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Different sensitivity of mayflies (Insecta, Ephemeroptera) to ammonia, nitrite and nitrate: linkage between experimental and observational data

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Abstract

Complex toxic effects of ammonia, nitrite and nitrate to aquatic animals are not well investigated. In rivers of southwestern Siberia, Russia, elevated ammonia and nitrite concentrations corresponded to significant reduction in species diversity of mayflies. The objectives of the study were to evaluate the sensitivity of six mayfly species to the mixture of ammonia, nitrite and nitrate in acute laboratory tests and to compare the sensitivity found with results of the first river bioassessment in the region considered and with species saprobic indexes. The rank of the species sensitivity was: *Baetis vernus* < *Potamanthus luteus* < *B. fuscatus* = *Cloeon bifidum* < *Ephemerella lenoki* < *Heptagenia sulphurea* ($p < 0.05$). The experiments revealed variation in sensitivity among the species by a factor of 7.5. Comparison of the tests results and the available field data shows that species which exhibit higher tolerance in the tests inhabit comparatively greater amount of sites include contaminated places. Final conclusion is addressed in comparison of the results found with more spatially and temporally extensive observations. Saprobic indexes of the species and their acute tolerances (LC50s) tend to be positively correlated ($r = 0.93$, $p = 0.02$).

Introduction

Ammonia, nitrite and nitrate are common toxic substances in various aquatic ecosystems in the world. In water these nitrogen-containing compounds are interrelated through the process of nitrification. Ammonia is converted into nitrate through a two-step process with nitrite produced as an intermediate product. Many studies of the aquatic toxicity of these chemicals have employed toxicity tests of the single contaminants to various animal species, including mayflies (Alcaraz & Espina, 1995; Khatami et al., 1998; USEPA, 1999; Scott & Crunkilton, 2000). Complex toxic effects of ammonia, nitrite and nitrate have not been well investigated. Although acute and chronic effects of nitrogen mixtures have been documented for many aquatic organisms (Rubin & Elmaraghy, 1977; Alcaraz et al., 1999; Schuytema & Nebeker, 1999; Berenzen et al., 2001), the effects of these mixtures on aquatic insects are poorly understood (see Beketov, 2003a). It is necessary to investigate the toxicity of these contaminants in mixtures because of their combined action. For example, toxicity to *Gammarus pulex* (Crustacea, Amphipoda) occurred at concentrations 10× lower in ammonia, nitrate and nitrite mixtures than in exposures of ammonia alone (Berenzen et al., 2001).

Mayflies (Ephemeroptera) play an important role in almost all freshwater ecosystems. They are widely accepted as bioindicators of water quality and ecological integrity (USEPA, 1998; Barbour et al., 1999; Bauernfeind & Moog, 2000). High sensitivity of mayfly taxa to various contaminants

including metals, ammonia and other chemicals was demonstrated in both observational and experimental studies (Hubbard & Peters, 1978; Hickey & Clements, 1998; Hickey et al., 1999; Clements et al., 2002). Mayflies frequently exhibit significant variation in sensitivity between species in the same family or at order level both in laboratory and field studies. Acute values of ammonia toxicity to different mayfly species can vary by a factor of 3.3 (USEPA, 1999). Such field-derived characteristics as saprobic index or Hilsenhoff's biotic index may also varied significantly among different mayfly species (Hilsenhoff, 1987; Moog, 1995). Correlation mode between acute tolerances (studied in toxicological tests) and susceptibilities that mayflies demonstrate in the field has not been investigated.

This study had two major objectives. The first one was to evaluate and compare the sensitivity of six mayfly species to the mixture of ammonia, nitrite and nitrate in acute laboratory tests. The second objective was to detect whether the observed distribution of mayflies is a result of the direct effect of the contaminants. Such characteristics as concentration of the toxicants in media where the species occur and their relative distribution were compared with the acute values. Due to the crude biomonitoring data preliminary conclusions only were expected here.

Additionally, species-specific acute sensitivity values derived in this study were compared with saprobic indexes found on the basis of extensive field studies (Moog, 1995). As saprobic index generally shows the level of species' chronic tolerance to organic pollution as well as elevated ammonia, nitrite and nitrate concentrations (Abakumov, 1983), such comparison could clarify a conformity between acute and chronic tolerances of the species.

Materials and methods

Experimental organisms

The species chosen were *Baetis vernus* Curtis, *Baetis fuscatus* L., *Potamanthus luteus* L., *Cloeon bifidum* Bengtsson, *Ephemerella lenoki* Tshernova, and *Heptagenia sulphurea* (Müller). They are typical river inhabitants in the region studied; most of them have a wide geographic distribution as well (Beketov & Kluge, 2003). All the larvae were collected in an unpolluted site of Inya river (Novosibirsk region, Russia) during summer of 2002. The larvae were approximately late-instars, not immediately ready to emerge. Number of instars does not appear to be constant for the species, so age was not identified. Specimens were collected just before each test using handnets. The organisms were sorted by small net in water-filled plastic dishes in the laboratory, with only active and externally undamaged individuals being selected.

Toxicity test method

Static, non-renewal toxicity tests were conducted with all six species. Duration of each test was 96 h. Test solutions were prepared with river water that was taken just after collecting the organisms. The exposures were carried out in glass beakers containing 1 l of water oxygenated through glass pipettes. Ten larvae per beaker and three replicates per concentration (including control) were used. Small pebbles obtained from the Inya river were used as a substrate. Mortality was monitored daily, the criterion for death being an absence of response to mechanical stimulation. Larvae were not fed during the experiments.

Chemical parameters of dilution water and real exposure concentrations were measured before and right after preparation of the tested solutions respectively (Table 1). Nominal and measured toxicant concentrations did not differ significantly ($p < 0.05$) (not shown). After 96 h of exposure the levels of the substances in the mixture were not measured. The most important parameters that influence the relative concentrations of two principal forms of total ammonia (relatively nonpoisonous ammonium ions NH_4^+ and extremely toxic un-ionized ammonia NH_3) are pH and temperature (USEPA, 1999). Accurate measurements of these two parameters performed daily showed no significant difference between tests with different species ($p < 0.05$) (Table 1). All the analyses were done by Western Siberian Center for Environmental Monitoring (Novosibirsk, Russia) by standard methods (Semenova, 1977). A mixture of 59.0% ammonia, 1.5% nitrite and 39.5% nitrate (percentage given as masses of nitrogen) was used

Table 1. Mean ± standard error of quality parameters of dilution water used in tests on *B. vernus*, *P. luteus*, *B. fuscatus C. bifidum*, *E. lenoki*, and *H. sulphurea* larvae

Parameter	*B. vernus*	*P. luteus*	*B. fuscatus*	*C. bifidum*	*E. lenoki*	*H. sulphurea*
Total ammonia ($mg\,l^{-1}$)	0.39 ± 0.02	0.36 ± 0.09	0.38 ± 0.03	0.37 ± 0.05	0.38 ± 0.03	0.36 ± 0.08
Nitrite ($mg\,l^{-1}$)	0.007 ± 0.001	0.007 ± 0.001	0.007 ± 0.002	0.007 ± 0.002	0.007 ± 0.001	0.007 ± 0.001
Nitrate ($mg\,l^{-1}$)	0.73 ± 0.28	0.70 ± 0.15	0.74 ± 0.18	0.72 ± 0.15	0.74 ± 0.18	0.70 ± 0.15
Orthophosphate ($mg\,l^{-1}$)	0.12 ± 0.01	0.10 ± 0.02	0.12 ± 0.04	0.12 ± 0.04	0.12 ± 0.01	0.10 ± 0.02
Hardness (mg $CaCO_3\,l^{-1}$)	185.20 ± 5.25	180.40 ± 5.12	184.18 ± 6.00	188.10 ± 5.22	185.20 ± 5.25	184.18 ± 6.00
Oxygen ($mg\,l^{-1}$)	7.08 ± 1.11	7.20 ± 1.15	7.10 ± 1.11	7.10 ± 1.17	7.08 ± 1.11	7.10 ± 1.18
BOD^5 ($mg\,l^{-1}$)	4.66 ± 1.00	4.54 ± 1.05	4.66 ± 1.55	4.39 ± 1.48	4.66 ± 1.55	4.66 ± 1.55
PH	7.5 ± 0.70	7.4 ± 0.72	7.5 ± 0.45	7.5 ± 0.45	7.5 ± 0.45	7.5 ± 0.45
Temperature (°C)	20.5 ± 2.66	20.6 ± 2.48	20.8 ± 2.12	20.8 ± 2.30	520.8 ± 2.12	20.8 ± 2.63

as a toxicant in all tests conducted. Proportion chosen reflects the approximate ratio of the contaminants in the rivers studied. Test concentrations of 1, 3, 9, 27, 81, and 243 $mg\,l^{-1}$ ($N - NH_3 + NH_4^+ + NO_3^- + NO_2^-$) were prepared. Ammonia chloride (NH_4Cl), sodium nitrate ($NaNO_3$) and sodium nitrite ($NaNO_2$) salts were used for preparing tested solutions. All concentrations reported here are expressed as N (nitrogen).

Observational data

In 2002 the first bioassessment using macroinvertebrate assemblage was conducted in the Upper Ob' River basin in Novosibirsk region, southwestern Siberia, Russia (54–56° N lat. and 82–84° E long.). During the ice-free period 171 samples were collected at 10 sites located at four rivers (Ob' River and its tributaries: Inya, Berd' and Tula Rivers). Samplings were made three times during the period: in late May–June, in July, and in late August–September. Invertebrates were collected using D-frame net (500 μm mesh) on the major stream habitat types include pebbles, submerged macrophytes, debris, and fine sediments. Rivers were sampled and analyzed for water quality every two months by Western Siberian Center for Environmental Monitoring by standard methods (Semenova, 1977).

Factor analyses and Pearson's correlation analyses were performed using STATISTICA® 99 (StatSoft, Tulsa, OK, USA) software to estimate the association between water quality parameters and the biological metrics. For the comparison of the toxicity data and results of the bioassessment, the following observational parameters were chosen: number of sites, where the species were found and mean maximum concentration of nitrogen from ammonia, nitrite and nitrate in water where they occurred (mean value between maximum concentrations found in different sites) (Beketov, 2003b).

Austrian saprobic indexes were used as species-specific characteristics which reflect the tolerance to the wide range of contaminants (include ammonia, nitrite and nitrate) in conditions of real ecosystems (Abakumov, 1983; Moog, 1995). As reviewed by Moog et al. (1997) different European saprobic systems for Ephemeroptera do not vary significantly. I used here the Austrian saprobic system because of its contemporaneity and comprehensiveness. The Eastpalaearctic species *E. lenoki* is not known from Europe therefore its saprobic index is not defined (Beketov & Kluge, 2003).

Statistical analysis

Trimmed Spearman–Karber method (Hamilton et al., 1977) was used in the computer program SPEARMAN® (Montana State Univ., USA) for calculating LC50 values (median lethal concentrations) and 95% confidence intervals. That is a non-parametric statistical procedure that especially effective in case of non-monotonous mortality proportions (USEPA, 1993). Survival data from the replicates (separate vessels) at each concentration were summarized before calculation of the LC50s. Values of LC50 were calculated for total nitrogen in the mixture, toxicity of separate

contaminants was not analyzed. Analyses of correlations and significance tests were performed using STATISTICA® 99 (StatSoft, Tulsa, OK, USA) software.

Results

Acute toxicity of ammonia, nitrite and nitrate mixture significantly differed among the tested species (Table 2). The rank of species sensitivity was *B. vernus* < *P. luteus* < *B. fuscatus* = *C. bifidum* < *E. lenoki* < *H. sulphurea* ($p < 0.05$). Analyses of confidence intervals overlaps showed that there were statistical differences between median lethal concentrations (LC50) derived for all species except *B. fuscatus* and *C. bifidum*. In all experiments control mortality was less than 10%.

It is clear from Table 2 that there is a considerable difference between LC50s obtained and maximum concentrations of the contaminants in waters. However, the mean maximum concentration of nitrogen in water where *B. vernus* occurred is within the 95% confidence interval of median lethal concentration derived for *H. sulphurea*. Hence, peak concentrations of the nitrogen-containing pollutants undoubtedly could result in local dying out of this sensitive species.

The biomonitoring studies have shown the high toxicant concentrations corresponded to reduction in species richness and diversity of mayflies (Table 3). Significant negative correlations ($p < 0.05$) were found for elevated ammonia and nitrite concentrations and Shannon's diversity index ($H' = -\Sigma p_i \ln p_i$) (Pesenko, 1982) for mayflies and for total invertebrates. High nitrite levels also correlated with EPTO (Ephemeroptera, Plecoptera, Trichoptera, and Odonata) taxa richness (Beketov, 2003b). Such habitat characteristics as current velocity, temperature, and size of a water body did not correlate with the biological metrics. The small number of sites sampled precluded conducting revealing multivariate analyses.

Comparison of the tests results and the field-observational data reveals that species which exhibit higher tolerance in the tests inhabit a greater amount of water bodies and these habitats include comparatively polluted ones. In contrast, more sensitive species inhabit relatively uncontaminated sites and they are represented in smaller number of rivers (Tables 2 and 3). Significant positive correlations were found between the obtained LC50s and numbers of sites where the species were found ($p = 0.03$) and mean maximum concentrations of nitrogen from ammonia, nitrite and nitrate in water where they occurred ($p = 0.003$) (Tables 2 and 4).

Saprobic indexes also correlated with the derived LC50s ($p = 0.02$) (Tables 2 and 4). As saprobic index generally shows the level of species' chronic tolerance (to organic pollution and elevated ammonia, nitrite and nitrate concentrations) (Abakumov, 1983), it is clear from the results

Table 2. Values of LC50 and 95% confidence intervals (in parentheses) of nitrogen from ammonia, nitrite and nitrate ($mg\,l^{-1}$), European saprobic indexes – SI, number of sites where the species were found – NS, and mean maximum nitrogen concentration from ammonia, nitrite and nitrate in waters where the species occurred – MMNC (standard error in parentheses, n = NS, $mg\,l^{-1}$)

Tested species	LC50 (95% CI)	SI	NS	MMNC
B. vernus	(32.14–43.72) 37.49	2.3	6	3.8 (1.4)
P. luteus	(25.80–34.64) 29.90	2.2	8	3.6 (1.7)
B. fuscatus	(17.18–24.38) 20.47	2.2	5	1.9 (0.7)
C. bifidum	(15.99–22.71) 19.05	2.2	5	1.9 (0.7)
E. lenoki	(12.42–17.25) 14.63	–	4	0.9 (0.1)
H. sulphurea	(2.76–8.86) 4.95	2.0	2	0.8 (0.1)

Table 3. Mean ammonia and nitrite concentration (standard error in parentheses, $n = 6$), Shannon's diversity index and species richness of mayflies – H′ and N Ephemeroptera, and mean abundance of the six mayfly species at 10 sites (standard error in parentheses, n – number of samples for the each site)

Variables	Sites									
	1	2	3	4	5	6	7	8	9	10
Ammonia	(0.18)	(0.29)	(0.30)	(0.24)	(0.28)	(0.01)	(0.20)	(0.32)	(0.42)	(0.45)
($N\text{-}NH_4^+$ mg l^{-1})	0.49	0.59	0.67	0.51	0.68	0.056	0.68	0.86	1.03	1.41
Nitrite	(0.001)	(0.001)	(0.002)	(0.003)	(0.005)	(0.004)	(0.006)	(0.008)	(0.008)	(0.007)
($N\text{-}NO_2^-$ mg l^{-1})	0.005	0.008	0.016	0.013	0.05	0.008	0.01	0.051	0.075	0.039
H′ Ephemeroptera	2.608	2.056	2.543	2.105	1.267	1.286	1.069	0.965	0.451	0
N Ephemeroptera	23	21	18	15	7	5	4	3	2	1
B. vernus	–	(0.03)	–	(2.22)	–	(0.46)	(0.45)	–	(0.03)	(3.24)
		0.11		5.00		1.00	0.89		0.09	6.70
P. luteus	(1.01)	(0.02)	(0.26)	(0.11)	–	(0.04)	(0.12)	(0.16)	(0.21)	–
	2.14	0.06	0.59	0.27		0.08	0.33	0.36	0.45	
B. fuscatus	(1.11)	(0.37)	(0.42)	(0.65)	(0.02)	–	–	–	–	–
	3.46	0.83	1.00	1.80	0.07					
C. bifidum	(0.41)	(2.20)	(0.21)	(0.92)	(1.02)	–	–	–	–	–
	1.04	5.72	0.50	2.40	2.20					
E. lenoki	(0.32)	(0.04)	(0.46)	(0.18)	–	–	–	–	–	–
	0.71	0.11	0.98	0.40						
H. sulphurea	(0.05)	–	(0.56)	–	–	–	–	–	–	–
	0.14		1.40							
n	28	18	32	15	15	12	9	11	11	20

Table 4. Correlation matrix of variables LC50, SI, NS, and MMNC (explanations in Table 3), values of r (Pearson's coefficient of correlation) and p are given

	LC50	SI	NS
LC50	1		
SI	0.93, $p = 0.02$	1	
NS	0.855, $p = 0.03$	0.735, $p = 0.1$	1
MMNC	0.953, $p = 0.003$	0.976, $p = 0.001$	0.664, $p = 0.15$

presented that acute and chronic tolerances of the tested species tend to be positively correlated. In addition, correlation between mean maximum nitrogen concentrations and saprobic indexes ($p = 0.001$) shows that bioassessment results are consistent with studies conducted for deriving the saprobic indexes (Table 4).

Discussion

Mechanisms of combined toxic effect of ammonia, nitrite and nitrate are not well understood at present. Alcaraz et al. (1999) reported joint effect of ammonia and nitrite to *Penaeus setiferus* (Crustacea, Decapoda) postlarvae as synergistic at 48 h of exposure and antagonistic after 72 h. Combined effect of these two toxicants to mayfly larvae was verified as an independent action for *E. lenoki* and antagonistic effect was found for *Cloeon dipterum* (Beketov, 2003a). Additive action was found in nitrite and nitrate toxicity to *C. dipterum* (Beketov, 2003a). Therefore results of the present study are not directly comparable to previously published investigations of these

nitrogen-containing compounds' separate toxicity (Hickey & Vickers 1994; Khatami et al., 1998; USEPA, 1999).

Synergistic toxic effect of ammonia, nitrite and nitrate was found in the present study that is consistent with previously published data (Berenzen et al., 2001). Acute median lethal concentration (LC50 for 96 h) of total ammonia ($N–NH_4^+ + NH_3$) derived for *E. lenoki* at pH 8.2 was 59.50 (44.59–79.39) mg l^{-1} (unpublished data). LC50 of the mixture (containing 59% of total ammonia) found in the present study for this species at pH 7.5 is equal to 14.63 (12.42–17.25) mg l^{-1} (Table 2). Hence, in this study 50% mortality occurred at approximately 7-fold lower ammonia concentration. This difference should be even more pronounced if the single ammonia toxicity was estimated at higher pH level because of the relatively lower amount of more toxic un-ionized ammonia (NH_3) (USEPA, 1999). Berenzen et al. (2001) reported a significant chronic effect of ammonia, nitrite and nitrate mixture to *Gammarus pulex* (Crustacea, Amphipoda) at 10-fold lower concentrations when compared to single ammonia toxicity tests which is quite comparable with the results shown here.

Notable variation in sensitivity among the species tested (Table 2) is of particular interest because all the species are the common representatives of mayfly assemblages in unpolluted streams in southwestern Siberia. All the specimens were taken in the same place therefore, it is unlikely that different sensitivity was caused by species-specific tolerance acquired through previous exposures. Numerous investigations devoted to aquatic ammonia toxicity revealed a considerable diversity in acute sensitivity of different taxa include differences across species in similar groups (USEPA, 1999). Thus, in Cladocera species it varies approximately by a factor of 6.5 and in mayflies by 3.3 (LC50s of un-ionized ammonia found at similar pH levels). The species studied here varied in sensitivity by a factor of 7.5 that is similar to the results for Cladocera.

Experimental approaches are frequently used in cause inferring studies in bioassessment (USEPA, 2000; Clements et al. 2002; Suter et al., 2002). Manipulation of stressors in experimental conditions allows researcher to assign direct causation to observed response. Although artificial conditions of the most experiments often limit the extrapolation of the results found to natural systems, comparison of experimental and observational data are very effective in causation in biomonitoring studies.

Correlations between experimental results and observational data presented in this study (Table 4) show that direct influence of the contaminants could be a cause of the observed species distribution. Due to the limited biomonitoring data final conclusions should be addressed in spatially and temporally extensive observations. Thus, Clements et al. (2002) supported a 10-year large-scale bioassessment by experiments focused on metals influence on mayfly assemblage. In that study integrating of descriptive and experimental approaches resulted in strong casual arguments. Species investigated in the present study have a wide geographic distribution (Beketov & Kluge, 2003) and so comparison of the acute values with biomonitoring data from different palaearctic regions will be of interest to confirm or disprove the correlations found.

Although the species investigated have relatively close saprobic indexes (beta-meso-saprobity), variation in these indexes significantly correlated with the acute median lethal concentrations. As observations conducted for deriving the saprobic indexes were not restricted spatially or temporally (in contrast to the first bioassessment in region studied) this finding shows the reliable positive correlation between acute (found in laboratory) and chronic (field-derived) tolerances. Available data on several Cladocera species (*Ceriodaphnia acanthina*, *C. dubia*, and *Daphnia magna*) show that acute and chronic ammonia sensitivity do not correlate in these similar taxa (USEPA, 1999). Summarized in the USEPA's (1999) report, acute–chronic ratios for ammonia significantly varied among different taxonomic groups and also show a lack of the correlation there. Limited data on complex effect of ammonia, nitrite and nitrate do not allow comparison of the results found with analogous examples.

Conclusions

The experiments revealed significant variation in sensitivity among the species tested. Combined

effect of the mixture on *E. lenoki* resulted in approximately sevenfold higher toxicity when compared to single ammonia effect. Results of the present study are hardly comparable with previously published investigations because most of them were devoted to the effects of the single toxicants.

Correlations between experimental and observational results show the direct influence of the contaminants could be a cause of the observed species distribution. Further bioassessment is necessary to make final conclusions. Saprobic indexes of the species tested and their acute tolerances (LC50s) tend to be positively correlated.

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