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BUMBLEBEES (BOMBIDAE) ALONG POLLUTION GRADIENT – HEAVY METAL ACCUMULATION, SPECIES DIVERSITY, AND *NOSEMA BOMBI* INFECTION LEVEL

ABSTRACT: Pollinator crisis (Kearns *et al.* 1998) and its possible causes has become a worldwide issue during the last two decades. Although pollution is among the possible causes of the widely observed pollinator loss, it is still poorly investigated and no studies are known, so far to test the effects of heavy metal contamination in bumblebees (Bombidae) – the second most important group of managed pollinators after honey bees (*Apis mellifera* Linneaus).

We have tested heavy metal (Pb, Cd, and Zn) accumulation, species diversity and parasite load (focusing on the common *Nosema bombi* Fantham and Porter, Microsporidia: Nosematidae) in bumblebees. For this purpose, we have chosen three heavy metal gradients (Guryevsk, Belovo and Olkusz) and two additional control sites (Kouznetskiy Alatau and Gornaya Shoria). All gradients were approximately 20 km long, starting in close proximity (1.3 km or less) of an active zinc or metal smelter, and each consisting of 5 sites located on semi-natural or degraded meadows in various distance from the smelter. On each site min. 50 bumblebees were caught by sweep nets, each individual identified to species level and next, its abdomen homogenized and used for assessment of *N. bombi* infestation. Heavy metal levels in soil of the tested gradients varied between (Pb: 13.6–814.2 mg kg^{-1}, Cd: 0.14–20.3 mg kg^{-1}, Zn: 67.0–889.3 mg kg^{-1})

Bumblebees accumulated Pb and Cd (Pb: 0.21–3.3 mg kg^{-1}, Cd: 0.002–0.069 mg kg^{-1}) in their bodies. The content of these metals in bumblebee bodies correlated with their content in soil (Pb: P <0.01, Cd: P = 0.002). However, no correlation was found between the Zn contents in bumblebees (Zn: 74.7–81.9 mg kg^{-1}) and the soil.

We have also found that the metal contents in soil or in the bodies of bumblebees caused no changes in species diversity or dominance on polluted sites, irrespective of type and the level of contamination. The variation of Shannon diversity (H'), as well as Simpson's diversity (D) were similar in all studied sites and ranged from 0.543 to 0.81 and from 0.152 to 0.484 respectively.

The proportion of infected individuals was generally not higher than 0.29 and did not differ significantly among the studied sites. Incidentally, based on variation in the small subunit ribosomal RNA (SSU-rRNA) gene, we have found a new strain of *Nosema bombi* in the Kouznetskiy Alatau and Gornaya Shoria (West Siberia, Kemerovo Region) samples. The new small subunit RNA sequence in the new strain of *N. bombi* was named *N. bombi* WS2 (West Siberia) SSU rRNA. Based on the obtained results we conclude, that bumblebees can withstand or even successfully deal with heavy metal contamination at certain levels.

KEY WORDS: bumblebees, species abundance, heavy metal contamination, *Nosema bombi*

1. INTRODUCTION

During the last decades more attention has been focused on the possible causes of the global pollinator crisis (Kevan 1991, Kearns *et al.* 1998, Biesmeijer *et al.* 2006, Goulson *et al.* 2008, Grixti *et al.* 2008). Among pollinators, bumblebees provide a considerable amount of natural pollination service on the Northern Hemisphere. Thanks to their relatively large and sturdy body, bumblebees are excellent pollinators in colder regions, where smaller wild bees are less frequent. In the Eurasian tundra they account for up to 85–95% of the total number of bees; while in the taiga zone of Europe and Western Siberia they make up 55–70% of the bee fauna (Panfilov 1968 after Bolotov and Kolosova 2006), and probably are responsible for a similar fraction of pollination services. Therefore, they play a key role in maintaining the normal functioning of these ecosystems (Chapin *et al.* 1997).

There is a growing number of agents recognized as having generally negative effects on pollinators (see for review: Kearns *et al.* 1998, Cane and Tepedino 2001). Most are caused by human activities such as urbanization (Eremeeva and Suchchev 2005), land use changes (Steffan-Dewenter *et al.* 2002) and also by the introduction of managed pollinator species to the natural environment (Kenta *et al.* 2007, Krauss *et al.* 2011) or the use of pesticides (Brittain *et al.* 2010). Pollution with heavy metals, however, is still poorly investigated, although it is well studied in other groups of invertebrates (for a review see Tyler *et al.* 1989). So far, mostly honeybees, or rather their product – honey – has been studied in the context of heavy metal pollution (Jones 1987, Malone e*t al.* 2001). A notable exception is a recent study conducted on red mason bees (*Osmia rufa* L.) along a heavy metal gradient near the zinc/lead smelter in Olkusz (Moroń *et al.* 2010). This study showed a clear negative correlation between the heavy metal contamination level of pollen found in red mason bee nests and the reproductive success of females. It also revealed that immature males are more sensitive to pollution and die before eclosion more often than females. Unfortunately, bumblebees have never been studied in this respect and nothing is known about whether or how they manage the possible effects of heavy metal pollution in the environment. Based on the results of the aforementioned study on red mason bees (Moroń *et al.* 2010), it can be expected that heavy metal pollution present in pollen, due to the transport of airborne particles of pollution through the air and into open flowers, may also affect bumblebees. Our pilot field observations confirmed that even in a highly polluted environment bumblebees were found in considerable numbers, but how they manage to survive and what negative effects can be observed in their species structure or there immune system remain unknown.

Studies conducted on other invertebrates have shown that various species deal with the effects of pollution in different ways. Some species are exceptionally sensitive to pollution, some accumulate heavy metals, while others actively regulate the level of pollutants in their bodies by excretion. Quite often different strategies are found in the same taxonomic group and in closely related species or even among various populations of the same species (for a review see: Tyler *et al.* 1989).

Both non-essential metals (in our case Pb and Cd – in all amounts) and essential metals (Zn – given in excess) can weaken an organism by changing the conformation or causing the denaturation of enzymes. Heavy metals can also corrupt the functioning of the immune system as shown by Sorvari *et al.* (2007) in ants. This study was the first to demonstrate that contamination causes a generally weaker immune response in ants, although the exact mechanisms were not described. As a result, pathogens and parasites can more easily enter and induce heavy infections in individuals living in a contaminated environment. Among bumblebees, one of the most common and widely studied intestinal parasites is a Microsporidia, *Nosema bombi* (McIvor and Malone 1995, Imhoof and Schmid-Hempel 1999, Brown *et al.* 2000, Pollinator Parasite Project 2006). It is a common parasite shown to have detrimental effects on bumblebees. Additionally, *N. bombi* is not species-specific and can infect most or possibly all bumblebee species (MacFarlane *et al.* 1995, Schmid-Hempel and Loosli 1998, Tay *et al.* 2005, Larsson 2006). This characteristic enables a comparison of infection levels of various species of bumblebee exposed to heavy metal contamination in the environment.

In this study we address the following questions: 1) does an excess of heavy metal pollution cause a measurable accumulation of both non-essential metals (heavy metals such as Pd and Cd that are dangerous at any amount) and essential metals (in this case Zn) in bumblebees or do they manage to regulate the level of these metals in their bodies; 2) does pollution cause a measurable change in species diversity (eliminating the weaker ones and thus decreasing diversity and/or increasing dominance); 3) do heavy metals weaken the immune system of bumblebees and cause higher parasite levels in populations living in a polluted environment?

2. STUDY SITES

Three heavy metal pollution gradients near various smelters were chosen for this study. We have chosen one metallurgic plant (Guryevsk – G gradient) and one zinc smelter (Belovo – B gradient) in the Kemerovo region in Russia and one zinc smelter in southern

Table 1. Location of bumblebee collection sites, habitat type and number of caught (during one hour by 4 people on 15–20 ha/site) individuals and species: (G1–G5) gradient near Guryevsk metallurgic plant, Russia, (B1–B5) gradient near Belovo zinc smelter, Russia, (O1–O5) gradient near Olkusz zinc smelter, Poland, additional control sites for Russian bumblebee fauna in the Mountains of Kouznetskiy Alatau (KA) and Gornaya Shoria (GS).

Site	Distance from the smelter (km)	GPS coordinates of sites	Habitat type	No. caught individuals (species)
G1	0.63	54°16'41.87"N, 85°56'30.50"E	degraded steppe	166 (13)
G2	4.6	54°19'11.61"N, 85°56'37.39"E	degraded meadow	126 (11)
G3	4.7	54°17'28.34"N, 86° 0'7.97"E	dry steppe	102 (15)
G4	8.7	54°21'21.40"N, 85°54'23.45"E	xerothermic meadow	109 (13)
G5	16.4	54°23'59.37"N, 85°47'18.39"E	xerothermic meadow	148 (12)
B1	0.3	54°27'9.85"N, 86°19'11.21"E	degraded meadow	136 (12)
B2	4.9	54°27'2.61"N, 86°23'51.96"E	degraded meadow	113 (12)
B3	6.9	54°24'29.56"N, 86°14'29.57"E	degraded meadow	128 (12)
B4	11.2	54°31'50.51"N; 86°25'26.85"E	degraded meadow	173 (13)
B5	20.4	54°35'34.95"N, 86°31'1.29"E	degraded meadow	108 (8)
O1	1.3	50°17'9.02"N, 19°27'53.16"E	xerothermic meadow	56 (8)
O2	4.0	50°18'18.91"N, 19°30'28.31"E	xerothermic meadow	48 (6)
O3	3.5	50°16'46.59"N, 19°31'24.25"E	xerothermic meadow	57 (7)
O4	8.5	50°20'6.71"N, 19°32'57.62"E	xerothermic meadow	50 (7)
O5	20.0	50°25'36.93"N, 19°37'39.11"E	xerothermic meadow	61 (7)
KA	app. 120 from both smelters	54° 6'30.69"N, 87°52'12.27"E	barren taiga	379 (8)
GS	app. 220 from both smelters	52°49'23.98"N, 88°22'39.94"E	barren taiga	276 (10)

Poland near Olkusz (O gradient). The chosen regions are all known for their intense mining and industrial activity and also a generally high level of heavy metals in their surrounding environment. Pollution gradients starting from the smelters were established by analyzing the heavy metal contamination of soil (all gradients) and of bumblebee bodies (on the G and B gradients) at these sites.

On each gradient 5 sites were chosen, the closest site being 0.3 to 1.3 km and the farthest sites 16 to 20 km from the smelter. In addition, two independent control sites with no industrial activity were chosen in Russia. These sites included the Gornaya Shoria (GS) and the Kouznetskiy Alatau (KA) Mountains in the southern part of the Kemerovo region. All sites were located on open meadows (steppe, xerothermic or degraded meadows, barren taiga) (for more details see Table 1).

3. MATERIAL AND METHODS

Samples were caught by sweep nets on sites during the peak of bumblebee abundance (between 21–26 August 2007 on gradients G and B, between 21–23 August 2004 on gradient O, and between 20 June to 3 July 2008 on KA and GS). On each site a team of four people collected specimens for approximately one hour, on an area covering approximately 15–20 ha at each site. Additionally, on each site, we tried to reach a minimum of 50 individuals (in Poland) or 100 individuals (in Russia) which we succeeded during the one hour time frame on each site (Table 1).

All collected bumblebees were identified to species level and two biodiversity indices were calculated for each site. Shannon diversity (H') (Shannon, 1948) was used to measure species diversity, while Simpson's diversity (D) (Simpson, 1949) was used as an estimator of species dominance. The latter is also often used in pollution monitoring studies as a good measure of the effects of pollution, because it is more sensitive to changes in dominance of species in a given habitat, than Shannon`s diversity. Such changes can appear when the population of a sensitive species decreases due to pollution. Both indices were calculated by using the programme BioDiversity Professional (2007). A statistical assessment of species abundance at the tested sites (on gradients and in the control areas) was performed using Wilcoxon's paired test with a Bonferroni correction to account for multiple comparisons (significant *P* level for the Russian gradient was set at $P < 0.00265$ and for the Polish gradient at $P < 0.005$).

To assess how pollution was distributed along the established gradient, samples of the upper layer of soil were taken from five points at each of the sites, mixed and analyzed for the presence of Zn, Pb, and Cd. In order to correlate the soil pollution level with the level of heavy metals in bumblebee bodies, their heads and thoraxes were sacrificed for an assessment of the same heavy metals as in the soil samples. Abdomens were collected and used for measuring parasite level.

Analyses of both the soil and the bumblebee body samples were done as follows: material was dried in 105°C, dissolved in nitric acid (HNO_3) and homogenized. Zn concentration was measured using flame, whereas Pb and Cd concentrations were assessed using graphite furnace atomic absorption; measurements were taken using a PerkinElmer model AAnalyst 800 Spectrometer.

Abdomens of collected bumblebees were used to check for the presence of *N. bombi.* In total 595 individuals were checked for infestation out of 2236 collected samples (for details see Table 2). Total DNA was isolated from ethanol-fixed bumblebee intestines using the QIAGEN DNeasy Blood & Tissue Kit. A fragment of the small subunit ribosomal RNA (SSU-rRNA) gene was amplified using primers *SSUrRNA-fl* (5'- CACCAGGTTGATTCTGCCT -3') *and SSUrRNA-rcl* (5'-GTTACCCGTCACTGCCTTG - 3') from Tay *et al.* (2005). PCR amplification was performed using 0.1 μg of genomic DNA in a 20-μl volume of 10 mM Tris-HCl (pH 8.9), 1 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 μM each of four dNTPs, 0.5 μM primers, and 2.5 units of Taq polymerase. After an initial denaturation step for 3 min at 94°C, the PCR reactions were subjected to 30 cycles of amplification consisting of 30 sec. of denaturation at 94°C, 40 sec. annealing at 52°C, and 40 sec. extension at 72°C. PCR results were assayed by 1% agarose gel electrophoresis. 200 ng of the PCR product was used in a 10 μl cycle sequencing reaction with the ABI BigDye Terminator Kit on an ABI 377 DNA sequencer. The obtained

Table 2. Bumblebees tested for *N. bombi* infestation on two heavy metal gradients, one near the Guryevsk metallurgic plant (sites G1–G5), the other near the Belovo zinc smelter (sites B1–B5), and two additional control sites in the Mountains of Kouznetskiy Alatau (KA) and Gornaya Shoria (GS) (see Table 1).

Site	No. tested	No. infected	% of infected
G1	82	5	6.1
G2	36	2	5.6
G3	37	3	8.1
G4	49	1	2.0
G5	25	1	4.0
B1	16	2	12.5
B2	22	0	0
B3	22	0	0
B4	36	5	13.9
B5	29	3	10.3
GS	132	22	16.7
KA	109	32	29.4

sequences were deposited in GenBank under accession numbers HM370543–HM370552.

Nemerow`s synthetical pollution index (Nemerow 1985) (PI) was adapted to our dataset and calculated based on mean residual values of pollution using the values measured on the least polluted i.e. the farthest (from the source of pollution) sites to calculate the residuals.

Pollution index (PI) was calculated according to a formula (1):

PI = [(Zn level of the polluted/ farthest site)/Zn level of the farthest site) +

(Cd level of the polluted/ farthest site)/Cd level of the farthest site) +

(Pb level of the polluted/ farthest site)/Pb level of the most further site)]/3

The pollution index (PI) was calculated in order to obtain a single value describing overall pollution at each site and thus allowing a comparison of diversity indices across sites. Infestation of bumblebees were compared by using G-test. All statistical analyses were done using Statistica v.8. (StatSoft, Inc. 2008).

3. RESULTS

3.1. Heavy metal pollution

The soil pollution levels for three gradients were slightly different. The pollution indices (formula 1) showed that with increasing distance, pollution levels were generally lower on all three gradients. They were approximately 7–9 times higher near the tested smelters than at the farthest site (Table 3). The only exception was G5 in which unexpectedly high Zn levels were discovered.

The level of heavy metals in bumblebee bodies exhibited an interesting phenomenon. On both measured gradients (Belovo and Guryevsk), the levels of Pb and Cd decreased along the gradient, as expected, but were found to be higher along the Guryevsk gradient than on the Belovo gradient, although the levels in the soil were higher in the latter. Zinc levels were fairly similar at all sites. The levels of Pb and Cd in bumblebee bodies correlated with the soil contamination levels on both gradients (G: Pb, P = 0.002, R^2 = 0.97; Cd, P = 0.009, R^2 = 0.93; B: Pb, P = 0.002 R^2 = 0.97, Cd P= 0.001, R^2 = 0.98) (Fig. 1).

Zn levels in bumblebee bodies oscillated between 74.7–81.9 mg kg^{-1} and did not show a significant correlation with soil concentrations on the tested gradients (G: P = 0.61, R^2=0.1; B: P = 0.36, R^2 = 0.28).

3.2. Diversity analysis

Representatives of 22 *Bombus* species were found on the Guryevsk and Belovo gradients including the most typical species for this area: *B. lucorum, B. veteranus* and

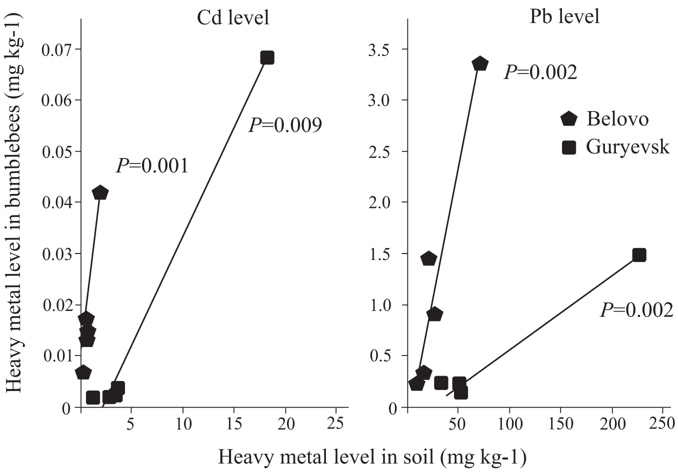

Fig. 1. Relationship between concentrations of Cd and Pb in soil and contents of these metals in bumblebees bodies. Bumblebees were collected along heavy metal gradients at the Guryevsk metallurgic plant and Belovo zinc smelter (see Table 1).

B. pascuorum. Around Guryevsk there were 21 species present among the 651 individuals caught (average 13 species per site), while in the vicinities of Belovo 20 species among the 658 individuals were caught and identified (average 11 species per site). Representatives of 10 *Bombus* species were found on two control sites from Gornaya Shoria and Kouznetskiy Alatau (average 9 species per site). In total, 655 individuals were collected and identified on these control sites. As on gradients B and G two species, *B. lucorum* and *B. pascuorum* dominated (~64% of the total number of individuals) on sites GS and KA. On both gradients and control sites the dominant species were also the most abundant species in the region (Table 4). In Olkusz (Poland) 12 species were present among 272 collected individuals (average 7 species per site) with numerous representatives of *B. terrestris* and *B. lapidarius.*

Altogether 2236 individuals were caught, identified and analysed on the three heavy metal polluted gradients and the two additional control sites (Table 4).

We did not detect a significant difference in species abundance between the sites on the tested gradients, even in comparison with control sites, KA and GS (where we expected the highest diversity) with the sites near the Guryevsk and Belovo smelters. Furthermore, the calculated diversity parameters were similar on all sites irrespective of pollution or geographic localization. Shannon's H' diversity index ranged between 0.543 and 0.811, while Simpson's D dominance index was oscillating between 0.152 and 0.484 (Fig. 2).

3.3. *Nosema* infection level

Altogether 595 individuals of ten selected species (species with more than 5 representatives) from G and B gradients and KA and GS control sites were analysed. Surprisingly, no infected individuals were detected among any of 228 investigated samples of *B. veteranus* and *B. pasquorum*. The other eight species contained infected individuals, although the level of infection varied at different sites. Due to the small number of individuals of most species from particular sites, we could compare only the sites as a whole (summing up all individuals for a site) (Table 2).

The percentage of infected individuals on the Russian sites oscillated between 2% and 29%, except for sites B2 and B3 where infection was not detected in 22 examined individuals per site. The percentage of infected bumblebees was similar on all sites, except for G1 and KA, where significantly more infected individuals were found on the distant control site (KA) than on the site near the Guryevsk smelter (percentage of infected individuals at G1 – 6.1%, at KA – 29.4%, $P < 0.0025$). On sites along the two gradients, the percentage of infected individuals did not exceed 15%, while this value amounted to 17% and 29% for the two control sites GS and KA, respec-

Table 3. Soil contamination levels of Zn, Cd, and Pb and a pollution index (formula 1) calculated for each site along gradients: (G1–G5) Guryevsk metallurgic plant, (B1–B5) Belovo zinc smelter, and (O1–O5) Olkusz zinc smelter (see Table 1).

Site	Zn (mg kg^{-1})	Cd (mg kg^{-1})	Pb (mg kg^{-1})	Pollution index
G1	311.63	2.14	74.72	9.55
G2	95.88	0.32	26.73	1.07
G3	120.09	0.25	25.17	1.16
G4	67.03	0.20	17.58	0.81
G5	552.14	0.14	13.64	0
B1	889.34	17.24	223.03	7.46
B2	377.10	2.29	52.01	0.88
B3	399.62	2.41	52.66	0.81
B4	322.64	1.96	48.49	0.25
B5	196.95	0.85	32.80	0
O1	350.00	20.334	814.16	7.81
O2	389.73	5.564	318.31	2.47
O3	390.08	5.012	231.24	1.96
O4	259.29	3.164	153.09	0.93
O5	154.00	1.54	74.43	0

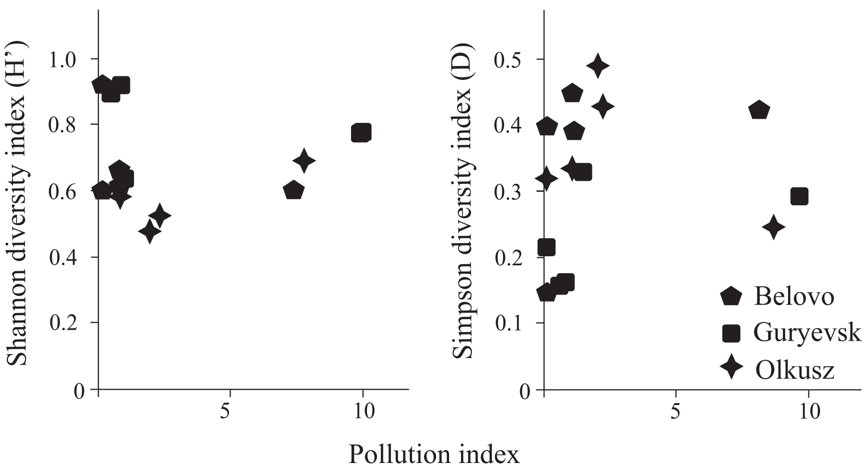

Fig. 2. Relationship between diversity indexes calculated for bumblebee assemblages and pollution index (formula 1) at sites along heavy metal gradients in (A) Guryevsk (G1–G5), B), Belovo (B1–B5), and C) Olkusz (O1–O5) (see Table 1).

tively. Parasite levels were not correlated with pollution levels on the two gradients (G: $R^2 = 0.1171$, $P = 0.7033$; B: $R^2 = 0.0553$, $P = 0.5730$).

3.4. Nucleotide sequence analysis

78 SSU rRNA PCR fragments (202 bp in length) belonging to eight *Bombus* species were sequenced and analyzed along with homologous sequences of closely related species examined earlier by other researchers, namely *N. bombi* (AY741110, AY741111), *N. apis* (DQ235446), and *N. ceranae* (DQ486027). Fourteen of the newly obtained sequences were identical to the SSU rRNA nucleotide sequences of *N. bombi*. The rest of the sequenc-

Table 4. Species identified and number of caught (during one hour by 4 people on 15-20 ha/site) individuals on each site along heavy metal gradients near (A) Guryevsk (G1–G5), (B) Belovo (B1–B5), and (C) Olkusz (O1–O5); and at two additional distant control sites: (D) mountains of Kouznetskiy Alatau (KA) and Gornaya Shoria (GS) (see Table 1).

(A) *Bombus* or *Psythirus* species	G1	G2	G3	G4	G5
B. armeniacus scythes Radoszkowski	8	0	0	0	0
B. confusus Schenk	3	1	0	0	2
B. consobrinus Dalbom	11	0	0	0	0
B. distinguendus Morawitz	0	3	1	2	2
B. hortorum Linnaeus	1	1	1	1	2
B. lapsus Morawitz	0	1	0	1	0
B. lucorum Linneaus	83	23	38	12	7
B. muscorum Linneaus	0	5	3	0	0
B. pascuorum Scopoli	14	1	2	12	0
B. ruderarius Müller	0	2	1	0	0
B. semenoviellus Skorikov	0	0	0	1	0
B. serrisquama Kirby	0	19	0	0	1
B. sichellii Radoszkowski	13	1	3	7	3
B. soroensis Fabricius	6	8	13	29	52
B. sporadicus Nylander	4	0	0	0	0
B. subterraneus Linneaus	0	5	2	10	12
B. veteranus Fabricius	0	28	60	6	45
P. bohemicus Seidl	2	2	0	1	0
P. campestris Panzer	1	0	0	1	8
P. quadricolor Lepeletier	4	0	2	26	8
P. rupestris Fabricius	16	2	0	0	6
(B) *Bombus* or *Psythirus* species	B1	B2	B3	B4	B5
B. confusus Schenk	0	0	4	6	0
B. consobrinus Dalbom	2	0	0	1	0
B. distinguendus Morawitz	17	5	2	11	4
B. hortorum Linnaeus	2	0	0	2	1
B. hypnorum Linnaeus	7	1	1	0	0
B. lapsus Morawitz	0	7	4	5	0
B. lucorum Linneaus	4	6	4	17	15
B. muscorum Linneaus	1	0	0	0	0
B. pascuorum Scopoli	5	0	3	0	0
B. ruderarius Müller	0	0	0	1	0
B. semenoviellus Skorikov	1	1	0	0	0
B. serrisquama Kirby	0	7	3	15	6
B. sichellii Radoszkowski	0	3	4	13	3
B. soroensis Fabricius	2	10	3	17	8
B. subterraneus Linneaus	6	9	9	34	6
B. veteranus Fabricius	88	77	75	50	65
P. bohemicus Seidl	0	0	0	1	0
P. campestris Panzer	1	0	0	0	0
P. quadricolor Lepeletier	0	1	1	0	0
P. rupestris Fabricius	0	1	0	0	0
(C) *Bombus* or *Psythirus* species	O1	O2	O3	O4	O5
B. cryptarum Linneaus	4	0	5	0	1
B. humilis Illiger	0	13	2	0	0
B. lapidarius Linneaus	3	20	5	17	0
B. lucorum Linneaus	8	2	5	3	1

(continued)

B. pratorum Linneaus	0	3	1	1	18
B. pascuorum Scopoli	0	0	0	0	1
B. ruderarius Müller	0	1	0	1	0
B. subterraneus Linneaus	1	0	0	0	0
B. sylvarum Linneaus	1	14	0	0	0
B. terrestris Seidl	38	3	30	4	4
P. campestris Panzer	0	0	0	1	7
P. rupestris Fabricius	1	1	0	23	29

(D) *Bombus* or *Psythirus* species	KA	GS
B. consobrinus Dalbom	7	35
B. hortorum Linnaeus	0	2
B. hypnorum Linnaeus	8	12
B. lucorum Linneaus	167	73
B. pascuorum Scopoli	153	80
B. pratorum Linneaus	1	3
B. saltuarius Skorikov	23	18
B. schrenki Morawitz	82	45
B. sporadicus Nylander	1	6
B. sichelii Radoszkowski	0	3

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                          ....|....|....|....|....|....|....|....|
N. bombi (B. lapidarius)  GACGTAGACGCTATTCCCTAAGATTAACCCATGCATGTTT
N. bombi (B. terrestris)  GACGTAGACGCTATTCCCTAAGATTAACCCATGCATGTTT
N. bombi WS1              GACGTAGACGCTATTCCCTAAGATTAACCCATGCATGTTT
N. bombi WS2              GACGTAGACGCTATTCCCTAAGATTAACCCATGCATGTTT
N. apis                   GACGTAGACGCTATTCCCTAAGATTAACCCATGCATGTCT
N. ceranae                GACGTAGACGCTATTCCCTAAGATTAACCCATGCATGTTT

                                 50         60         70         80
                          ....|....|....|....|....|....|....|....|
N. bombi (B. lapidarius)  TTGAAGATT– ATTATCTGAAAAATGGACTGCTCAGTAATA
N. bombi (B. terrestris)  TTGAAGATT– ATTATCTGAAAAATGGACTGCTCAGTAATA
N. bombi WS1              TTGAAGATT– ATTATCTGAAAAATGGACTGCTCAGTAATA
N .bombi WS2              TTGAAGATTTATTATCTGAAAAATGGACTGCTCAGTAATA
N. apis                   TTGACGTACTATGTACTGAAAGATGGACTGCTCAGTAATA
N. ceranae                TTGA––––––––CATTTGAAAAATGGACTGCTCAGTAATA

                                 90        100        110        120
                          ....|....|....|....|....|....|....|....|
N. bombi (B. lapidarius)  CTCACTTTATTTTATGTGCACCGCAGAT- AACTACGTTAA
N. bombi (B. terrestris)  CTCACTTTATTTTATGTGCACCGCAGAT- AACTACGTTAA
N. bombi WS1              CTCACTTTATTTTATGTGCACCGCAGAT- AACTACGTTAA
N. bombi WS2              CTCACTTTATTTTATGTATACAGTAGAT- AACTACGTTAA
N. apis                   CTCACTTTATTTGATGTACATTATACAT- AACTACGTTAA
N. ceranae                CTCACTTTATTTTATGTAAATTT TTAATTAACTACGTTAA

                                130        140        150        160
                          ....|....|....|....|....|....|....|....|
N. bombi (B. lapidarius)  AGTGTAGATAACATGTGTACAGTAAGAGTGAGACCTATCA
N. bombi (B. terrestris)  AGTGTAGATAACATGTGTACAGTAAGAGTGAGACCTATCA
N. bombi WS1              AGTGTAGATAACATGTGTACAGTAAGAGTGAGACCTATCA
N. bombi WS2              AGTGTAGATAACATGTATACAGTAAGAGTGAGACCTATCA
N. apis                   AGTGTAGCTAACATATGTACAGTAAGAGTGAGACCTATCA
N. ceranae                AGTGTAGATAAGATGTTTACAGTAAGAGTGAGACCTATCA

                                170        180
                          ....|....|....|....|
N. bombi (B. lapidarius)  GCTAGTTGTTAGGGTAATGG
N. bombi (B. terrestris)  GCTAGTTGTTAGGGTAATGG
N. bombi WS1              GCTAGTTGTTAGGGTAATGG
N. bombi WS2              GCTAGTTGTTAAGGTAATGG
N. apis                   GCTAGTTGTTAAGGTAATGG
N. ceranae                GCTAGTTGTTAAGGTAATGG
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Fig. 3. Alignment of the partial nucleotide sequences of the newly discovered SSU rRNA (*Nosema bombi* WS2) of *Nosema bombi* isolated from bumblebee samples taken in Kouznetskiy Alatau (KA) and Gornaya Shoria (GS) in Russia with similar sequences of *N. bombi* (from *Bombus lapidarius* and *terrestris*), *N. apis* and *N. ceranae* deposited in GenBank.

es were identical to each other and contained seven nucleotide substitutions in comparison with the original *N. bombi* SSU rRNA sequence (Fig. 2). A Blast search did not reveal the presence of the newly identified sequence in the databases. This sequence, named *N. bombi* WS2 (West Siberia), was found in all investigated species from Gornaya Shoria and Kouznetskiy Alatau. It is necessary to note that the original variant of *N. bombi* was also present in *B. lucorum* from the same populations. The *N. bombi WS2* sequence was not found in *Bombus* species from the Belovo and Guryevsk populations (Fig. 3).

4. DISCUSSION

The three gradients showed pollution levels that corresponded with their smelting profile. The gradients near the two Zn smelters (Olkusz and Belovo) contained high levels of both Zn and Cd contamination in the soil, while the metal smelter in Guryevsk had a relatively lower pollution level for all tested pollutants. The distribution of pollution near the smelters was similar, besides site G5, where an exceptionally high level of Zn pollution was detected (552.14mg kg^{-1}). This is probably a sampling artefact caused by some high point pollution at this site, especially since this value is not followed by a higher Cd level, which is a Zn smelting by-product and should show a similar change in levels as Zn.

Generally, contamination at the G and B sites were lower than at the O site. Nevertheless both Pb and Cd pollution levels were higher than the background levels measured at the Stations of Complex Background Monitoring in the Russian Federation (UN Env. Prog. Chemical Branch DTIE, Review of scientific information on cadmium, Review of scientific information on lead, 2008). At sites near the Belovo and Olkusz smelters, all three metals exceeded the maximum level permitted for this type of soil according to Polish standards (Official Journal of Polish Laws 165/1359 Act of soil standards and quality, 2002. 09. 02.).

Analysis of changes in the concentration of Zn in soil did not have an effect on the concentration of this metal in the bodies of the collected bumblebees. Zn is an essential trace element and its level can be partially controlled through methallotionein reserves in the organism, which can explain the lack of a correlation between external and internal concentrations of this metal. Nevertheless, such control mechanisms are costly (Sibly and Calow 1989). Maintaining physiological levels of Zn in the organism, both at excessive and inadequate levels, requires the activation of various regulatory mechanisms. In some species it is deposited in various parts of the body in the form of inactive molecules, in others surplus Zn can be actively excreted, e.g. in faeces. These regulatory mechanisms allow for relative control over Zn levels.

On the contrary to Zn, concentrations of both Pb and Cd showed a clear correlation between levels found in soil and in bumblebee bodies. Both elements are toxic for organisms (besides diatoms, which use Cd instead of Zn in carbonic anhydrase: Lane and Morel 2000) and impair the functioning of various enzymes. Moroń *et al.* (2010) showed that pollution with Zn, Pb and Cd decreased the number of offspring in solitary red mason bees (*Osmia rufa*), especially male offspring, in a gradient near the Olkusz zinc smelter. Bumblebees collected on pollution gradients do not seem to have effective regulatory mechanisms for non-essential metals. The level of non-essential metals is clearly correlated with the level present in their environment. This would also suggest that bumblebees should be more sensitive to pollution, however this does not seem to be the case.

Generally, sites near the source of emission were less polluted than expected. Observed contamination levels can be expected to cause changes in the diversity of species, eliminating the more sensitive ones and thus favouring the less sensitive, but such differences were not observed. Pollution with the three tested metals did not change the observed diversity or species dominance. One possible explanation of this phenomenon is simply that such pollution levels do not significantly affect the observed species. Bumblebees are widely distributed in various environments, probably because they are tolerant to changes in their surroundings and low levels of contamination. One of the factors that can facilitate higher tolerance may result from their social structure: a hierarchy in the nest protects reproducing individuals (queens) from pollution, therefore allowing the colony to reproduce. This phenomenon was already described in ant colonies, in which individuals had lower levels of pollutant in their bodies' concomitant with higher positions in the nest hierarchy (Maavara *et al.* 2007).

Is it really the case then that bumblebees are more tolerant to pollution with no side effects, or are there are other costs that we have not detected? We have no data on the effects of contamination at an individual level such as life-expectancy, number of offspring or the proportion of sexes in offspring, but we do have data on parasite levels for these populations. Parasite level should reversely correspond to the activity of the immune system, which should be lower in a stressful environment. Interestingly, parasite levels were also unchanged along the gradient and moreover, there was a tendency for bumblebees to harbour fewer parasites on pollution gradients than on the two distant control sites. There are two possible explanations for this unexpected result: one may involve the time of sampling. The samples were collected in different years, which may have affected the general infection levels. However, the KA control site was significantly more infected than the polluted site near the metal smelter (G1). This difference can be either the effect of different sampling time, especially since a significant difference was not observed between the other control site, GS, and the gradients. The other explanation involves the genetic analysis of the collected *Nosema* samples. It is possible that on the two separate control sites the new *N. bombi* subspecies (WS2) caused higher infection levels, due to its novelty, than the one present on the gradient and widely distributed in bumblebees in Europe and Asia. However, further studies are needed to clarify this unexpected difference between the gradients and the control site in Kouznetskiy Alatau.

In summary, we did not detect significant differences in species diversity along the pollution gradients, and in the light of our data we can consider bumblebees as "tough pollinators" able to function in a pollution-stressed environment with no visible changes in their species diversity and parasite levels.

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