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EFFECT OF NANOSILVER ON METABOLISM IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): AN INVESTIGATION USING DIFFERENT RESPIROMETRIC APPROACHES

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Abstract: Nanosilver (nAg) has been incorporated into many consumer products, including clothing and washing machines, because of its antimicrobial properties. Consequently, the potential for its release into aquatic environments is of significant concern. Documented toxic effects on fish include altered gene expression, gill damage, and impaired gas exchange, as well as mortality at high nAg concentrations. The present study reports the effects of nAg on the metabolism of rainbow trout (*Oncorhynchus mykiss*). Fish were exposed to environmentally relevant concentrations (0.28 ± 0.02 μg/L) and higher (47.60 ± 5.13 μg/L) for 28 d, after which their standard metabolic rate (SMR), forced maximum metabolic rate (MMR_f), and spontaneous maximum metabolic rate (MMR_s) were measured. There was no effect observed in SMR, MMR_f, or MMR_s, suggesting that nAg is unlikely to directly affect fish metabolism. On average, MMR_s tended to be greater than MMR_f, and most MMR_s occurred when room lighting increased. The timing of MMR_f chase protocols was found to affect both MMR_f and SMR estimates, in that chasing fish before respirometric experiments caused higher MMR_f estimates and lower SMR estimates. Although compounded effects involving nAg and other environmental stressors remain unknown, the present study indicates that the tested range of nAg is unlikely to constrain fish metabolism. *Environ Toxicol Chem* 2017;36:2722–2729. © 2017 SETAC

Keywords: Ecotoxicology Respirometry Salmonid Stress Maximum metabolic rate

INTRODUCTION

Nanosilver (nAg) is a type of nanoparticle commonly used in many consumer products for its antimicrobial properties [1]. Because many of these products include clothing, cleaning products, and washing machines [2], nAg is entering wastewater at an increasing rate and has significant potential to affect the aquatic environments that receive municipal waste streams. Once in the aquatic environment, nAg may dissolve into silver ions (Ag^+) or aggregate with compounds or other nAg particles [3]. Concentrations of nAg in the aquatic environment are not well known; however, most studies estimate environmental concentrations in the nanograms per liter range [4,5], and concentrations as high as 0.32 μg/L [6] and 1.4 μg/L have been reported [7] for some aquatic habitats.

Toxicity from nAg is thought to result from a combination of the effects of both the nAg and the Ag^+ it releases [8], with nAg's main mode of toxicity being through oxidative stress and Ag^+s' main mode being through inhibition of the Na^+/K^+ sodium-potassium adenosine triphosphatase (ATPase) pump in gill cells leading to osmoregulatory impairment [9]. Toxic effects from nAg include altered gene expression, gill damage, and impaired gas exchange, as well as mortality at high concentrations [10–12].

Sublethal effects of nAg, such as changes in gene expression [12,13] and impairment of gas exchange [10,12,13], may affect the metabolism of fish, which encompasses all costs arising from basic bodily functions (e.g., respiration, osmoregulation), activity, and digestion/absorption/processing of food (specific dynamic action). Increased metabolic costs can lead to reduced growth [14,15], fecundity [16], and potentially survival [17], all of which reflect individual fitness under the constraint of natural selection. As such, measures of metabolic rate provide a useful link between the physiological effects of contaminants and their effects on fish at both the organismal and population levels [18,19].

The objectives of the present study were to investigate the effects of nAg on metabolism in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) through nAg exposure at environmentally relevant levels and higher. We examined potential toxic effects of nAg on standard metabolic rate (SMR), maximum metabolic rate (MMR), and aerobic scope. Standard metabolic rate is defined as the minimal energy requirement of ectothermic, unstressed, and resting animals at a specific temperature, which is made up of all of the basic functions of cellular metabolism, such as protein synthesis and ATP turnover, that keep a fish alive [20]. Maximum metabolic rate defines the maximal aerobic metabolic rate attainable [21]. These 2 rates encompass the aerobic scope, which describes an organism's scope of activity—the aerobic amount a fish can increase its metabolic activity above maintenance levels [22]. We hypothesized that sublethal physiological stress related to nAg exposure would potentially increase SMR (from such effects as changes in gene expression) and decrease MMR (from such effects as gill damage causing an impairment in gas exchange), thereby causing a decrease in aerobic scope.

The dataset was also examined to investigate the effects of using different respirometric methods, specifically diverse ways to obtain MMR and the effects of changes in timing of MMR

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methods. This analysis is important to highlight the constraints and limitations of these methods and how different methods have the potential to affect results. Respirometric methods are used in a growing number of studies, and this analysis is relevant not just to the nAg results of the present study but to all studies that use these techniques.

MATERIALS AND METHODS

Experimental animals

Oncorhynchus mykiss were obtained from Lyndon Fish Hatcheries (New Dundee, Ontario, Canada) on 11 February 2014 ($n = 200$) and 29 April 2014 ($n = 200$). All fish were female diploid fish and weighed approximately 1 g at arrival. Fish were quarantined for 1 wk in large (160–200 L) tanks, and throughout the experiment were kept at a water temperature of 14 °C. Fish were exposed to a 12:12-h diurnal lighting regime with gradual light changes (lights turned on and off in 3 stages to mimic dawn and dusk) throughout the quarantine and experiment. After quarantine, 11 fish were placed in each of the 24 exposure tanks (40 L each) and acclimated for 1 wk. Seven of these fish were used for cortisol and morphometric sampling at various time points (results reported in Murray et al. [23]), and 4 fish from each tank were used in the respirometric experiments, for a total of 94 fish included in respirometric experiments (there were 2 mortalities, bringing the total down from the 96 allocated). There were also several issues with equipment failure and deviations from protocol that lowered the number of fish included to 80 for SMR, 93 for forced maximum metabolic rate (MMR_f), 89 for spontaneous maximum metabolic rate (MMR_s), 79 for forced aerobic scope (AS_f), and 80 for spontaneous aerobic scope (AS_s). At the time that respirometric experiments were initiated, fish weighed on average 2.91 ± 1.14 g.

Exposure experiment

Nanosilver exposure was achieved using a flow-through setup, where a peristaltic pump transported nAg solution to the tanks. There were 2 exposure trials, with treatments comprised of a control and nominal exposure concentrations of 1 μg/L and 200 μg/L in the first trial, and a control and nominal exposure concentrations of 300 μg/L and 600 μg/L in the second trial. Four replicate exposures were conducted for each treatment, giving 24 total exposures over both trials. However, it was observed that measured nAg concentrations were much lower than nominal concentrations and quite variable, and that concentrations within the higher exposure tanks (200, 300, and 600 μg/L nominal) overlapped and were not significantly different [23]. As a result, respirometric data for these exposure groups were combined into a single group (high). Therefore, for statistical analysis, we considered 3 treatment levels over both trials: the controls (from both trials), a low exposure (1 μg/L nominal concentration from trial 1; mean exposure concentration = 0.28 ± 0.02 μg/L), and a high exposure (200 μg/L nominal concentration from trial 1, and 300 and 600 μg/L nominal concentrations from trial 2; mean exposure concentration = 47.61 ± 5.13 μg/L).

Exposures were conducted for 28 d before respirometric experimentation. Silver (Ag) concentrations in randomly selected exposure tanks were taken every 4 d. Detailed methods of Ag measurements in water are described in Murray et al. [23]. Briefly, total Ag was analyzed by inductively coupled plasma–mass spectrometry (ICP–MS, XSeries 2; Thermo Scientific). Concentrations of nAg in comparison with ionic Ag were analyzed by collecting water samples in centrifugal filter tubes (Amicon Ultra-3K; EMD Millipore), and then centrifuging samples to separate the retentate from the dissolved fraction. The 2 fractions were then analyzed by ICP–MS to determine the total Ag in the retentate (nAg) and the dissolved fraction (ionic Ag).

Respirometric experiments

Respirometric experiments were conducted at the end of the exposures on 4 fish from each of the 24 exposure tanks that had not been fed for at least 24 h. Three metabolic rates were measured for each fish: SMR, MMR_f, and MMR_s. Both SMR and MMR_s were estimated from fish held in respirometric chambers for a 24-h period, whereas MMR_f was estimated as the maximal respiration after a chase trial (see the section *MMR_f*).

Four cylindrical chambers (length = 12.3 cm; diameter = 3.3 cm; and volume = 92 mL each) were installed in a 400-L tank with recirculating water. Each chamber was sealed and monitored for oxygen using an oxygen probe. Temperature was kept at 14 ± 0.1 °C with water baths, and water was kept oxygenated and mixed with air stones and pumps. Fish were exposed to the same 12:12-h diurnal cycle as for the exposure experiment (with gradual light changes) and shielded from any external stimuli with a dark curtain draped around all edges of the tank. Activity in the room where experiments were conducted was also minimized. Equipment in the tank was kept to a minimum to reduce bacterial respiration and potential adhesion of nanoparticles. Oxygen probes were calibrated every week or when inconsistencies in oxygen measurements were observed. Before each respirometric trial, nAg stock solution was added to the tank to match nominal exposure concentrations; similar to exposure experiments, water samples were taken near the end of the experiment to ascertain actual Ag concentrations. As with the exposures, total Ag measured in respirometric tanks was also lower than nominal [24]. To minimize background bacterial respiration and contamination of nAg between experiments, the tank was drained and all surfaces cleaned and flushed repeatedly with water between experiments. After respirometric experiments were completed, the fish were measured, weighed, and euthanized by cervical dislocation. Muscle tissue was also taken for determination of total Ag concentration.

Oxygen consumption rates (MO_2) were measured by automatic intermittent-flow respirometry [25] using an AutoResp system (Loligo Systems). In the respirometric system, water was pumped continuously by a flow pump (Eheim Universal Hobby Pump 1046) from each chamber to the fiber optic probe (PreSens) and back into the chamber. Each respirometric cycle lasted for 8.5 to 9 min and consisted of a flushing period of 3 min where water in the respirometer was renewed with water from the holding tank, a 1-min wait period where the respirometer was closed but no oxygen concentration measurements were taken, and a 5.5- to 6-min measure period when oxygen concentrations were measured. The measurement period was shortened to 5.5 min to ensure that oxygen conditions remained normoxic in the chambers. The rate of decline in oxygen concentration during this period permitted estimation of MO_2 [26].

For each respirometric trial, background MO_2 values (i.e., no fish inside the respirometer chamber) from before and after each respirometric trial were measured with 20-min measure periods for each respirometer and averaged, and the average was subtracted from all whole-fish MO_2 (mg O_2/h) estimates [27]. Individual MO_2 estimates with an r^2 lower than 0.9 were determined to reflect poor linear estimates of MO_2 [28] and were eliminated. Estimates of MO_2 in the respirometers with low O_2

concentrations were also eliminated to ensure fish were not oxygen-limited (<73.1% air saturation or ~15.2 kPa as per Tang et al. [29]). This processing of data resulted in the removal of a small amount of MO_2 measurements (0.08% of measurements excluded as a result of low oxygen concentration in the respirometric chamber, 0.16% of measurements not used because of low r^2, for a total of 0.24% of the 13 120 measurements removed from the estimation of MO_2).

Subsequently, mass-specific oxygen consumption rates were calculated as

$$_{spec}\dot{M}O_2 = \frac{\dot{M}O_2}{M^b} \quad (1)$$

where $_{spec}MO_2$ is the mass-specific oxygen consumption rate (mg O_2/kg/h), M is the fish body mass (kg), and b is the mass exponent adjustment. For MMR_f, $b = 0.88$, but it was 1 for all other respirometric rates (description of mass exponent adjustment estimation can be found in the *Estimation of mass-adjusted metabolic rates* section).

SMR and MMR$_s$

Standard metabolic rate was estimated as the mean of the 10 lowest observed MO_2 estimates during the 24-h experiment [30,31]. This method was chosen after estimating SMR using various methods [32] and finding that it best represented the data when examining MO_2 plots and comparing the SMR estimates achieved with the various methods to the position of the horizontal band of low MO_2 values. Forced maximum metabolic rate was defined as the single highest MO_2 of the 3 estimates observed immediately after the chase procedure [33]. Spontaneous maximum metabolic rate was calculated as the highest single MO_2 observed during the 24-h experiment (excluding MMR_f measurements; similar to Svendsen et al. [31]). Aerobic scopes between both SMR and MMR_f (AS_f), and SMR and MMR_s (AS_s) were calculated by subtracting the SMR from the MMR_f and MMR_s, respectively [34].

MMR$_f$

To estimate MMR_f, one fish at a time was placed in a circular bucket and exhausted through a standardized 5-min manual chase method: 1-min chase, 3 min turning the fish over, and 1 min holding the fish out of water [35,36]. At the end of this 5-min chase, fish were not capable of burst swimming, a sign of exhaustion [37]. Immediately after chasing, fish were placed into a respirometric chamber to measure MMR. For a subset of fish (the first 3 tanks tested out of 24), MMR_f was conducted at the end of the SMR measurements. Having observed higher rates of metabolism (MMR_s) during SMR trials than observed during MMR_f (see the *Results* section), the protocol was then changed to test MMR_f at the beginning of the respirometric trial, in an attempt to obtain higher MMR_f measurements by potentially capturing the immediate stress of transfer to a novel environment (the respirometric chamber) in addition to the chase methods. The influence of the timing of MMR_f estimation on other respirometric variables was evaluated to ensure this change in methods did not influence results.

Estimation of mass-adjusted metabolic rates

To mass-adjust metabolic rates for the size of fish appropriately, it was first determined whether rates were isometric with body size [15] by examining $\log_{10}$-transformed relationships between each rate with $\log_{10}$-transformed body mass, including control, low, and high treatments, as a grouping variable. For each rate, differences in slopes among treatments were examined and in all but one case, slopes were not significantly different among treatments (test for heterogeneity of slopes, all $p > 0.05$). In the case of MMR_f, a single outlier in the low treatment with high leverage resulted in a more elevated slope compared with the other 2 treatments (test for heterogeneity of slopes, $p = 0.045$). Following removal of this single outlier value, slopes were not significantly different among treatments ($p = 0.055$), and analysis proceeded without the outlier.

Next, for each metabolic rate, analysis of covariance (ANCOVA) was used to determine a common slope value for instances where differences existed among treatments in the relationship between $\log_{10}$(metabolic rate) and $\log_{10}$(body mass). For both SMR and MMR_f, the effect of treatment was not significant (ANCOVA, SMR: $F_{1,2} = 0.34$, $p = 0.71$; MMR_f: $F_{1,2} = 0.20$, $p = 0.82$). In the case of MMR_s, treatment was a significant effect in the model (ANCOVA: $F_{1,2} = 3.61$, $p = 0.03$), with the high treatment significantly lower by weight than the control (Tukey honest significant difference test, $p = 0.04$). In this case, the common slope from the ANCOVA was taken and evaluated against a value representing isometry (slope of 1) using a one-sample t test.

Where there was no significant treatment effect in the ANCOVA models, treatment was ignored and the slope of each metabolic rate was estimated with body mass for all observations (each $\log_{10}$-transformed). In all but one case, the slope of each rate was not significantly different from 1 (one-sample t test, SMR $t_{78} = 1.50$, $p = 0.54$; MMR_s $t_{86} = -0.56$, $p = 0.49$). For these rates, it was concluded that metabolic rates were isometric with a mass exponent equal to 1. In the case of MMR_f, the slope of the log–log relationship of mass-relative rates against body size resulted in a slope of 0.88, which was significantly different from a slope of 1 (one-sample t test, $t_{90} = -1.72$, $p = 0.045$). Residual plots of the models were examined for normality and homogeneity among treatments and in all cases were found to meet assumptions. Relationships between metabolic variables and fish body mass are graphed in Supplemental Data, Figure S1.

Statistical analysis

Analyses were conducted in R Ver 3.1.3 [38]. Respirometric data (SMR, MMR_f, MMR_s, AS_f, and AS_s) were analyzed using a mixed-effect model (lmer). The full model tested for effects was

$$Respirometry\ variable = Treatment + Tank + Chamber + Time\ of\ MMR_f \quad (2)$$

where treatment was a fixed effect, and tank, chamber, and timing of MMR_f (conducting MMR_f procedure before or after SMR experiments) were random effects. To determine the significance of nAg exposure on *O. mykiss* metabolic rates, log-likelihood tests were used to compare the full model (see Equation 2) with a reduced model (the full model without the treatment effect).

The effect of timing of MMR_f chase procedure on respirometric variables was analyzed using a Welch's 2-sample t test. For MMR_f, a significant difference was found between trial controls (likely caused by the change in timing of the MMR_f procedure). Therefore, trial was included in these models as a random effect. No significant differences were found for all other respirometric variables between the controls of each trial

(analysis of variance, all $p > 0.05$); consequently, controls were combined into a single treatment and trial was not considered a factor in the analysis. Residual plots of the models were examined for normality and homogeneity among treatments and in all cases were found to meet assumptions. Where appropriate, post hoc analyses were conducted with a Tukey honest significant difference test.

RESULTS

Exposure concentrations of Ag

Most of the Ag measured in the exposure tanks was in particulate form (nAg), with only 0 to 0.18% in ionic form (Ag^+; average 0.05% ionic). Concentrations of total Ag were lower than nominal and quite variable, ranging from 0.18 to 0.38 μg/L in the low treatment and from 4.46 to 159 μg/L in the high treatment (Supplemental Data, Table S1). Additional details on concentrations of total Ag can be found in Murray et al. [23].

Effects of nAg on metabolic rates

The mixed effects analysis of metabolic rates revealed no difference in SMR among nAg treatments, which had an overall mean of 119 mg O_2/kg/h (Figure 1A, Table 1; log-likelihood test, $\chi^2_{(df=2)} = 2.682$, $p = 0.262$). There was also no difference among treatments for MMR_f, which had an overall mean of 470 mg O_2/kg/h (Figure 1B, Table 1; log-likelihood test, $\chi^2_{(df=2)} = 0.132$, $p = 0.936$), or MMR_s, which had an overall mean of 495 mg O_2/kg/h, although the H treatment appeared lower than other treatments (Figure 1C, Table 1; log-likelihood test, $\chi^2_{(df=2)} = 5.279$, $p = 0.071$) and was significantly lower when mass-adjusted rates were compared with mass as a covariate (see the *Methods* section). Finally, no difference by treatment was found for either AS_f, with an overall mean of 357 mg O_2/kg/h (Figure 1D, Table 1; log-likelihood test, $\chi^2_{(df=2)} = 0.081$, $p = 0.960$), or AS_s, with an overall mean of 376 mg O_2/kg/h (Figure 1E, Table 1; log-likelihood test, $\chi^2_{(df=2)} = 3.409$, $p = 0.182$).

Maximum metabolic rates

Spontaneous maximum metabolic rate was higher than MMR_f for 59% of the 87 measurements, with MMR_f being on average 95% of MMR_s. Interestingly, MMR_s most often coincided with the change in the room lighting, particularly when the room lighting increased in the morning (63.3% of MMR_s measurements). An example of the typical pattern of MO_2 in the experiments can be seen in Figure 2, with the timing of lighting increasing and decreasing marked.

Forced maximum metabolic rate and AS_f were found to be higher if the MMR_f chase procedure was conducted before the respirometric experiment (Figure 3; 2-sample t test, MMR_f, $t_{11.01} = -3.015$, $p < 0.012$; AS_f, $t_{8.029} = -5.738$, $p < 0.001$). The mean for MMR_f when fish were chased at the beginning of the respirometric trial was 481.44 ± 10.60 ($n = 82$), and 378.49 ± 32.45 ($n = 10$) when chased after the trial. The mean AS_f when fish were chased before the trial was 368.54 ± 11.91 ($n = 73$), and 220.36 ± 22.91 ($n = 6$) when chased after. Standard metabolic rate was found to actually be

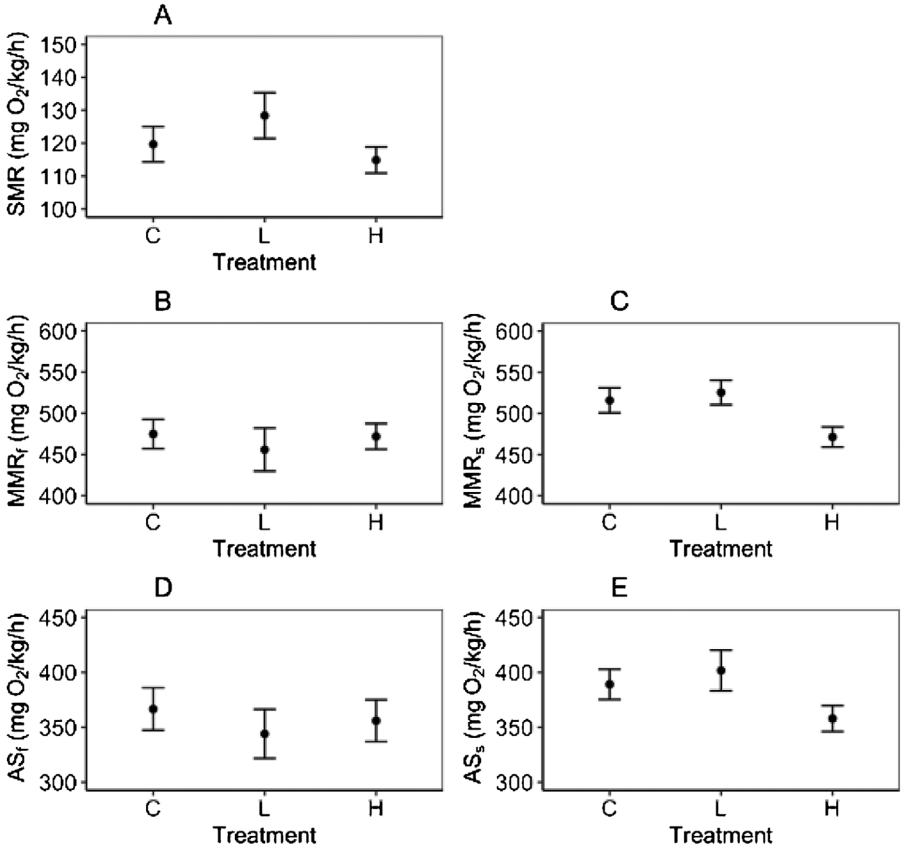

Figure 1. Metabolic variables for different treatment levels. Data expressed as means ± standard errors: (**A**) standard metabolic rate (SMR); (**B**) forced maximal metabolic rate (MMR_f); (**C**) spontaneous maximum metabolic rate (MMR_s); (**D**) forced aerobic scope (AS_f); and (**E**) spontaneous aerobic scope (AS_s). C = control; L = low (0.28 ± 0.02); H = high (47.61 ± 5.13).

Table 1. Means, standard errors, and sample sizes for metabolic variables in different nanosilver exposure treatments (mg O_2/kg/h)

	SMR		MMR_f		MMR_s		AS_f		AS_s	
Exposure levels	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*
Control	119.66 ± 5.33	25	474.82 ± 17.58	31	515.79 ± 15.26	29	366.70 ± 19.31	25	389.17 ± 13.82	25
Low	128.35 ± 6.95	15	455.88 ± 26.06	15	525.43 ± 14.94	15	344.06 ± 22.30	14	401.79 ± 18.59	15
High	114.84 ± 3.97	40	471.92 ± 15.52	47	471.36 ± 12.12	45	356.04 ± 19.02	40	358.05 ± 11.82	40
Overall	118.88 ± 2.91	80	470.25 ± 10.57	93	494.95 ± 8.59	89	357.29 ± 11.97	79	375.65 ± 8.28	80

SE = standard error; *n* = sample size; SMR = standard metabolic rate; MMR_f = forced maximum metabolic rate; MMR_s = spontaneous maximum metabolic rate; AS_f = forced aerobic scope; AS_s = spontaneous aerobic scope.

lower if fish were chased before the trial (Figure 3; *t* test, $t_{77.812} = 3.117$, $p < 0.003$). The mean SMR when chased before the respirometric trial was 116.43 ± 2.88 ($n = 73$), and 144.44 ± 11.22 ($n = 7$) when chased after. No significant difference was found for spontaneous metabolism (MMR_s and AS_s) based on the timing of the chase procedure.

DISCUSSION

nAg effects on metabolism

Once random effects were taken into account, results showed no significant effect of nAg on metabolic variables of SMR and MMR_f in *O. mykiss* following 28 d of exposure. Measures of MMR_s were significantly depressed relative to controls using body size as a covariate; however, this relation became insignificant once random effects were taken into account. These results as well as outcomes from other studies seem to suggest that metabolic rate may be largely insensitive to nAg exposure, even at very high exposure concentrations. Similar to our results, Bilberg et al. [10] did not observe an effect of nAg on SMR of Eurasian perch (*Perca fluviatilis*) at nominal concentrations from 63 to 300 μg/L over 24 h, but nAg did decrease hypoxia tolerance at 300-μg/L exposure. In the same study, bulk Ag (in the form of Ag nitrate) at a nominal concentration of 386 μg/L increased SMR and decreased hypoxia tolerance. The present study results confirm this insensitivity over a similar nominal exposure concentration, but over a more biologically relevant time period when evaluating potential risk to aquatic ecosystems (28 d in the present study vs 24 h). Similarly, in zebrafish (*Danio rerio*) embryos, no effect of nAg on the metabolic rate was observed when exposed from 24 through 48 h to very high levels of nAg (20 000–140 000 μg/L) [39].

In parallel with the present study, Murray et al. [23] examined plasma cortisol and nAg accumulation in *O. mykiss*.

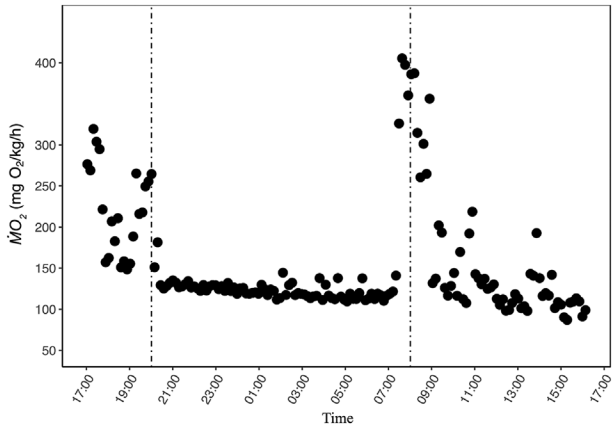

Figure 2. Standard metabolic rate experiment for a typical fish in the respirometric trials, showing oxygen consumption rate (MO_2) over 24 h. Timing of changes in lighting is marked with dashed/dotted lines. Lights turned off at 20:00 h and turned on at 08:00 h.

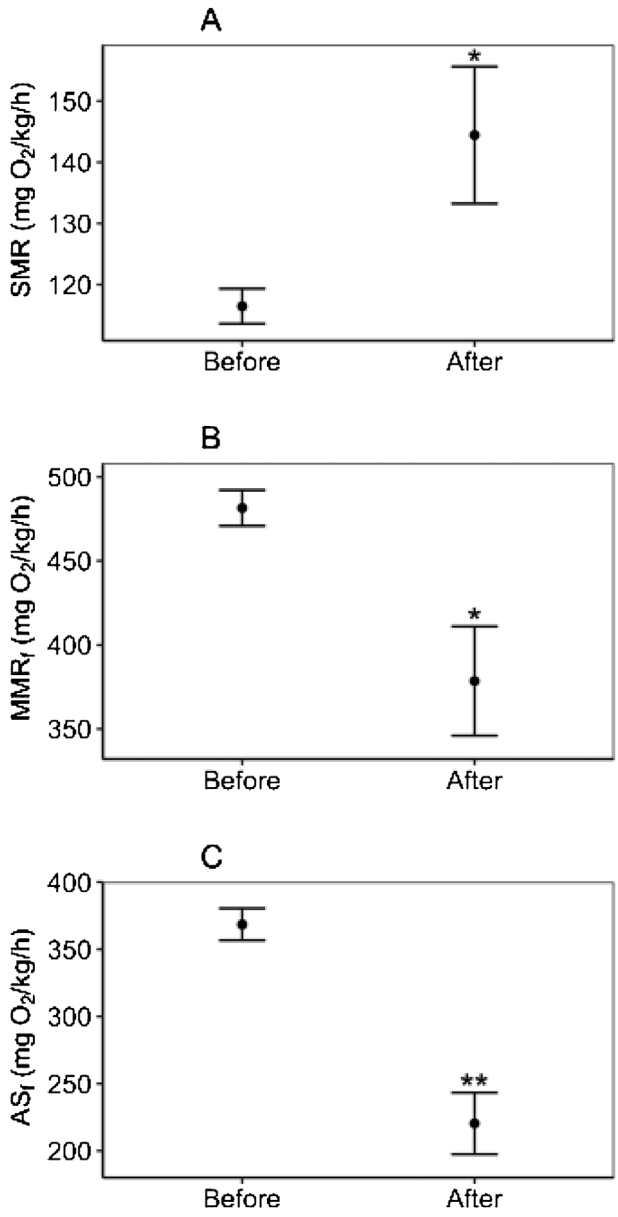

Figure 3. Differences in metabolic variables by timing of forced maximum metabolic rate (MMR_f) procedure before and after standard metabolic rate (SMR) experiment: (**A**) SMR; (**B**) MMR_f; and (**C**) forced aerobic scope (AS_f). Asterisks denote significant differences among groups. * = $p < 0.05$; ** = $p < 0.001$.

Fish exposed to nAg for 28 d showed a significant cortisol (stress) response to nAg exposure, and were found to have accumulated significant Ag in muscle tissue at the highest exposures. These significant direct cortisol effects are in contrast to the general lack of an effect of nAg on SMR in the present study and other studies. Other research has reported significant correlations between cortisol elevation and SMR [40,41]. In addition, other findings have shown an effect of nAg exposure on metabolism when fish were under additional stress (hypoxia)——potentially as a result of gill damage that nAg has been observed to cause in fish [11,13]. The present study did reveal some evidence that MMR_s may be depressed at a high concentration of nAg, which may indicate some effect of nAg on maximum metabolic rates, but only at very high levels of exposure unlikely to be encountered in natural systems.

It is possible that the inability to detect significant changes in metabolic rates may be attributable to adaptation to chronic exposures of the contaminant under investigation. Although, to our knowledge, no additional studies have been conducted on the effects of other nanoparticles on fish metabolism, a variety of studies have analyzed the effect of ionic metals on metabolic rate where a phenomenon of increasing metabolic insensitivity with exposure time has been observed. For example, Pb decreased MMR_f during acute exposure (24 h), but not in chronic exposures of 33 to 57 d in fathead minnow (*Pimephales promelas*), indicating that fish were able to recover from the effects of Pb on metabolism [42]. It is possible that this same phenomenon of recovery with chronic exposure to a contaminant may be at work in the present study, and may be why no significant effect on metabolic rates was seen after 28 d, whereas a significant effect on metabolism was observed when fish were under additional stress (hypoxia) following their shorter (24 h) exposure [10]. A pattern of recovery of other toxic effects from nAg over time also supports this. For example, gill epithelial proliferation was more distinct at 14-d exposure in comparison with 35-d exposure [13] and altered gene expression was often greater at 7- or 21-d exposure versus 28-d exposure [12]. Short-term effects could be important in natural settings where intermittent decreases in physiological performance could play a role in fish reproduction and survival, although this hypothesis remains to be tested. However, such intermittent effects are not representative in all cases of metal exposure; the effect of chronic Al on *O. mykiss* in acidic water exposed for 36 d was found to decrease MMR_f and AS_f by approximately one-half, and there was a trend for increased SMR [43]. These studies do however generally support the hypothesis of an increase in SMR and decrease in MMR attributable to contaminant exposure, although this pattern appears to depend on exposure time (chronic vs acute) [20].

Another potential challenge in detecting an effect of nAg exposure on SMR is the observation that the respirometric procedure appears to be inherently stressful, indicated by elevated cortisol levels in fish that were involved in respirometric experiments in comparison with fish that were not in such experiments [44]. An elevated metabolic response caused by respirometry may make more subtle differences in SMR as a result of contaminant exposure more difficult to detect. This potential elevated standard metabolism may not affect MMR_s, but elevated SMR would certainly affect aerobic scope estimates.

Comparison of methods in estimating maximum metabolic rates

Forced maximum metabolic rate was lower than MMR_s for 59% of the measurements. This indicates that the chase protocol of 5 min (consisting of 1-min chase, 3 min turning fish over, and 1-min air exposure) was insufficient to obtain maximum metabolic rates that were higher than the spontaneous rates observed in the chambers during a 24-h observation period. Whereas critical swimming speed trials can also be used to obtain MMR, chase protocols are widely used as well and give results similar to swim trials [45]. In another study, swim trials were found to reveal the highest MMR estimates, with chase trials reflecting 36% lower MMR than swim trials and only 23% lower when an air-exposure element was added to the chase trial [36]. Therefore, the recommendations of these authors [36] were followed to use a chase with an air-exposure element because swim trials were not possible in the present study. The results of the present study support the idea that chase trials may not obtain true MMR (because our MMR_s was higher than MMR_f in most cases).

Although MMR_s is less often used as a metabolic metric than MMR_f, it has been used in other studies, and there is evidence that it is perhaps more ecologically relevant than MMR_f as a measure of exhaustive energy expenditure [31]. Animals may not exercise at maximal intensity very often under natural conditions, and spontaneous maximal performance is likely a better reflection of peak metabolic expenditure in natural settings and thus how it may be influenced by stressors such as nAg exposures [46].

In the present study, MMR_s was usually caused by light disturbances in the room. The effect of light changes on metabolic rates has been observed before, and because of this many studies expose fish to only dim lighting during respirometric trials or otherwise shield fish from large changes in light intensity [29,33,40]. This was not done in the present study; fish were exposed to the same lighting regime as they had been throughout the 28-d exposure trials. This lighting regime employed gradual light changes, similar to light increases in nature during sunrise and sunset, but appears to have still been sufficient to elicit a metabolic reaction in fish in the form of peak activity. It is also possible that this observation simply reflects natural patterns of metabolic expenditure because fish are traditionally known to be most active during the crepuscular periods of sunrise and sunset [47].

Forced maximum metabolic rate was approximately 21% higher when fish were chased before the SMR experiments, likely because of the increased stress fish were experiencing from being in the new environment of the respirometric chambers, combined with exercise stress when the chase protocol was conducted. This implies that, in general, it may be beneficial to conduct chase protocols before SMR trials, to increase the maximum metabolic rate estimate that is achieved. Conducting chase protocols was also found to decrease SMR measurements by approximately 20%. The reason for this difference is unknown because it was expected that the timing of the chase protocol would either have no effect or potentially increase SMR if done before SMR trials (as a result of excess postexercise oxygen consumption [35,48]). Potentially, this effect was seen as a result of fish that were chased beforehand being forced into a more active coping style toward stress, in that fish that are known to have active coping styles (have a more active response to stress—measured by having a higher number of escape attempts from a stressor, and a lower latency period of the first escape attempt) have been found to have lower SMRs than fish with passive coping styles [49]. Individuals who react actively to stress may evacuate their stress more effectively and thus generate lower estimates of SMR [50]. However, additional research is needed to draw solid conclusions because forcing a

fish to swim in a chase trial, as was done in the present study, may not be equivalent to the inborn personality traits of fish. Regardless, any differences within the present study were accounted for in analysis through inclusion of the before–after variable in the mixed-model analysis used.

Respirometric results from the present study suggest that care must be taken when using chase trials to obtain MMR estimates because they may result in MMR estimates that are lower than the actual maximum rate. In addition, conducting chase trials before respirometric trials appears to provide benefits to estimating both MMR_f and SMR [44]. This is useful information because conducting chase trials before is more efficient and results in less handling of fish; however, with this method there have been concerns that the recovery period from the chase may increase SMR estimates [35]. The present study results imply that SMR estimates are not affected detrimentally, and consequently the chase can be performed beforehand.

The present study provides information on nAg exposure that will be helpful to regulators and ongoing whole-ecosystem experiments by demonstrating that the metabolic effects of nAg at environmentally relevant concentrations were undetectable over a significant (28 d) exposure period. Overall, the effects of nAg exposure to *O. mykiss* metabolism were absent or, at best, only marginal in the present study, and only at the highest exposure concentrations, despite the observation of elevated plasma cortisol concentrations and Ag accumulation in muscle as a result of nAg exposure in a companion study [23]. Although adaptation to contaminant exposure has been proposed in other studies [13], it is unlikely that this was entirely the case in the present study, where fish exposed to nAg were found to have elevated stress (indicated by higher blood cortisol concentrations) throughout the study. This information will provide valuable context when evaluating changes in energetics and growth of fishes exposed to nAg in the environment.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3827.

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Data availability—Data can be accessed by contacting the corresponding author (lamurray12@gmail.com).

REFERENCES

1. Wijnhoven SWP, Peijnenburg WJGM, Herberts CA, Hagens WI, Oomen AG, Heugens EHW, Roszek B, Bisschops J, Gosens I, van de Meent D. 2009. Nano-silver—A review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology* 3:109–138.
2. Pulit-Prociak J, Stokłosa K, Banach M. 2014. Nanosilver products and toxicity. *Environ Chem Lett* 13:59–68.
3. Choi O, Clevenger TE, Deng B, Surampalli RY, Ross L, Hu Z. 2009. Role of sulfide and ligand strength in controlling nanosilver toxicity. *Water Res* 43:1879–1886.
4. Gottschalk F, Sun TY, Nowack B. 2013. Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies. *Environ Pollut* 181:287–300.
5. Sun TY, Gottschalk F, Hungerbühler K, Nowack B. 2014. Comprehensive probabilistic modeling of environmental emissions of engineered nanomaterials. *Environ Pollut* 185:69–76.
6. Blaser S, Scheringer M, Macleod M, Hungerbühler K. 2008. Estimation of cumulative aquatic exposure and risk due to silver: Contribution of nano-functionalized plastics and textiles. *Sci Total Environ* 390: 396–409.
7. Liu J, Chao J, Liu R, Tan Z, Yin Y, Wu Y. 2009. Cloud point extraction as an advantageous preconcentration approach for analysis of trace silver nanoparticles in environmental waters. *Anal Chem* 81: 6496–6502.
8. Lapresta-Fernández A, Fernández A, Blasco J. 2012. Nanoecotoxicity effects of engineered silver and gold nanoparticles in aquatic organisms. *TrAC* 32:40–59.
9. Farkas J, Christian P, Urrea JAG, Roos N, Hassellöv M, Tollefsen KE, Thomas KV. 2010. Effects of silver and gold nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat Toxicol* 96: 44–52.
10. Bilberg K, Malte H, Wang T, Baatrup E. 2010. Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). *Aquat Toxicol* 96:159–165.
11. Farmen E, Mikkelsen HN, Evensen O, Einset J, Heier LS, Rosseland BO, Salbu B, Tollefsen KE, Oughton DH. 2012. Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low μg/L concentrations of Ag nanoparticles. *Aquat Toxicol* 108:78–84.
12. Pham CH, Yi J, Gu MB. 2012. Biomarker gene response in male Medaka (*Oryzias latipes*) chronically exposed to silver nanoparticle. *Ecotox Environ Safe* 78:239–245.
13. Griffitt RJ, Brown-Peterson NJ, Savin DA, Manning CS, Boube I, Ryan RA, Brouwer M. 2012. Effects of chronic nanoparticulate silver exposure to adult and juvenile sheepshead minnows (*Cyprinodon variegatus*). *Environ Toxicol Chem* 31:160–167.
14. Rennie MD, Collins NC, Shuter BJ, Rajotte JW, Couture P. 2005. A comparison of methods for estimating activity costs of wild fish populations: More active fish observed to grow slower. *Can J Fish Aquat Sci* 62:767–780.
15. Rennie MD, Purchase CF, Shuter BJ, Collins NC, Abrams PA, Morgan GE. 2010. Prey life-history and bioenergetic responses across a predation gradient. *J Fish Biol* 77:1230–1251.
16. Djawdan M, Sugiyama TT, Schlaeger LK, Bradley TJ, Rose MR. 1996. Metabolic aspects of the trade-off between fecundity and longevity in *Drosophila melanogaster*. *Physiol Zool* 69:1176–1195.
17. Speakman JR. 2005. Body size, energy metabolism and lifespan. *J Exp Biol* 208:1717–1730.
18. Handy RD, Depledge MH. 1999. Physiological responses: Their measurement and use as environmental biomarkers in ecotoxicology. *Ecotox Environ Safe* 8:329–349.
19. McKenzie DJ, Garofalo E, Winter MJ, Ceradini S, Verweij F, Day N, Hayes R, van der Oost R, Butler PJ, Chipman JK. 2007. Complex physiological traits as biomarkers of the sub-lethal toxicological effects of pollutant exposure in fishes. *Philos Trans R Soc Lond B Biol Sci* 362:2043–2059.
20. Sokolova I, Lannig G. 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: Implications of global climate change. *Clim Res* 37:181–201.
21. Norin T, Malte H. 2012. Intraspecific variation in aerobic metabolic rate of fish: Relations with organ size and enzyme activity in brown trout. *Physiol Biochem Zool* 85:645–656.
22. Fry FEJ. 1947. *Effects of the Environment on Animal Activity*. University Toronto Studies, Biological Services, No. 55. Publications of the Ontario Fisheries Research Laboratory, No. 68. University of Toronto Press, Toronto, Canada.
23. Murray LM, Rennie MD, Enders EC, Pleskach K, Martin JD. 2017. Effect of nanosilver on cortisol release and morphometrics in rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 36:1606–1613.
24. Murray L. 2015. Effect of nanosilver particles on metabolism and cortisol release in rainbow trout (*Oncorhynchus mykiss*). Master's thesis. University of Manitoba, Winnipeg, Manitoba, Canada.
25. Steffensen JF. 1989. Some errors in respirometry of aquatic breathers: How to avoid and correct for them. *Fish Physiol Biochem* 6:49–59.
26. Rosewarne PJ, Wilson JM, Svendsen JC. 2016. Measuring maximum and standard metabolic rates using intermittent-flow respirometry: A student laboratory investigation of aerobic metabolic scope and environmental hypoxia in aquatic breathers. *J Fish Biol* 88:265–283. DOI: 10.1111/jfb.12795
27. Svendsen JC, Banet AI, Christensen RHB, Steffensen JF, Aarestrup K. 2013. Effects of intraspecific variation in reproductive traits, pectoral fin use and burst swimming on metabolic rates and swimming performance in the Trinidadian guppy (*Poecilia reticiulata*). *J Exp Biol* 216:3564–3574.
28. Norin T, Malte H. 2011. Repeatability of standard metabolic rate, active metabolic rate and aerobic scope in young brown trout during a period of moderate food availability. *J Exp Biol* 214:1668–1675.
29. Tang M, Boisclar D, Mènard C, Downing J. 2000. Influence of body weight, swimming characteristics, and water temperature on the cost of

swimming in brook trout (*Salvelinus fontinalis*). *Can J Fish Aquat Sci* 57:1482–1488.
30. Schurmann H, Steffensen JF. 1997. Effects of temperature, hypoxia and activity on the metabolism of juvenile Atlantic cod. *J Fish Biol* 50:1166–1180.
31. Svendsen JC, Genz J, Anderson GW, Stol JA, Watkinson DA, Enders EC. 2014. Evidence of circadian rhythm, oxygen regulation capacity, metabolic repeatability and positive correlations between forced and spontaneous maximal metabolic rates in lake sturgeon *Acipenser fulvescens*. *PLoS One* 9:e94693.
32. Chabot D, Steffensen JF, Farrell AP. 2016. The determination of standard metabolic rate in fishes. *J Fish Biol* 88:81–121.
33. Svendsen JC, Steffensen JF, Aarestrup K, Frisk M, Etzerodt A, Jyde M. 2012. Excess posthypoxic oxygen consumption in rainbow trout (*Oncorhynchus mykiss*): Recovery in normoxia and hypoxia. *Can J Zool* 11:1–11.
34. Clark TD, Sandblom E, Jutfelt F. 2013. Aerobic scope measurements of fishes in an era of climate change: Respirometry, relevance and recommendations. *J Exp Biol* 216:2771–2782.
35. Norin T, Clark TD. 2016. Measurement and relevance of maximum metabolic rate in fishes. *J Fish Biol* 88:122–151. DOI: 10.1111/jfb.12796
36. Roche DG, Binning SA, Bosiger Y, Johansen JL, Rummer JL. 2013. Finding the best estimates of metabolic rates in a coral reef fish. *J Exp Biol* 216:2103–2110.
37. Milligan CL. 1996. Metabolic recovery from exhaustive exercise in Rainbow trout. *Comp Biochem Phys A* 113:51–60.
38. R Development Core Team. 2015. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
39. Cowart DA, Guida SM, Shah SI, Marsh AG. 2011. Effects of Ag nanoparticles on survival and oxygen consumption of zebra fish embryos, *Danio rerio*. *J Environ Sci Heal A* 46:1122–1128.
40. Morgan JD, Iwama GK. 1996. Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr. *Fish Physiol Biochem* 15:385–394.
41. O'Connor CM, Gilmour KM, Arlinghaus R, Matsumura S, Suski CD, Philipp DP, Cooke SJ. 2011. The consequences of short-term cortisol elevation on individual physiology and growth rate in wild largemouth bass (*Micropterus salmoides*). *Can J Fish Aquat Sci* 68:693–705.
42. Mager EM, Grosell M. 2011. Effects of acute and chronic waterborne lead exposure on the swimming performance and aerobic scope of fathead minnows (*Pimephales promelas*). *Comp Biochem Phys C* 157:7–13.
43. Wilson RW, Bergman HL, Wood CM. 1994. Metabolic costs and physiological consequences of acclimation to aluminum in juvenile Rainbow trout Oncorhynchus mykiss). 2: Gill morphology, swimming performance, and aerobic scope. *Can J Fish Aquat Sci* 51:536–544.
44. Murray L, Rennie MD, Svendsen JC, Enders EC. 2017. Effects of respirometry methods on cortisol release in rainbow trout *Oncorhynchus mykiss*. *J Fish Biol*, in press. DOI: 10.1111/jfb.13292
45. Cutts CJ, Metcalfe NB, Taylor AC. 2002. Juvenile Atlantic salmon (*Salmo salar*) with relatively high standard metabolic rates have small metabolic scopes. *Funct Ecol* 16:73–78.
46. Husak JF. 2006. Does survival depend on how fast you can run or how fast you do run? *Funct Ecol* 20:1080–1086.
47. Helfman GS. 1981. Twilight activities and temporal structure in a freshwater fish community. *Can J Fish Aquat Sci* 38:1405–1420.
48. Lee CG, Farrell AP, Lotto A, Hinch SG, Healey MC. 2003. Excess post-exercise oxygen consumption in adult sockeye (*Oncorhynchus nerka*) and coho (*O. kisutch*) salmon following critical speed swimming. *J Exp Biol* 206:3253–3260.
49. Martins CIM, Castanheira MF, Engrola S, Costas B, Conceicao LEC. 2011. Individual differences in metabolism predict coping styles in fish. *Appl Anim Behav Sci* 130:135–143.
50. Careau V, Thomas D, Humphries MM, Reale D. 2008. Energy metabolism and animal personality. *Oikos* 117:641–653.