measuring Nka-α-subunits in both intestine and kidneys, similar to that used in gills. We are currently in the process of testing expression profiles at the mRNA and protein level for relevant transporters in the kidneys[38,51,60] and intestine[35,50] to further increase the available repertoire of tools to evaluate smoltification. We want to highlight the importance of adopting the optimized protocol both in the aquaculture sector and in other research institutes. To our knowledge, no standardized sampling protocol for all three tissues exists and widespread standardization will further aid in validating the method across research topics within fish physiology.

Using the NKA enzyme activity assay in all three organs may give the opportunity for a more holistic evaluation of smolt quality. Indeed, using this holistic approach may aid in better understanding the fish's physiological status. Nevertheless, the optimized protocol should be used together with new emerging biomarkers and techniques. For example, the NKA assay and expression of ion transporters can be further integrated with other physiological and molecular markers, including markers for immune response,[104] growth, metabolism and energy status[105,106] and stress response[107,108] in the same individual fish. However, such approaches need to be applicable in modern aquaculture facilities.

4 | CONCLUDING REMARKS AND PERSPECTIVES

We have modified the NKA micro assay protocol by McCormick[10] to be used in gills, intestine and kidneys in smolts and larger post-smolts. Collectively, repeated and concomitant analysis of the NKA enzyme activity in all three organs are required to better time and evaluate correct transfer of smolt to SW. Enzyme activity can be accompanied by analysis of NKA isoforms, in all three organs together with new developments of alternative biomarkers. This would presumably facilitate a more accurate and better assessment of the physiological consequences of new intensive production strategies of smolts and large smolts (>250 g) currently used in salmonid aquaculture. Applying the NKA assay in all three organs have the potential to detect disturbances in overall osmoregulatory ability and how it may respond to environmental changes (temperature, salinity, photoperiod, suboptimal water quality, etc.). Although, this review focus on the salmonid aquaculture industry the method should also be considered in other aquaculture species. Comparative studies may shed light on species-specific differences that can broaden our understanding of fish physiology. Apart from being a good proxy for peak smolt development, it may be useful in several different fields of fish physiology and biology, including: (1) nutrition and digestion (effect on changes in feed composition, uptake of macro/micro molecules); (2) toxicology (effect of toxic substances); (3) stress physiology (stress response); (4) fish health and welfare (disease and physiological disturbances); (5) acid–base regulation (hypercapnia/hypocapnia and hyperoxia/hypoxia); and (6) water chemistry (suboptimal water quality). Indeed, the NKA enzyme is indirectly involved in secondary transport of fluids and many different solutes and if used correctly can give useful insight in all the above fields.

AUTHOR CONTRIBUTIONS

Marius Takvam: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; software; formal analysis; data curation; resources. **Kristina Sundell:** Funding acquisition; methodology; validation; writing – review and editing; resources; formal analysis; investigation; conceptualization; data curation; project administration. **Henrik Sundh:** Conceptualization; validation; methodology; formal analysis; investigation; data curation. **Naouel Gharbi:** Funding acquisition; resources; supervision; validation. **Harald Kryvi:** Visualization; validation; software. **Tom Ole Nilsen:** Conceptualization; investigation; funding acquisition; writing – review and editing; visualization; validation; methodology; project administration; resources; supervision; formal analysis; data curation.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in https://bora.uib.no/bora-xmlui/handle/1956/23216.

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