

Metabolic Perturbations in Persian Sturgeon (*Acipenser persicus*) Embryos Exposed to Silver Nanoparticle-Embedded Water Filtration Systems: An NMR-Based Metabolomics Approach

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Abstract

The widespread application of silver nanoparticles (AgNPs) in aquaculture raises concerns about their sub-lethal toxicological effects on early life stages of valuable fish species. However, the underlying metabolic mechanisms of AgNP toxicity in sturgeon embryos remain largely unknown. This study was designed to address this knowledge gap by employing a ^1H NMR-based metabolomics approach to investigate the metabolic alterations in Persian sturgeon (*Acipenser persicus*) embryos following indirect exposure to water passed through zeolite-silica filters coated with AgNPs (1% w/w). Among six fabricated filters, two optimal filters with (F2) and without (F1) 3-aminopropyl-triethoxysilane (APTES) exhibited complete antifungal efficacy against *Saprolegnia* spp. The released silver ion (Ag^+) concentrations after 96 h were 1.56 ± 0.25 and 5.92 ± 1.88 $\mu\text{g/L}$ for F1 and F2, respectively. Multivariate analysis (PCA and OPLS-DA) of the ^1H NMR spectra revealed a clear time- and dose-dependent metabolic discrimination between control and treated embryos. Significant metabolic perturbations included: (i) elevated branched-chain amino acids (BCAAs), alanine, glutamate, and aspartate, indicating enhanced protein catabolism for energy compensation; (ii) increased lactate and succinate accompanied by altered acetate levels, suggesting a shift from aerobic to anaerobic respiration and a slowdown in the TCA cycle; and (iii) decreased choline with concomitant increases in phosphocholine and betaine (particularly in F1 at day 4), implying cell membrane remodeling and osmotic stress responses. Notably, the higher Ag^+ release from F2 induced more severe metabolic disturbances than F1. Our findings demonstrate that NMR-based metabolomics is a powerful, non-targeted tool for early detection of AgNP-induced sub-lethal toxicity in sturgeon embryos, providing critical insights into the energy metabolism, osmoregulation, and membrane integrity disruptions that may compromise later life stages.

Highlights

1. ^1H NMR-based metabolomics was applied to assess AgNP toxicity in sturgeon embryos for the first time.
2. PCA and OPLS-DA revealed clear time- and dose-dependent metabolic discrimination.
3. AgNP exposure induced a shift from aerobic to anaerobic metabolism (elevated lactate and succinate).
4. Enhanced protein catabolism was evidenced by elevated BCAAs, alanine, glutamate, and aspartate.
5. Decreased choline with increased phosphocholine and betaine indicated membrane/osmoregulatory disruption.
6. Differential betaine response ($\uparrow$ in F1 vs. $\downarrow$ in F2) reflected the organism's capacity to cope with stress.
7. NMR-based metabolomics is a powerful tool for early detection of sub-lethal AgNP toxicity.
8. Identified biomarkers (lactate, succinate, betaine, BCAAs) can serve as early warning signals.
9. Higher Ag^+ release from APTES-containing filters (F2) caused more severe metabolic disturbances.

10. Findings emphasize the need for comprehensive risk assessment of nanomaterials in aquaculture.

1. Introduction

Nanotechnology has revolutionized various industrial and commercial sectors, including medicine, electronics, and aquaculture, due to the unique physicochemical properties of engineered nanoparticles (Nie et al., 2007; Sozer & Kokini, 2009). Among these, silver nanoparticles (AgNPs) are the most widely commercialized nanomaterials, primarily owing to their potent broad-spectrum antimicrobial activity (Shahverdi et al., 2007; Tran et al., 2013). In aquaculture, AgNPs have shown promising potential for controlling waterborne pathogens, such as *Saprolegnia* spp., a major fungal threat in fish hatcheries, often by integrating them into filtration systems (Johari et al., 2014; Johari et al., 2016; Banan et al., 2016).

However, the same antimicrobial properties that make AgNPs beneficial also raise significant ecotoxicological concerns. The continuous release of AgNPs and their ionic counterparts (Ag⁺) into aquatic environments poses a risk to non-target organisms (Benn & Westerhoff, 2008; Elzey & Grassian, 2010). Numerous studies have documented the toxic effects of AgNPs on various fish species, including oxidative stress, neurotoxicity, and genotoxicity (Asharani et al., 2008; Garcia-Reyero et al., 2015; Oh et al., 2016). Fish embryos are particularly vulnerable, as they are highly sensitive to environmental stressors, and embryonic toxicological studies have a high correlation (over 80%) with acute stress responses in adult fish (Braunbeck & Lammer, 2006). Moreover, embryonic testing is cost-effective and ethically more acceptable than adult fish testing (Ringwood et al., 2010). Studies have demonstrated developmental abnormalities and cytotoxicity in embryos of zebrafish (*Danio rerio*) (Asharani et al., 2008), medaka (*Oryzias latipes*) (Cho et al., 2013), and sturgeon species (*Acipenser persicus* and *A. stellatus*) (Banan et al., 2016) following AgNP exposure.

While these traditional toxicological endpoints (mortality, hatching rate, malformations) are valuable, they often fail to capture the early, sub-lethal molecular perturbations that precede overt toxicity. This is where metabolomics, a comprehensive analysis of the low-molecular-weight metabolome, offers a distinct advantage (Nicholson & Lindon, 2008). Metabolomics provides a direct "snapshot" of the physiological state of an organism, reflecting the ultimate downstream consequences of genomic, transcriptomic, and proteomic changes in response to a stressor (Mathew & Padmanaban, 2013; Viant et al., 2003). NMR-based metabolomics, in particular, is a robust, reproducible, and non-destructive technique that requires minimal sample preparation, making it ideal for studying the holistic metabolic responses of fish embryos to toxicants (Lardon et al., 2013; Aru et al., 2016; Cappello et al., 2016; Li et al., 2015; Kim et al., 2011; Zhao et al., 2016; Bo et al., 2014; Zhang et al., 2011; Wu et al., 2013).

Despite the increasing application of AgNP-coated filters in aquaculture, a significant knowledge gap exists regarding their sub-lethal metabolic effects on the early life stages of endangered fish species, such as the Persian sturgeon (*Acipenser persicus*). While our previous work focused on the antifungal efficacy of such filters (Banan et al., 2016), the underlying biochemical mechanisms and potential

metabolic disruptions in exposed embryos remain largely unexplored. The present study was conducted to address this critical knowledge gap, as understanding the molecular mechanisms of AgNP toxicity in sturgeon embryos is essential for the sustainable application of nanotechnology in aquaculture and the conservation of this valuable species. Therefore, we hypothesized that chronic exposure to water passed through AgNP-coated filters would induce significant alterations in the embryonic metabolome, reflecting disruptions in energy metabolism, osmotic regulation, and cellular stress responses.

To test this hypothesis, we applied a ^1H NMR-based metabolomics approach combined with multivariate statistical analysis (PCA and OPLS-DA) to characterize the metabolomic fingerprints of Persian sturgeon embryos exposed to two optimal AgNP filters. Our specific objectives were to: (1) identify the major metabolic pathways perturbed by AgNP exposure, (2) compare the metabolic effects of the two different filter types (F1 and F2), and (3) assess the potential of this approach as a sensitive tool for early toxicity screening in sturgeon aquaculture.

2. Materials and Methods

2.1. Chemicals

Deuterium oxide (D_2O , 99.9%), potassium deuterioxide (KOD, 40 wt. % in D_2O , 99.5 atom % D), and sodium 3-trimethylsilyl-propionate-2,2,3,3- d_4 (TSP) were purchased from Sigma-Aldrich (Milan, Italy). All other chemicals were obtained from Merck (Darmstadt, Germany).

2.2. AgNP-Coated Water Filtration Systems

Six filtration systems were constructed using zeolite and silica substrates coated with different concentrations of AgNPs (0.2, 0.5, and 1 g AgNPs per 100 g substrate), with and without 3-aminopropyltriethoxysilane (APTES) as a binding agent. Based on preliminary antifungal screening (Banan et al., 2016), two filters containing 1% AgNPs were selected for the metabolomics study: Filter F1 (without APTES) and Filter F2 (with APTES). The release of silver ions (Ag^+) from these filters into water was measured using graphite furnace atomic absorption spectroscopy (Varian, GTA-120). After 48 h and 96 h, Ag^+ concentrations were 2.13 ± 0.97 and 7.84 ± 1.46 $\mu\text{g/L}$, and 1.56 ± 0.25 and 5.92 ± 1.88 $\mu\text{g/L}$ for F1 and F2, respectively. The selection of these concentrations was based on preliminary antifungal efficacy tests and represents environmentally relevant levels for potential AgNP release in aquaculture systems.

2.3. Embryo Sampling

Persian sturgeon broodstock were obtained from the Shahid Rajaee Propagation and Restocking Centre (Sari, Iran). Artificial fertilization was performed, and fertilized eggs were distributed among seven incubators receiving water from the control (no AgNP) and AgNP filters (F1 and F2). At two and four days post-fertilization (dpf), fifteen embryos were collected from each treatment group (three replicates per treatment, 15 embryos per replicate) and immediately flash-frozen in liquid nitrogen and stored at -80°C until analysis. All experiments were conducted in triplicate (three independent experiments), and each experiment included three biological replicates per treatment.

2.4. Sample Preparation for NMR Analysis

Metabolites were extracted from pooled embryo samples (50 mg wet weight) using a modified Folch method (Folch et al., 1957). Briefly, embryos were homogenized in 12 mL of chloroform-methanol (2:1, v/v). After addition of 4 mL of water and centrifugation (2000 g, 30 min, 4°C), the aqueous phase was separated, dried using a rotating evaporator (Eppendorf Concentrator Plus), and stored at -80°C. Prior to NMR analysis, the dried aqueous extract was dissolved in 670 µL of 0.1 M potassium phosphate buffer (pH 7.4) with 30 µL of TSP (10 mM, internal standard), centrifuged (13000 g, 5 min, 4°C), and 650 µL of the supernatant was transferred to a 5 mm NMR tube for analysis.

2.5. NMR Measurements and Data Processing

¹H NMR spectra were recorded at 300 K on a Varian Unity Inova 500 spectrometer (Agilent Technologies, CA, USA) operating at 499.839 MHz. Spectra were acquired with a 45° pulse, an acquisition time of 1.5 s, a relaxation delay of 4 s, and 256 scans. Water suppression was achieved using presaturation. After Fourier transformation (0.3 Hz line broadening), spectra were manually phased and baseline corrected using MestReNova (v. 8.1). The spectra were binned into 0.0025 ppm integral regions from 9.5 to 0.5 ppm, excluding the residual water region (δ 4.6–5.2). Bins were normalized to the total spectral area. The resulting data matrix was mean-centered and Pareto-scaled.

2.6. Multivariate Statistical Analysis

The NMR spectra were integrated (binned) using 0.0025 ppm integral regions between 9.5 and 0.5 ppm, excluding the portions with the residual water (δ 4.6–5.2) resonance. Bins were normalized to the sum of a total spectral area to compensate for the overall concentration differences. The final data set 64 × 3920 (samples × variables) was automatically reduced to ASCII files and converted into an Excel file. Multivariate analysis was performed using SIMCA 14 software (Umetrics, Umeå, Sweden). Principal Component Analysis (PCA) was initially applied to visualize natural clustering and identify outliers. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was employed to maximize the separation between groups (control vs. F1 vs. F2) and to identify the discriminant metabolites. Model quality was evaluated by R² (goodness of fit) and Q² (predictive ability). Metabolite identification was confirmed by comparing chemical shifts, multiplicities, and spin-spin coupling constants with literature data, the Chenomx NMR suite (v. 8.2), and the Human Metabolome Database (HMDB) (Wishart et al., 2007).

3. Results

3.1. NMR Spectra and Metabolite Identification

A total of 64 ¹H NMR spectra were recorded from the aqueous extracts of Persian sturgeon embryos across all treatment groups and sampling time points (2 and 4 days post-fertilization). Figure 1 shows a representative ¹H NMR spectrum with expanded regions, displaying the characteristic resonance signals of a wide range of metabolites.

Using a combination of literature data, the Chenomx NMR suite (v. 8.2), and the Human Metabolome Database (HMDB) (Wishart et al., 2007), a total of 27 metabolites were confidently identified (Table 1). These included: essential amino acids (valine, leucine, isoleucine [BCAAs], phenylalanine, methionine, threonine, lysine, histidine), non-essential amino acids (alanine, glycine, serine, tyrosine, glutamate, aspartate, proline), energy metabolism intermediates (lactate, succinate, acetate, pyruvate), osmolytes and membrane components (choline, phosphocholine, betaine, trimethylamine), and nucleotides (uracil, cytosine).

Table 1. List of metabolites identified in the ¹H NMR spectra of Persian sturgeon embryo extract. Chemical shifts (ppm) and multiplicities (s: singlet, d: doublet, t: triplet, dd: doublet of doublets, m: multiplet) are reported.

Compound	Group	ppm	Multiplicity ^a
Acetate(Ace)	β CH ₃	1.9	<i>s</i>
Alanine (Ala)	α CH	1.49	<i>d</i>
Aspartate (Asp)	α CH	2.68/2.80	<i>dd/dd</i>
Betaine (Bet)	N-CH ₃ / α CH ₂	3.26/3.39	<i>s/s</i>
Choline (Cho)	N-(CH ₃) ³⁺ N-CH ₂ O-CH ₂	3.21	<i>S</i>
Cytosine (Cyt)	C ₅ H	5.98	<i>d</i>
Formate (Form)	HCOO-	8.46	<i>s</i>
Glutamate (Glu)	γ CH ₂	2.38	<i>t</i>
Glycine (Gly)	CH ₂	3.57	<i>s</i>
Glycerol	α CH ₂	3.62	<i>dd</i>
Histidine (His)	C ₂ H, ring / C ₄ H, ring	7.79/7.07	<i>s/s</i>
Isoleucine (Ile)	γ' CH ₃ / δ CH ₃	0.94/1.02	<i>d/ t</i>
Lactate	β CH ₃	1.3	<i>s</i>
Leucine (Leu)	δ CH ₃ , δ' CH ₃	0.96	<i>d</i>
Lysine (Lys)	γ CH ₂ / δ CH ₂	1.43/1.73	<i>m/ m</i>
Methionine (Met)	S-CH ₃	2.14	<i>s</i>
Phenylalanine (Phe)	C _{2,6} H, ring / C _{3,5} H ring/ C ₄ H ring	7.42/7.42/7.33	<i>m/m/m</i>

Phosphocholine (P-Cho)	N-(CH ₃) ³⁺	3.24	s
Proline (Pro)	β'CH/ γ CH ₂	2.04/2.01	m/m
Serine (Ser)	α CH (C)/ β CH	3.86/3.96	dd/ dd
Succinate (Suc)	α, β CH ₂	2.41	s
Trimethylamine oxide (TMAO)	O-N-(CH ₃) ₃	3.24	s
Threonine (Thr)	γ CH ₃	1.34	d
Tryptophan (Trp)	C ₄ H, ring	7.72	d
Tyrosine (Tyr)	C _{2,6} H/ C _{3,5} H, ring	6.98/7.19	d/ d
Uracil (Ura)	C ₅ H, ring / C ₆ H, ring	5.81/7.54	d/d
Valine (Val)	γ'CH ₃ / γ CH ₃	1.04/0.99	d/ d

3.2. Overview of Global Metabolic Changes: PCA

To obtain an unbiased overview of the global metabolic differences between the experimental groups, Principal Component Analysis (PCA) was first applied to the entire spectral dataset. The PCA score plot (PC1 vs. PC2), which explained 57% of the total variance, is presented in Figure 2.

As can be seen in Figure 2, the PCA plot revealed a clear and distinct separation between the three experimental groups. The control samples clustered tightly on the negative side of PC1, while the F2 embryos shifted furthest towards the positive side of PC1, with F1 embryos occupying an intermediate position. This separation pattern indicates a dose-dependent metabolic response, with the higher Ag⁺ release from F2 (7.84 µg/L at 2 dpf and 5.92 µg/L at 4 dpf) inducing more pronounced metabolic alterations than F1 (2.13 µg/L at 2 dpf and 1.56 µg/L at 4 dpf). Additionally, a strong temporal response was observed, particularly in the control and F1 groups. For both groups, embryos sampled at 2 dpf were distributed along the negative side of PC2, while those sampled at 4 dpf moved towards the positive side of PC2. Interestingly, the F2 group exhibited minimal temporal separation, suggesting that the high Ag⁺ concentration caused a persistent and profound metabolic disruption that overshadowed normal developmental metabolic changes.

3.3. Identification of Discriminant Metabolites: OPLS-DA and S-Plot Analysis

To maximize the separation between groups and identify the specific metabolites responsible for the observed discrimination, Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was performed for pairwise comparisons. The metabolites with the highest discriminatory power (absolute p(cov) ≥ 0.05 and p(corr) ≥ 0.5) were selected for further analysis.

3.3.1. Temporal Metabolic Changes (2 dpf vs. 4 dpf)

First, OPLS-DA models were built for each group individually to analyze the dynamic metabolic changes over time (2 dpf vs. 4 dpf). The score plots for these models are shown in Figure S1 (Supporting Information), and the corresponding coefficient plots are presented in Figure 3.

All OPLS-DA models exhibited excellent performance, with high Q^2 values (>0.70), indicating strong predictive ability. The reliability of the models was further validated by random class permutation tests ($n=200$, data not shown). As shown in Figure 3, the temporal metabolic trajectory of the control group was characterized by a significant decrease in BCAAs, alanine, glutamate, and phosphocholine, accompanied by an increase in acetate and acetone. These changes reflect the normal developmental progression from reliance on anaerobic metabolism (early stage) towards aerobic metabolism (late stage). In contrast, the F1 group exhibited a milder temporal shift, with increased levels of BCAAs, methionine, glutamate, and succinate, and decreased acetate and lactate at 4 dpf. This suggests a stress-induced deviation from normal metabolic development. The F2 group showed the most drastic temporal alterations, with marked increases in BCAAs, lactate, alanine, acetate, methionine, succinate, and phosphocholine at 4 dpf, while acetone, choline, and gluconate decreased. These changes are indicative of severe metabolic stress, impaired aerobic respiration, and disrupted membrane integrity.

3.3.2. Treatment-Specific Metabolic Changes (Control vs. F1 vs. F2)

To identify the metabolites specifically altered by AgNP exposure, OPLS-DA models were built for Control vs. F1 and Control vs. F2 comparisons at each time point (2 and 4 dpf). The S-plots derived from these models are shown in Figure 4, and the corresponding score plots are provided in Figure S2 (Supporting Information). The metabolites with the highest discriminatory power (absolute $p(\text{cov}) \geq 0.05$ and $p(\text{corr}) \geq 0.5$) were selected for further analysis.

At 2 dpf (Figure 4A-B):

F1 vs. Control: F1 embryos showed increased levels of BCAAs, glycine, and acetate, while choline levels were decreased.

F2 vs. Control: F2 embryos exhibited a more pronounced elevation of BCAAs and glycine, with a stronger decrease in choline. Additionally, a slight increase in alanine was observed in F2.

At 4 dpf (Figure 4C-D):

F1 vs. Control: A notable shift occurred in F1 embryos, with significant increases in BCAAs, phenylalanine, methionine, lactate, and succinate, alongside decreased acetate, gluconate, phosphocholine, and acetone. Most notably, betaine levels were significantly increased (2.71-fold) in F1 embryos at 4 dpf (Table 2).

F2 vs. Control: F2 embryos displayed more severe and widespread perturbations. BCAAs, lactate, alanine, acetate, methionine, succinate, phosphocholine, and phenylalanine were all markedly elevated, while choline, acetone, and gluconate were depleted. The fold changes for all identified metabolites are summarized in Table 2.

The differential behavior of betaine between the two filters at 4 dpf is particularly noteworthy: while F1 showed a 2.71-fold increase, F2 showed a 1.75-fold decrease (Table 2). This suggests that the lower Ag⁺ concentration in F1 (1.56 µg/L) triggered a protective osmoregulatory response (increased betaine synthesis), whereas the higher Ag⁺ concentration in F2 (5.92 µg/L) overwhelmed this system, leading to a failure in betaine synthesis and osmoregulatory breakdown.

Table 2. Relative changes (fold increase or decrease) in metabolite concentrations in embryos exposed to AgNP filters (F1 and F2) relative to the control group at 2 and 4 days of incubation. Positive values indicate an increase, and negative values indicate a decrease in metabolite concentration.

Metabolites	Second incubation day		Fourth incubation day	
	Filter1	Filter2	Filter1	Filter2
Alanine	+1.64	1.35	-1.58	+1.55
leucine	+2.9	+2.2	+3	+4
Lysine	+1.56	+1.55	+1.22	-1.25
Methionine	+3.04	+2.92	+1.74	+1.83
Phenylalanine	+1.22	+1.48	+4.5	+5.16
Threonine	-3.22	-2.43	+1.04	+1.39
Tyrosine	+1.11	+1.17	+1.02	-1.42
BCAA	+2.16	+2.19	+2.16	+2.19
Histidine	+23.7	+5.46	+3.49	-2.85
Aspartate	+42	+86	+14.6	+32
Glutamate	+2.27	+2.6	-1.05	+2.2
Glycine	+1.9	+1.8	+1.19	-1.35
serine	-1.02	-1.4	-1.61	1.63
Beta-Alanine	+2.15	+11	+5.26	+9.82
Betaine	+1.83	-1.13	+2.71	-1.75
Organic acids:				
Formate	-6.66	-8.33	+3.17	-27.5
Lactate	-4	-3.44	+1.27	+1.29
Acetate	-4.76	-1.56	-1.47	+1.34
Succinate	+5.72	+15.84	+1.95	+2.58
Other metabolites:				
Choline	-14.28	-3.7	-1.66	-1.35
Phosphocholine	+4	+2.4	-1.92	+3.59
Acetone	-2.08	-6.6	-1.49	-57.5
Gluconate	-2.56	-5.26	-1.88	-6.38

3.4. Total Free Amino Acid (FAA) Pool

To complement the metabolomics data and assess the overall protein catabolism status, the total free amino acid (FAA) pool was calculated for each group. As shown in Figure 5, a distinct pattern emerged:

In the control group, the total FAA pool decreased significantly from 2 to 4 dpf ($P < 0.05$), reflecting the normal utilization of endogenous yolk protein reserves for organogenesis and energy production (Tyler et al., 1988). In contrast, in the F1 group, the total FAA pool increased from 2 to 4 dpf, while in the F2 group, it remained relatively stable. These findings indicate a stress-induced protein catabolism in AgNP-treated embryos, which is consistent with the elevated BCAAs and other amino acids observed in the metabolomics data (Figure 4 and Table 2). At 2 dpf, the total FAA pool was significantly lower in both F1 and F2 groups compared to the control ($P < 0.05$). However, at 4 dpf, the F1 group showed a significantly higher FAA pool compared to both the control and F2 groups, while F2 remained similar to the control (Figure 5).

4. Discussion

This study provides the first comprehensive metabolic snapshot of Persian sturgeon embryos exposed to water from AgNP-embedded filters, using a powerful ^1H NMR-based metabolomics approach. Our findings clearly demonstrate that even when AgNPs are not directly added to the water but integrated into a filtration system, they induce significant and measurable changes in the embryonic metabolome, revealing a time- and dose-dependent toxicity pattern. The results offer crucial insights into the underlying mechanisms of AgNP toxicity, particularly concerning energy metabolism, osmotic stress, and cellular membrane integrity.

4.1. Energy Metabolism: A Shift from Aerobic to Anaerobic Respiration

In the early life stages of fish, embryonic development primarily relies on the catabolism of endogenous free amino acids (FAAs) inherited in the yolk (Li et al., 2009). In our study, the control group showed a natural decline in total FAAs over time, indicating their utilization for organogenesis and energy production (Tyler et al., 1988). However, AgNP-exposed embryos (F1 and F2) exhibited either an increase or no significant decline in FAAs (Fig. 5), suggesting a stress-induced breakdown of yolk proteins to meet increased energy demands (Carrola et al., 2016; Feng et al., 2013). This is strongly supported by the elevated levels of BCAAs (leucine, isoleucine, valine) in both treated groups (Sowers, 2009; Lee et al., 2016). BCAAs are not synthesized *de novo* and must be derived from proteolysis; their accumulation is a classic biomarker of protein catabolism under stress conditions. The most compelling evidence for a metabolic shift comes from the alterations in lactate, succinate, and acetate. At 4 dpf, the significant increase in lactate and succinate in F2 and F1 embryos, compared to the control, is a strong indicator of anaerobic respiration and a slowdown of the TCA cycle (Lardon et al., 2013; Podrabsky et al., 2007). Under normal oxygen conditions, succinate is oxidized to fumarate in the TCA cycle. However, if the electron transport chain is inhibited by Ag^+ ions, this process is halted, leading to succinate accumulation. Simultaneously, a decline in aerobic metabolism reduces NAD^+ availability, forcing the cell

to regenerate NAD⁺ through the LDH-mediated conversion of pyruvate to lactate, thereby increasing lactate production (Southam et al., 2008). This shift towards anaerobic metabolism is a classic response to mitochondrial dysfunction, which has been previously reported for AgNPs in liver cells (Kim et al., 2011) and in hepatotoxic studies of other nanoparticles (Bo et al., 2014; Feng et al., 2013).

4.2. Amino Acid Perturbations and Oxidative Stress

The elevation of specific amino acids, such as glutamate and aspartate, points towards additional stress pathways. Glutamate is a key metabolite in ammonia detoxification and is a precursor for glutathione (GSH) synthesis, a crucial cellular antioxidant (Jaspers et al., 1985; Li et al., 2009). The increased glutamate in AgNP-exposed embryos suggests a demand for its role in both energy production through the TCA cycle and in combating oxidative stress by maintaining GSH levels (Bo et al., 2014; Kim et al., 2011). The significant elevation of aspartate, an important energy substrate for fish, further underscores the increased energy demand (Li et al., 2009), and aligns with the increased aspartate aminotransferase activity reported in rainbow trout treated with nanosilver (Monfared & Soltani, 2013). The increased phenylalanine and methionine, also seen in human keratinocytes and liver cells exposed to AgNPs (Carrola et al., 2016; Kim et al., 2011), further corroborate the oxidative stress and protein catabolism hypotheses. Methionine plays a vital role as an antioxidant and is a precursor for taurine and polyamines, both essential for stress responses (Levine et al., 1996; Wu et al., 2004).

4.3. Osmotic Stress and Membrane Integrity

The observed reduction in choline levels, accompanied by increases in phosphocholine and, most notably, betaine, suggests AgNP-induced disruptions in cell membrane structure and osmotic balance. Choline is an essential nutrient for the synthesis of phosphatidylcholine, a major component of cell membranes, and is also a precursor for the osmolyte betaine (Zeisel et al., 1991; Abro et al., 2014). The decrease in choline and increase in phosphocholine indicate membrane remodeling or damage (Li et al., 2015; Bu et al., 2010; Feng et al., 2010). A particularly interesting finding is the differential behavior of betaine between the two filters. Betaine is a crucial organic osmolyte and antioxidant that protects cells against osmotic stress by maintaining water balance and stabilizing protein structure (Anamul Hoque et al., 2007; Okuma et al., 2002). In F1-exposed embryos at 4 dpf, betaine significantly increased (2.71-fold). We hypothesize that this is a compensatory response to mild osmoregulatory stress caused by the lower Ag⁺ release (1.56 µg/L). At this concentration, the embryos can still activate their protective osmoregulatory mechanisms, increasing betaine synthesis to stabilize cellular osmolarity. In contrast, F2-exposed embryos (higher Ag⁺ release, 5.92 µg/L) showed decreased betaine (1.75-fold), suggesting that the severe oxidative stress and energy depletion induced by higher Ag⁺ concentrations overwhelm the system, leading to a failure in betaine synthesis and a breakdown in osmoregulation. This distinction highlights the utility of metabolomics in revealing not just the presence of toxicity, but the organism's capacity to cope with it.

4.4. Toxicological Relevance and Implications

The dose-dependent metabolic perturbations observed in this study have significant implications for the environmental risk assessment of AgNPs in aquaculture. The identification of specific metabolic biomarkers—such as elevated lactate, succinate, BCAAs, and the differential betaine response—provides a sensitive, early-warning system for sub-lethal AgNP toxicity in sturgeon embryos. These biomarkers could be used for routine monitoring of hatchery water quality and for evaluating the safety of new nanomaterial-based filtration systems. Furthermore, our findings emphasize the importance of considering the type of filter coating (with or without APTES) in the design of AgNP-based water treatment systems, as the APTES-containing filter (F2) caused more severe metabolic disruption due to higher Ag⁺ release.

5. Conclusion

In summary, our NMR-based metabolomics study has successfully identified the primary biochemical pathways perturbed in Persian sturgeon embryos by indirect exposure to AgNP filters. The dominant effects are a shift to anaerobic energy production, increased protein catabolism, and cellular membrane/osmoregulatory disruption. The degree of metabolic disturbance is correlated with the amount of Ag⁺ released from the filters, with F2 (APTES) causing more severe and widespread disruption than F1 (without APTES). These findings demonstrate that metabolomics is a highly sensitive and non-invasive tool for early risk assessment of nanomaterials in aquaculture. The metabolic biomarkers identified in this study, such as lactate, succinate, betaine, and BCAAs, can serve as early warning signals for sub-lethal toxicity before visible effects appear. This approach has far-reaching implications for the sustainable application of nanotechnology in aquaculture, emphasizing the need for a comprehensive understanding of the subtle, long-term impacts on the health and fitness of released juveniles. Future studies should monitor these metabolic biomarkers in later life stages to confirm whether these embryonic perturbations translate into long-term developmental or physiological deficits.

Declarations

Author Contribution

Statements and DeclarationsCompeting Interests: The authors declare that they have no competing interests.**Ethical Approval:** All animal procedures were performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. The study was approved by the Ethics Committee of Tarbiat Modares University (Tehran, Iran) **Data Availability:** The raw NMR spectra and multivariate analysis datasets generated during this study are available from the corresponding author upon reasonable request. Processed data are included in this published article and its supplementary information files.**Author Contributions:** [Kalbassi and Marincola] conceived and designed the study; [Soltankarimi] performed the experiments and analyzed the data; [Soltankarimi] and [Rahimi] wrote the original draft; [Kalbassi] and [Marincola] reviewed and edited the manuscript. All authors have read and approved the final manuscript.**Use of AI Tools:** No generative AI tools were used for content

creation. AI-assisted copy editing was used solely for readability and language polishing, and all authors take full responsibility for the final version.

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Figures

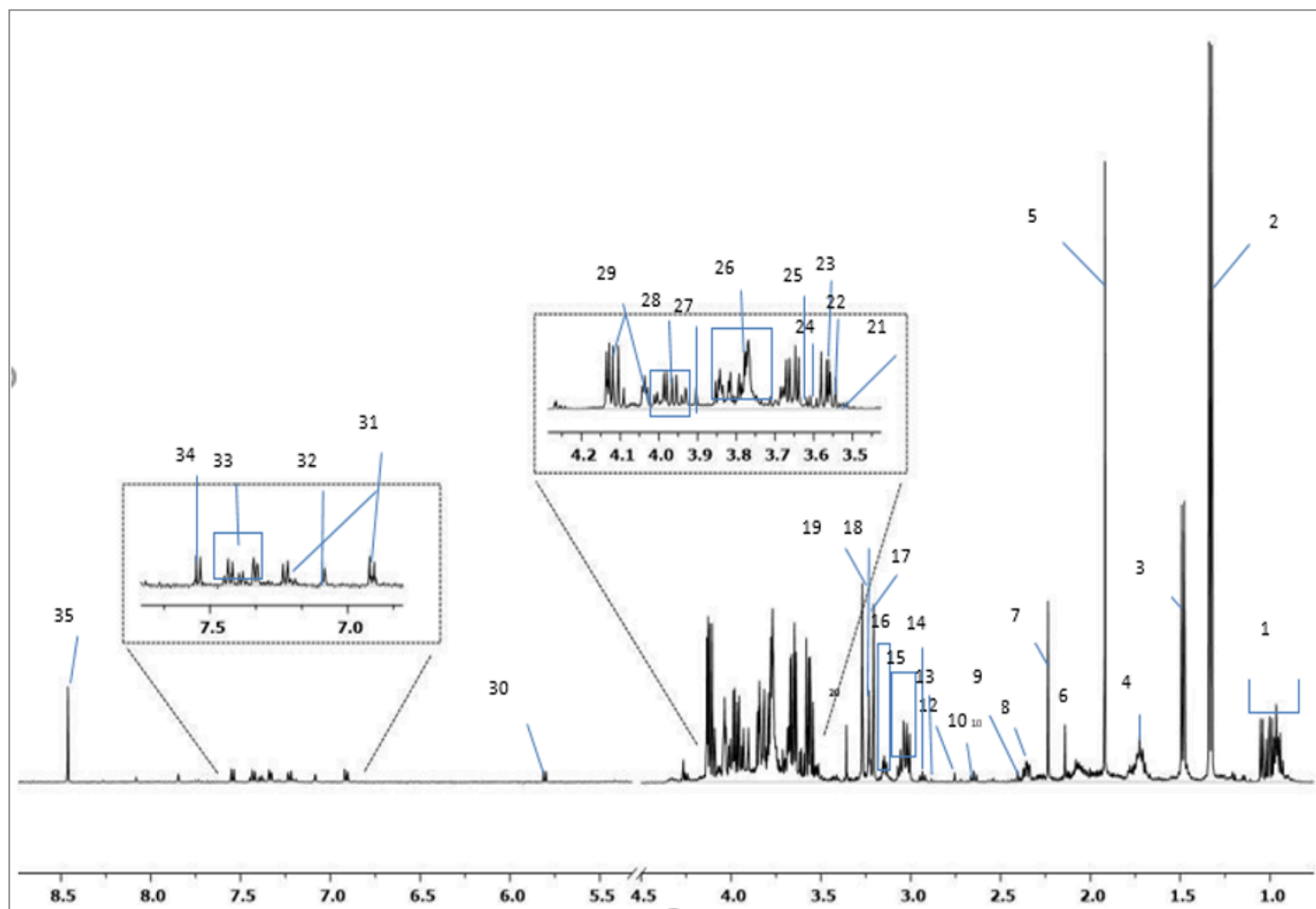


Figure 1

Representative 500 MHz ¹H NMR spectrum of the aqueous extract of Persian sturgeon (*Acipenser persicus*) embryo. Peaks are numbered as follows: (1) BCAA (isoleucine, leucine, and valine), (2) Lactate, (3) Alanine, (4) Leucine, (5) Acetate, (6) Methionine, (7) Acetone, (8) Glutamate, (9) Succinate, (10) Methionine, (11) DMA, (12) Trimethylamine, (13) Aspartate, (14) Lysine, (15) Histidine, (16) Proline, (17) O-Phosphocholine, (18) Choline, (19) Betaine, (20) Succinate, (21) Choline, (22) Glycine, (23) Glycerol, (24) Threonine, (25) Valine, (26) Gluconate, (27) Betaine, (28) Serine, (29) Gluconate, (30) Uracil, (31) Histidine, (32) Tyrosine, (33) Phenylalanine, (34) Uracil, and (35) Formate.

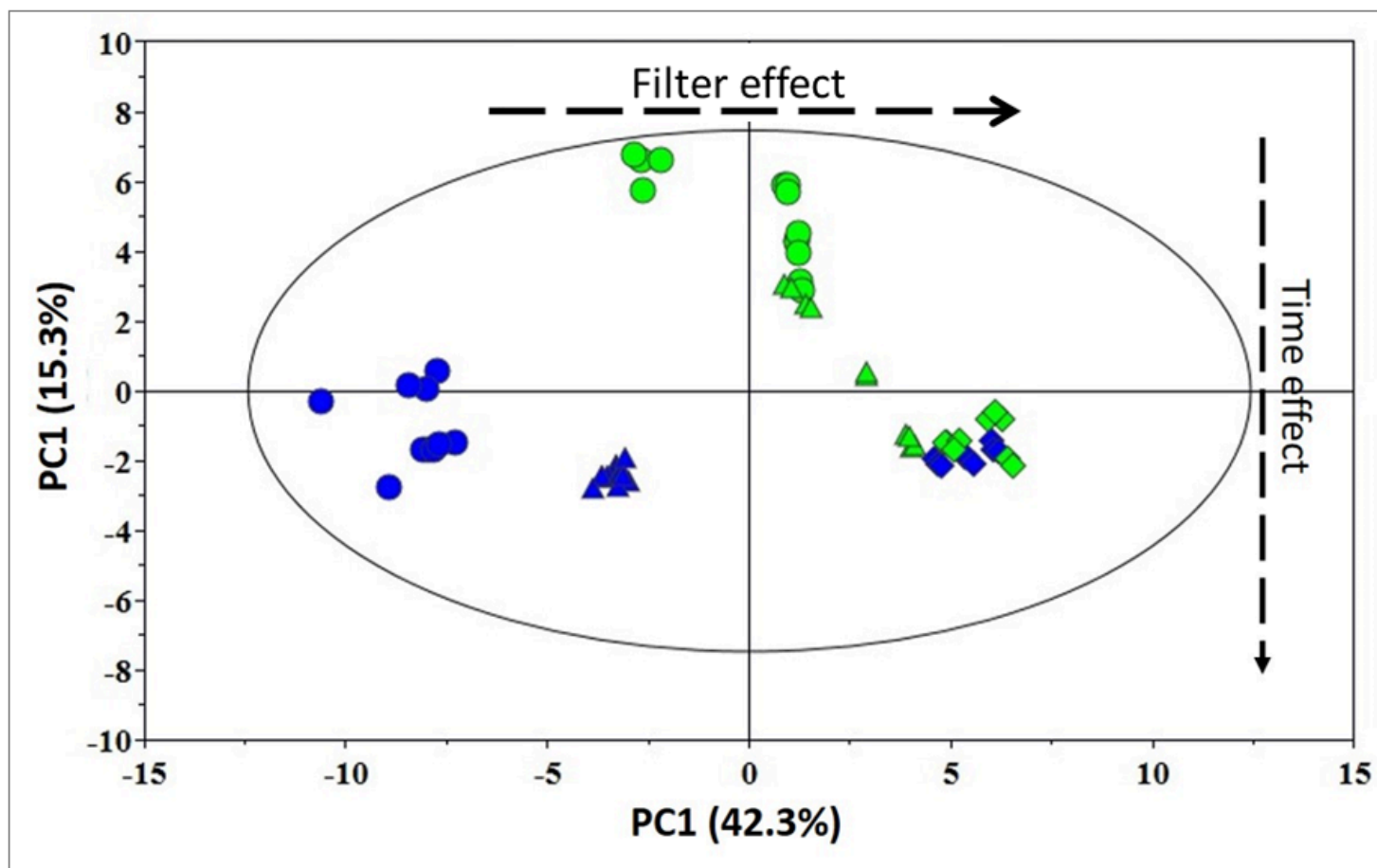


Figure 2

Principal Component Analysis (PCA) score plot of the overall ^1H NMR spectral data set of Persian sturgeon embryos. Control (●); AgNP-F1 (▲); and AgNP-F2 (■) exposed embryos. The scores are colored according to the sampling time point: 2 dpf (green) and 4 dpf (blue). The plot reveals clear time- and dose-dependent metabolic discrimination.

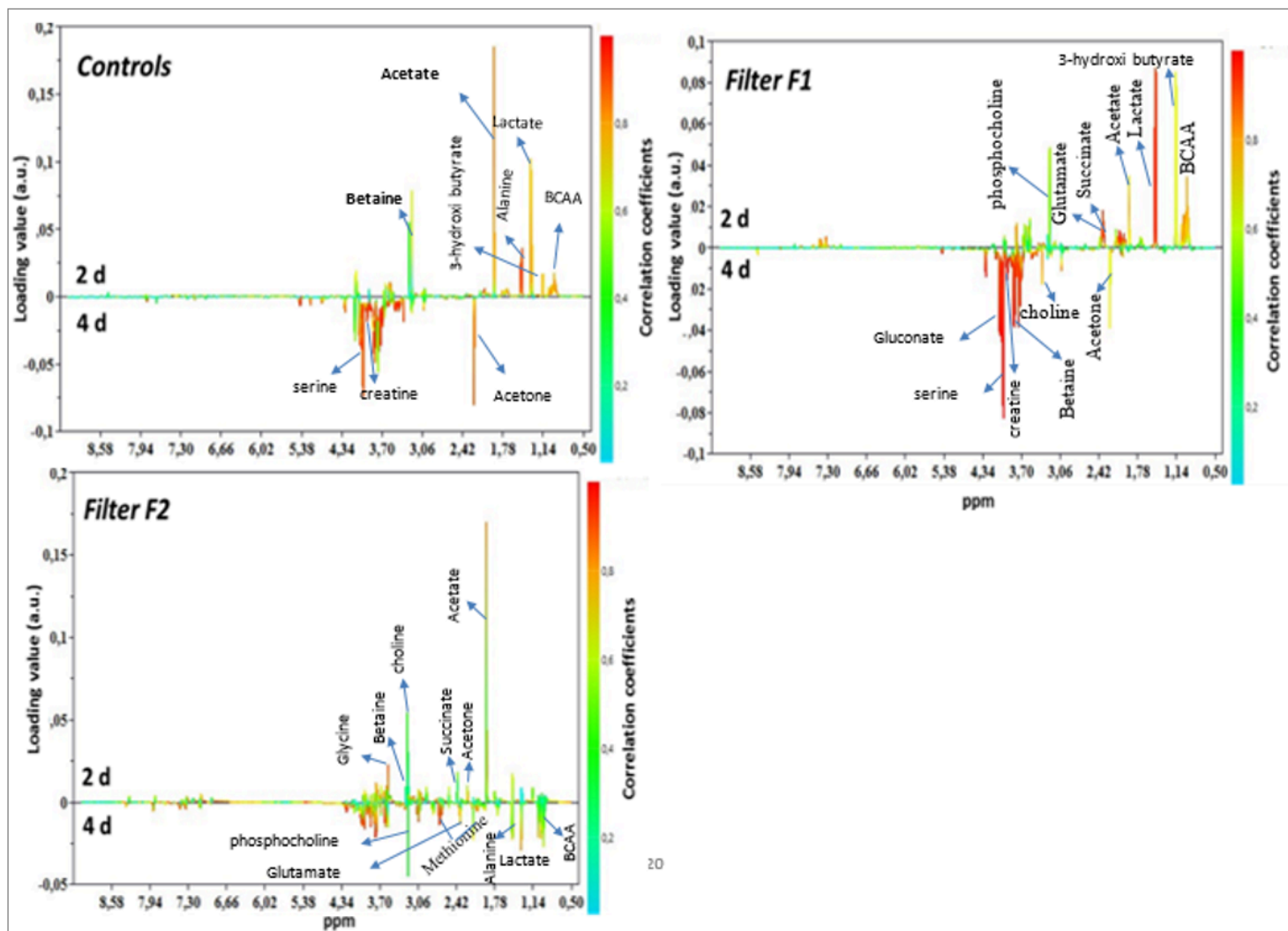


Figure 3

OPLS-DA coefficient plots for the models built from the ^1H NMR spectra of embryos relative to the pairwise comparisons between the two sampling time points (2 dpf vs. 4 dpf): (top) Controls ($R^2X = 0.659$, $R^2Y = 0.988$, $Q^2 = 0.976$); (middle) F1 group ($R^2X = 0.666$, $R^2Y = 0.977$, $Q^2 = 0.976$); (bottom) F2 group ($R^2X = 0.610$, $R^2Y = 0.778$, $Q^2 = 0.705$). Metabolites with positive coefficients are elevated at 4 dpf, while those with negative coefficients are elevated at 2 dpf.

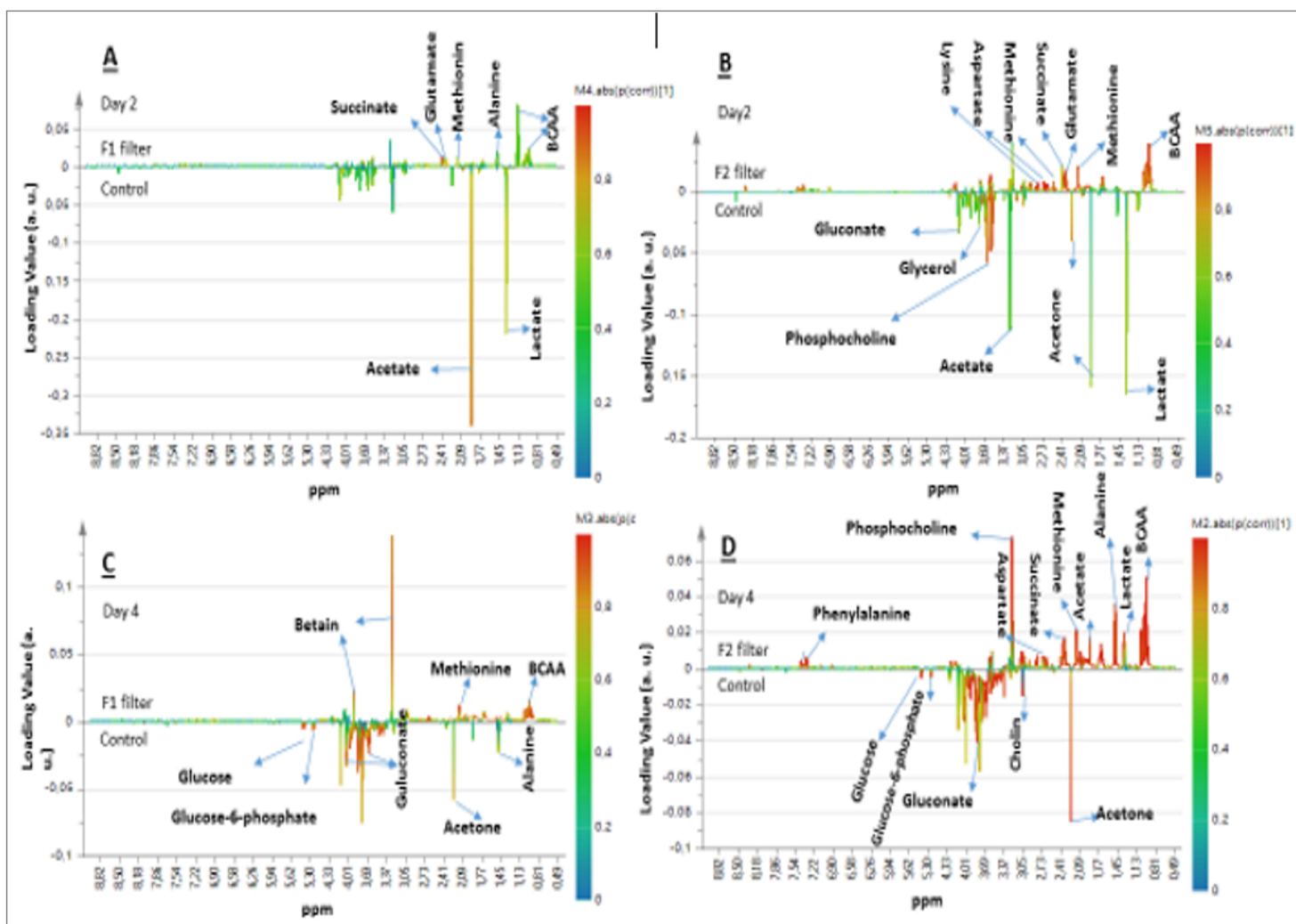


Figure 4

OPLS-DA S-plots for the models built from the ^1H NMR spectra of embryos relative to the pairwise comparisons between Control vs. F1 (left panels) and Control vs. F2 (right panels) at (A-B) 2 dpf and (C-D) 4 dpf time points. Each point represents a metabolite; those farthest from the origin (top-right or bottom-left) are the most discriminant. Metabolites in the top-right are elevated in the treated group, while those in the bottom-left are decreased.

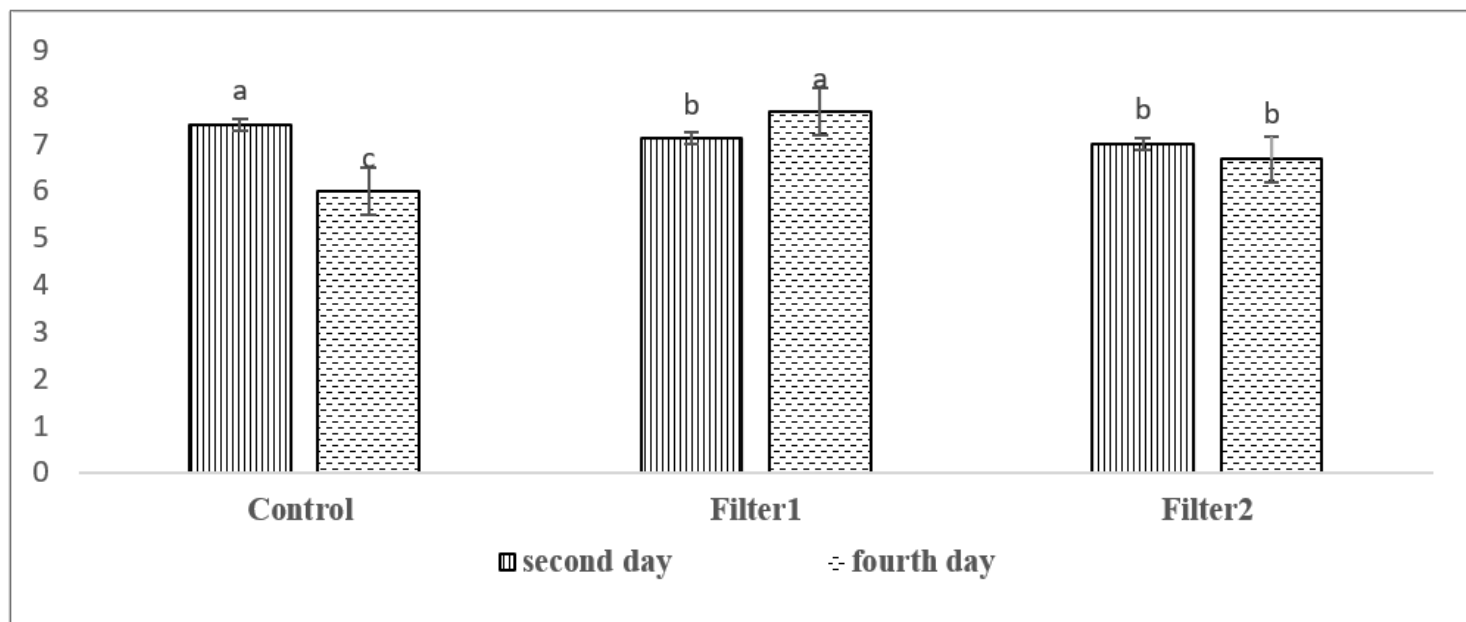


Figure 5

Total free amino acid (FAA) pool in Persian sturgeon embryos reared in control (without AgNPs), Filter 1, and Filter 2 groups at 2 and 4 days of incubation. The P-value from one-way ANOVA among the three groups is shown above each time point. Significant differences ($P < 0.05$) between any two groups are indicated by different letters (a, b, c).

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