SEM-EDX Analysis of Heavy Metals in Anal Papillae of Hydropsyche Angustipennis Larvae (Trichoptera, Insecta) as a Support for Water Quality Assessment

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SEM-EDX analysis of heavy metals in anal papillae of *Hydropsyche angustipennis* larvae (Trichoptera, Insecta) as a support for water quality assessment

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Abstract

Anal papillae of caddisflies are peripheral organs responsible for osmoregulation and detoxification. Investigation of morphological abnormalities in the anal papillae of *Hydropsyche angustipennis* enriched with using SEM-EDX analysis (scanning electron microscopy-energy dispersive X-ray analysis), was used to assess heavy metal pollution levels in urban streams receiving surface runoff. Heavy metal ions not previously detected in water and tissue samples were detected using SEM-EDX method. Morphological irregularities were most frequently observed in larvae from the most contaminated streams. Heavy metals were almost 10 times more concentrated in darkened papillae than in pale, normal-shaped papillae. The present study

confirms that SEM-EDX microscopy is an effective method as a support of standard heavy metal bioassays, especially if there is a necessity to detect trace elements in very low concentrations or incidental appearance of some ions in the water.

Key words: bioassay, bioindicator, contamination, morphological abnormalities, sublethal effects, caddisflies

1. *Introduction*

The assessment of pollution impact on aquatic ecosystems is usually based on both physical and chemical analysis of water quality and biomonitoring of living organisms. However, the emphasis in evaluation of the environment pollution of surface water has been recently shifted to monitoring of bioindicator species [1, 2]. Among traditionally used bio-indices, a majority are founded on taxonomic differentiation of aquatic invertebrates or multispecies assemblages' sensitivity in response to human-induced stressors [3]. Also morphological [4] or behavioural [5, 6] changes in comprehensively studied species, that are caused by modifications of environment quality, may turn out to be as exploitable as traditional biotic indices [7]. The using of individual taxons as one-species universal bioindicators is gaining more and more attention in assessment of aquatic pollution.

One of the main purposes of the current policy (e.g. Water Framework Directive (Directive 2000/60/EC) in EU) is the protection and improvement of aquatic environment in all types of surface waters. The protection programme also applies to highly modified, polluted urban streams, usually inhabited only by eurytopic species [8, 9]. The wide tolerance range of these organisms limits their usefulness in pollution assessment [10, 11]. On the other hand, such less sensitive species, resistant to substantial changes of water quality, may be used for detection of dangerous pollutants e.g. heavy metals, as non-destructive environmental bioindicators [12].

Evaluation of heavy metals pollution is often based on the assumption that metals can accumulate in organism tissues, which reflect the environment contamination [13, 14]. Such bioaccumulation-based methods may also effectively track elements, which are often in concentrations below detection limits [7]. However, the obtained results may not reflect the water quality conditions correctly, because organisms show diverse levels of assimilation, detoxification, metal tolerance and active absorption/removal of particular elements [14]. Discrepancies may appear when heavy metals are being excessively assimilated from sediment [15], adsorbed on the body surface [16] or accumulated in subsequent trophic levels [17]. Higher levels of heavy metals concentration are observed in early larval stages, in which the detoxification system is not fully developed. On the other hand, several larval stages in the life cycle result in lower levels of contamination [18].

Morphological abnormalities of bioindicator species offer another useful tool for pollution assessment, reflecting metal exposure conditions via element concentrations in organism tissues (*16*). Body deformations should be clearly visible, easy to compare with not contaminated individuals and irreversible even after ceasing the pollution, but simultaneously not lethal (therefore, they are often referred to as "sublethal effects") [19 – 21]. Depending on species, such changes may consist of irregularities e.g. in the head capsule structure, mouthparts, tracheal gills or anal papillae [3]. In case of caddisflies, morphological abnormalities are usually connected with changes in water chemistry and based on the analysis of tracheal gills and anal papillae structure [16, 22]. Larvae of Hydropsychidae seem to be a promising tool in biomonitoring of surface waters, especially for streams flowing through urbanized areas [7 – 9]. They are widely distributed and resistant to chemical and physical modifications of stream environments. The presence of heavy metals in water manifest itself in their accumulation in larvae tissues as well as in anal papillae abnormalities such as darkeninig and constricting [7, 16, 22]. It is suggested that anal papillae become darkened due to accumulation of heavy metals in the epithelium. The degree

of such morphological irregularities in response to water pollution changes gradually, which can be particularly informative [16].

In our previous studies, we have also observed suggested correlation between heavy metal contamination in water and tissue samples, and sublethal effects leading to morphological changes in anal papillae [7, 23]. However, due to small sizes of anal papillae (2-3 mm) it has not been tested for heavy metal accumulation so far. In the present study, our aim was to directly confirm that this particular organ (anal papillae) has an ability to accumulate heavy metals and depending on the level of contamination we will be able to record progressing changes in anal papillae. The method most often used for tissue analysis, such as AAS (Atomic Absorption Spectrometry) was not possible to apply in this research due to small sizes of the tested organ. Thus, we used SEM-EDX analysis, technique enabling qualitative identification of all trace elements in the tested sample, even such small sizes. By SEM-EDX microscopy analysis, we were also able to compare particular parts of anal papillae e.g. pale with darkened ones. We supplemented the analysis of particular elements presence with visual observations of anal papillae in search for possible morphological irregularities and further correlations. We aimed to prove that this organ can serve as an effective tool in water quality assessment, especially at the screening stage, as its state can be verified intravitally and it indicates the effect through accumulation of contaminants.

2.Results

2.1. Sublethal effects of anal papillae

Analysed individuals had various stages of irregularities. Subsequent darkening of anal papillae was observed as well as larvae with two out of four papillae darkened. Part of specimens had all papillae darkened partially – only at the top of papillae or black rings (circuited in black) around papillae (Fig. 2b, 3a). In larvae with all papillae darkened completely also structural deformation (rugosity/shrinking) of papillae was observed, which is the final mark of degradation (Fig. 2c).

Such changes were recorded in larvae from the L1-RC and O3-IC sampling sites. At the reference sites (B1-NS and G1-NS), in almost half of the individualsthe anal papilae were not even protruded outside the last abdominal segment (Fig. 5). Only a small percentage of larvae had partially (COLRAP +/-) or completely darkened (COLRAP) papillae (8-11 % and less than 10 %, respectively). An opposite situation was observed in samples from streams flowing through the city center and included in the sewage system, where most individuals had morphological abnormalities. In larvae collected from the Rivers Sokołówka and Olechówka (S1-IC and S2-NS, O1-RS and O2-IC, respectively) less than 30% of specimens had no changes in anal papillae, while this value for larve at the L1-RC sampling site amounted to only 2 %. Mean values of the observed abnormalities differed among sampling sites within each category: PALE ($F_{ANOVA(7,24)}$=3.7705 and p_{ANOVA}<0.0068), COLRAP +/- ($F_{ANOVA(7,24)}$=9.1353 and p_{ANOVA}<0.0000) and COLRAP ($F_{ANOVA(7,24)}$=2.2101 and p_{ANOVA}<0.0498) (Fig. 5). The values for individual categories in Fig. 5 did not sum up to 100%, as some of the larvae did not have any visible/exposed anal papillae..

2.2. Heavy metals in anal papillae

Analysis of heavy metal concetration in anal papillae revealed the presence of 11 elements. Summarised mean values of concentration of heavy metals in anal papilae cuticula did not exceed 10 % at any of sampled stations (Table 1). Heavy metal domination in anal papillae was presented in increasing order Fe>Mo>Mn≥Al>Cu>Pb≥Ni>Co≥As≥Ti≥V (Table 1). The lowest values for most elements were recorded for reference sites flowing beyond the city center (G1-NS and B1-NS). Iron reached maximum values (13%) in papillae from larvae collected at the S1-IC sampling site and dominated in the papillae of larvae from O2-IC and L1-RC (Table 1). Aluminium was detected in larvae papillae at all sampling sites (highest values L1-RC) and it was the only element recorded at reference site G1-NS, although sometimes below the accepted detection limit. Most of investigated elements, even nickel, were observed in anal papillae collected at the

sampling site localized near the railway siding O2-IC. Considering mean values of heavy metal concentrations in anal papillae, sampling sites differed significantly ($F_{ANOVA(7,51)}$=5.7109 and p_{ANOVA}<0.0001), which results from discrepancy among values of Fe, Mo, Mn and Al and total surface load of heavy metals (Table 1). In both pale and darkened papillae, similar elements were recorded, however, in different concentrations and dominance ($F_{ANOVA(2,56)}$=20.0931 and p_{ANOVA}<0.0000) (Fig. 6). For protruded, but not darkened papillae (PALE) mean values were ten times lower compared to completely darkened ones (COLRAP). In case of partially and totally darkened papillae, the order of leading heavy metals was similar and as follows: Fe>Mn>Mo>Al and Fe>Mo>Mn>Al, respectively. For PALE, the order was different: Al>Cu>Ni>Fe>Pb (Fig. 6).

3. ***Discussion***

Heavy metal concentration in hydropsychid larvae commonly corresponds to the contamination of water and sediment, regardless of whether the source of pollution was urbanization, industry [16, 24, 25] or agriculture [26]. *H. angustipennis*, like other representatives of this family [27, 28], are metallotolerant organisms and capable of accumulating heavy metals in the body tissues even if the elements‘ concentrations in the environment are below the detection level [7, 23]. Although there is usually a positive relationship between the presence of heavy metals in the water and the same elements accumulated in the body tissues, it may not always be true for all the elements detected. The presence of a particular heavy metal in the larval bodies may result from the rate of assimilation of the element and the removal efficiency when its concentration becomes toxic [18, 24, 29, 30], the duration of exposure to pollution [7, 23] and the feature of bottom sediments [31]. Exposed to pollution, organisms respond in various ways. For aquatic insect larvae, which possess anal papillae, the organ serves as a regulatory and supportive in detoxification. Their epithelium is covered with a structure of highly concentrated, specialized cells for osmoregulation via active and passive transport of ions [3, 32]. In these cells heavy metals can be bonded and precipitated by metallothioneins [33, 34]. Darkening and deformation

of anal papillae due to water contamination appear when heavy metal and metaloid concentrations in the environment exceed physiological ion exchange ability [16]. Morphological abnormalities in the anal papillae of *Hydropsyche* spp. in response to water pollution, also in contact with heavy metals, were observed under laboratory conditions [16, 22, 35] as well as in the field [7, 36]. Analysis of such deformities in natural conditions can be applied especially, when contamination persists for several days, regardless of its concentration [37], though even short-time high concentrations of heavy metals can cause morphological abnormalities [20]. The observed deformities of anal papillae progress gradually, dependent on the intensity of contamination, and are irreversible [19]. Aquatic invertebrates have other structures with a 'long memory' of water pollution as the epithelium of the middle intestine, the Malpighian tubules or adipose tissue [5] but only anal papillae, as an external organ, are possible to investigate intravitally. The appearance of visible changes in anal papillae of *H. angustipennis* larvae may be the first sign for further environmental investigation.

Physicochemical research conducted on streams within the Łódź city revealed the presence of various heavy metals in the water samples, Zn>Cu>Pb>Cd [7, 23, 38, 39], in the bottom sediments, Zn>Cu>Pb>Mn>Ni>Cr>Cd [40 – 43], and in the body tissues of aquatic insects, Fe>Zn>Pb>Cu>Mn>Ni>Cd>Cr [7, 23, 43]. In the present study, in anal papillae Al, Mo, Co, As, Ti and V were also recorded. None of these elements were detected in previously mentioned studies, thus it is difficult to assess if they were available in the water, might contribute by food uptake, are the result of omitting these elements at the stage of setting up the analysis device or of trace amounts not exceeding the detection threshold. However, in the mentioned studies different methodology was applied (AAS with flame atomization or non-flame atomization in a graphite tube atomizer). Taking into account that SEM-EDX is a qualitative method it is not possible to directly compare the results with quantitative methods such as AAS. Nevertheless, based on the obtained results, element dominance can be ordered, and this

dominance trend can be compared. The results are usually presented as a percentage of a given element in the examined tissue, which in the case of anal papillae should be understood as the dominance of the occurrence in the environment [44], which a larva was trying to neutralize in the body [33]. Some elements, even in very low concentrations, may be toxic to aquatic organisms, and therefore their detection is important from the monitoring point of view. This may be the case of the aluminum, arsenic and vanadium, which even in a low concentration in water, can be toxic and may contribute to various types of abnormalities in aquatic organisms [37].

SEM-EDX microscopy confirmed that darkened and deformed papillae had higher concentrations of heavy metals than pale one. This explains why the sub-lethal effects observed in these organs are more common in the contaminated waters [7]. SEM-EDX method proved to be a valuable support in environmental studies, especially the one, in which caddisfly larvae *H. angustipennis* meet most of expectations and can serve as a heavy metal bioindicator in urban streams. Using the representative of the same species, at the same stage of development, which has been exposed for a long time to contaminated water, the possible discrepancies of bioaccumulation [7, 45, 46] and underestimation related to increased temporary metallotolerance [47, 48] will not have such a significant impact on the objectivity of water quality assessment. Although SEM-EDX does not give quantitative results, it allows to indicate to which heavy metals and metalloids the body has been exposed. In anal papillae, heavy metals are accumulated to inactive form, while analyzing these elements in the tissues is burdened by their active management in the body. The SEM-EDX method was confirmed to be valuable also for various freshwater [49, 50], marine [51] and terrestrial insects [52]. It seems that this method could be particularly useful in the screening study, in which it enables to identify all the trace elements present in the sample and indicate the direction for further detailed studies. The SEM-EDX analysis could also indicate, point short-term discharges of sewage, which cannot always be

captured by taking a water sample for analysis, as after ceasing the pollution accumulated elements will be possible to trace in detoxification organs.

4. ***Materials and methods***

4.1. Study area

The study was conducted in streams located in the city of Łódź (central Poland). In contrast to most other large cities, Łódź is not situated on a single large river, but has a channel network consisting of almost 30 small streams. Sampling sites were established so that they do not differ in terms of basic environmental parameters, but only in the degree of human-mediated stream bed modification and the level of water pollution (Fig. 1). Detailed characteristics of the sampling sites were included in Tszydel et al. [7]. Seven out of the eight sampling sites were located within the city (on the rivers Bzura, Sokołówka, Łódka and Olechówka). Two reference sites were assigned for the river network of the Łódź agglomeration, one located on the outskirts of the Łódź city, in the Bzura River (B1-NS), and the other one in the Grabia River running outside the urban area (G1-NS) (Fig. 1). Alterations of streambeds as well as potential sources of pollution are shown in Fig. 1.

4.2. Heavy metal analysis in anal papillae

Samples were taken once a month from March till June 2011, just after winter thaws and spring heavy rains. At each sampling site 30 larvae individuals of *H. angustipennis* in the full-grown, fifth instar were taken (collected from stones and available objects immersed in water; hand sorted). Specimens were rinsed twice with deionized water in order to remove contamination from the body surface and stored in 70% ethanol. In the laboratory, before heavy metal evaluation, larvae were photographed (Nikon SMZ 1000 and OptaTech HD digital camera) for anal papillae morphological comparisons (Fig. 2a-c). In *H. angustipennis* there are always four anal papillae. In unpolluted streams, this organ is invisible, hidden in the last (9^{th}) abdomen segment [53]. In response to increasing contamination, papillae start to grow and begin to

protrude outside as white or pale finger-shaped structures (marked as PALE; Fig. 2a) to function more efficiently [32]. Larvae with partially darkened anal papilae were marked as COLRAP +/- (Fig. 2b) and those completely darkened as COLRAP (Fig. 2c). Presence of pale papillae, as well as its darkening and structural changes, were assigned as morphological abnormalities of anal papilae. For each category mean values of the percentage of larvae with irregularities compared to all collected specimens were indicated for particular sampling term and site. Only the most advanced/last larvae stage (5^{th} instar) was taken into the analysis. Larvae stages were identified based on our experience, however, in case of any doubts, we used the keys Edington and Hildrew [53] as well as Hildrew and Morgan [54], where particular species ranges of head capsule and other diagnostic features for each larval stadium are provided. In the temperate climate zone, 5^{th} instar stage is present in the water the longest and the larvae collected for the study were the last molting at least 3 months earlier. Thus, the process of the larval molting did not influence the assessment of darkening. After a visual observation and taking a photograph of the larvae, the last abdominal body segments were dissected for SEM-EDX analysis. We have randomly selected 10 individuals with visible anal papillae (at least one papillae). Samples were then placed in 2% glutaraldehyde and dehydrated in graded ethanol concentrations (30-96%) and acetone. In order to remove water from the samples and avoid deformation, critical point dryer (POLARON CPD), was used. For microscope examination, the samples were coated with carbon and gold in a vacuum sprayer (JEOL JEE 4C vacuum evaporator). Papillae were then mounted to the pins with double-sided adhesive carbon tape, keeping their position parallel to the pin surface. The approximate thickness of papillae at the analysis points is only 1 mm in diameter and thus it is not limiting the SEM-EDX analysis or causing analysis errors. Elemental analyses were carried out with FEI QUANTA 250 scanning electron microscope (FEI Company) equipped with energy-dispersive X-ray spectroscopy module (EDX) [55]. Measurements were carried out at three different measuring points (Fig. 3a), then the results were averaged for individual categories of

the sub-lethal effects. In case of papillae classified as COLRAP and COLRAP +/-, only darkened places were analyzed. The samples were analysed using always the same tilt angle – 0° (approx. 90° take-off angle) and with the accelerating voltage set to 30kV. We used 0.1% as the detection limit for the EDX analysis [56]. EDX detectors collected a spectrum for every pixel of the frame. The spectra were then processed into a set of element intensity maps and analysed using MultiSpec© [57], a multispectral image analysis software, in which a clustering algorithm is used to identify and quantify groups of mutually exclusive chemical compositions. EDX analysis is generally used for qualitative assessment of particular elements, however, the method can have wider application [56].

In our case, the system provided information on the qualitative chemical composition of a sample, showing the weight percentage and atomic percentage for each element of interest in a given point on the SEM image (Fig. 3b). For each point, three measurements were made and those from which a repetitive spectrum was obtained were implemented. The EDX spectra obtained for one of the point analyses are shown in Fig. 4. Before the EDX analysis of the biological material tested, the microscope operating conditions and microanalytical parameters were set. These included specifying microscopic parameters related to detector geometry and system calibration. Calibration was performed whenever the curve in the HPD (Halographic Peak Deconvolution) profile did not coincide with the spectrum profile for the main peaks. The calibration method uses spectral lines for AlK and CuK. To identify the spectrum, the HPD function was used to collect spectra and generate a theoretical spectrum for comparison with the resulting set of spectra. Only peaks in which the collected peaks were superimposed on the theoretical peaks were accepted (Fig. 4).

4.3. Data analysis

All analyses were performed with the use of STATISTICA software system version 10 [58]. Kolmogorov-Smirnov test with Lilliefors correction were used to test for normality. To

standardize and stabilize the distribution of variance the logarithmic transformation of variables ($\log_{10}$ (x+1)) was made [59, 60]. One-way ANOVA test (preceded by Levene's test) was performed in comparisons: between concentration of heavy metals in anal papillae as well as deformities of anal papillae. In all statistical analyses, the level of significance was equal to $p < 0.05$ [61].

The dataset generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Fig. 1 Study area and location of the sampling sites. Graphic symbols show the type of pollution being the main source of heavy metals in the studied rivers**.** Sampling sties were encoded as follows: the first letter of the code is the name of a stream (B – Bzura, S – Sokołówka, L – Łódka, O – Olechówka and G – Grabia), the next is a number of the station, the third mark means the degree of riverbed naturalness (N – natural, R – regulated, I -isolation by covering the bottom with concrete and/or bricks). The last letter indicated the relation of the river to the municipial sewage system (C- stream included in the sewage system, S – stream in areas of

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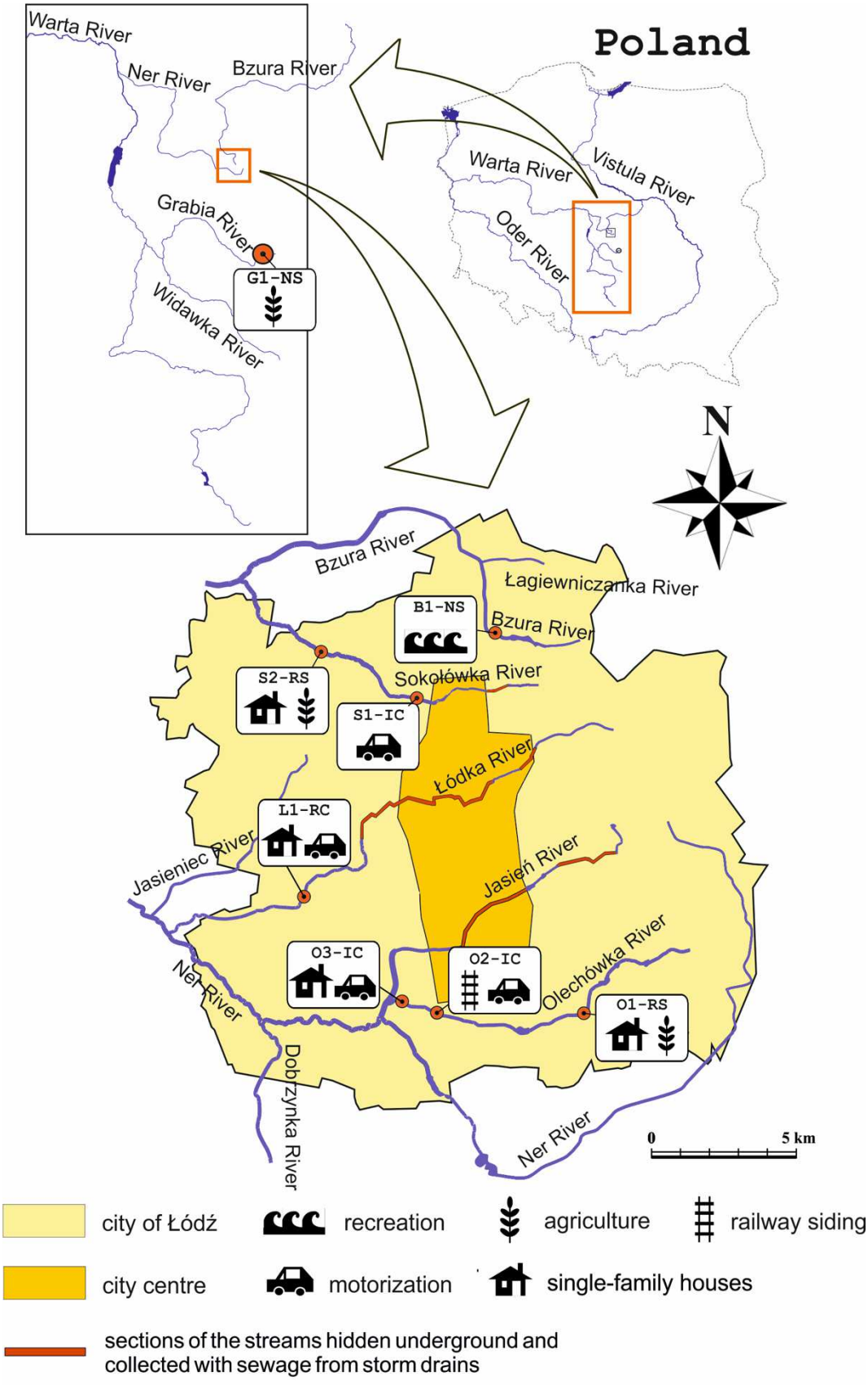

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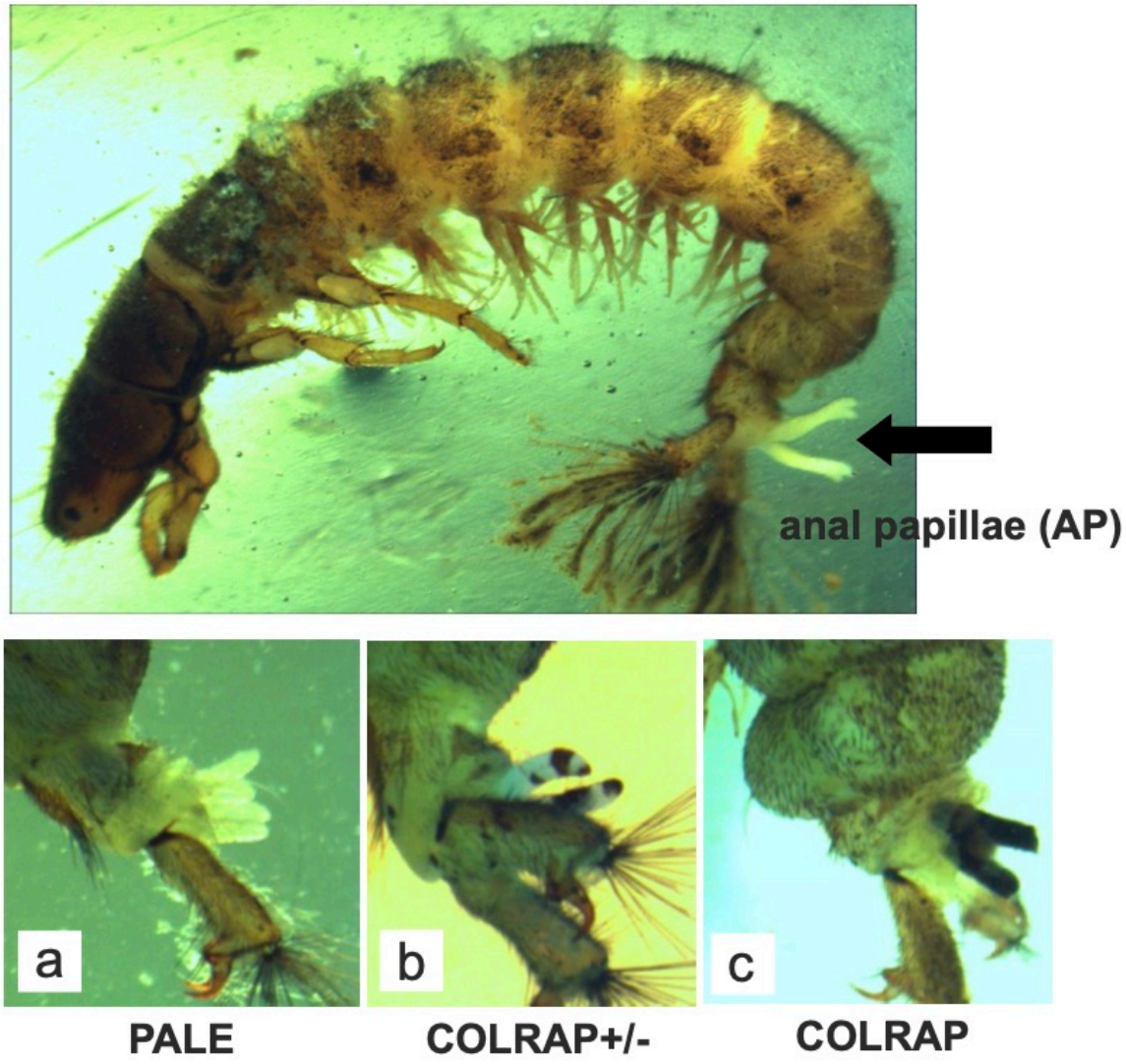

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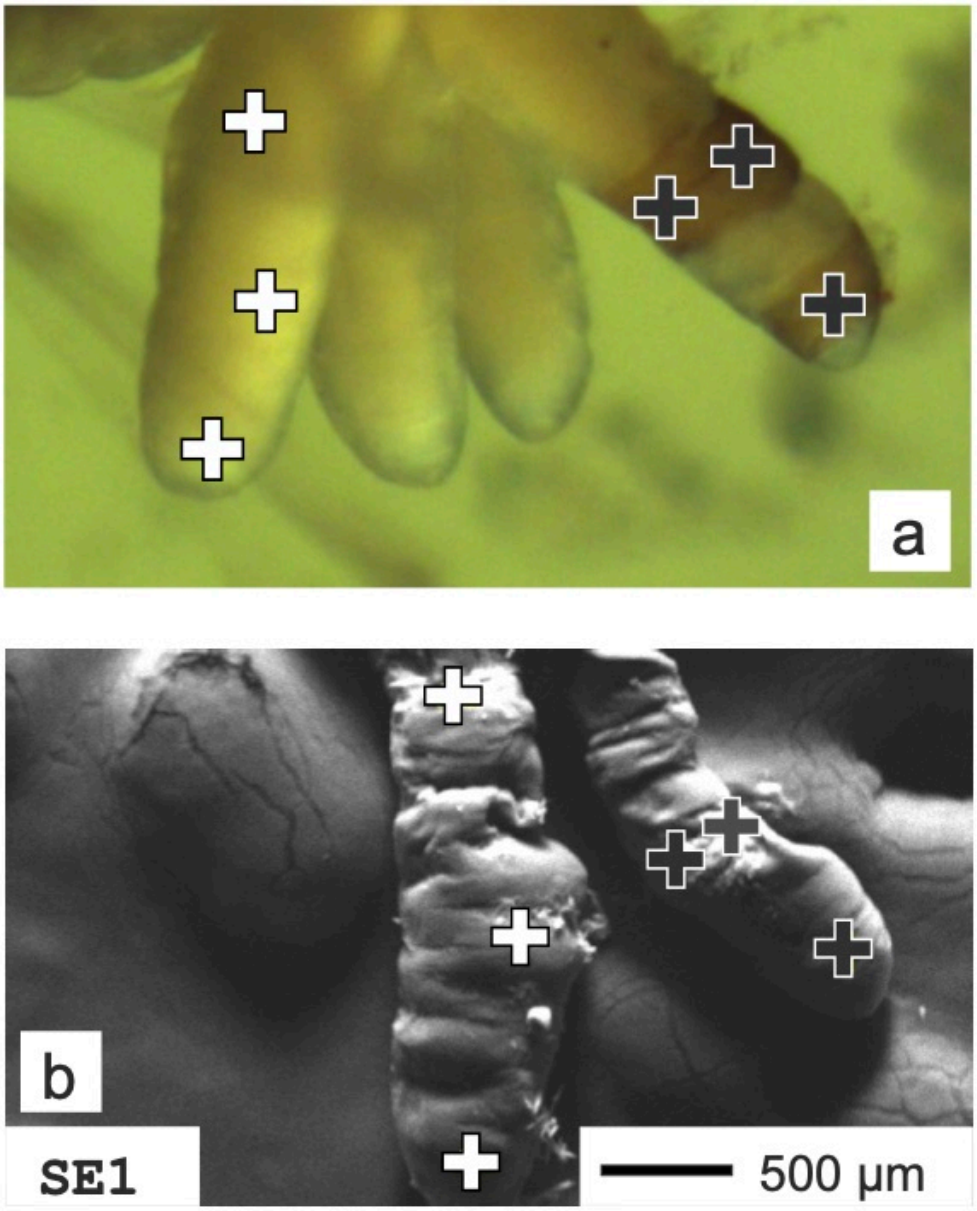

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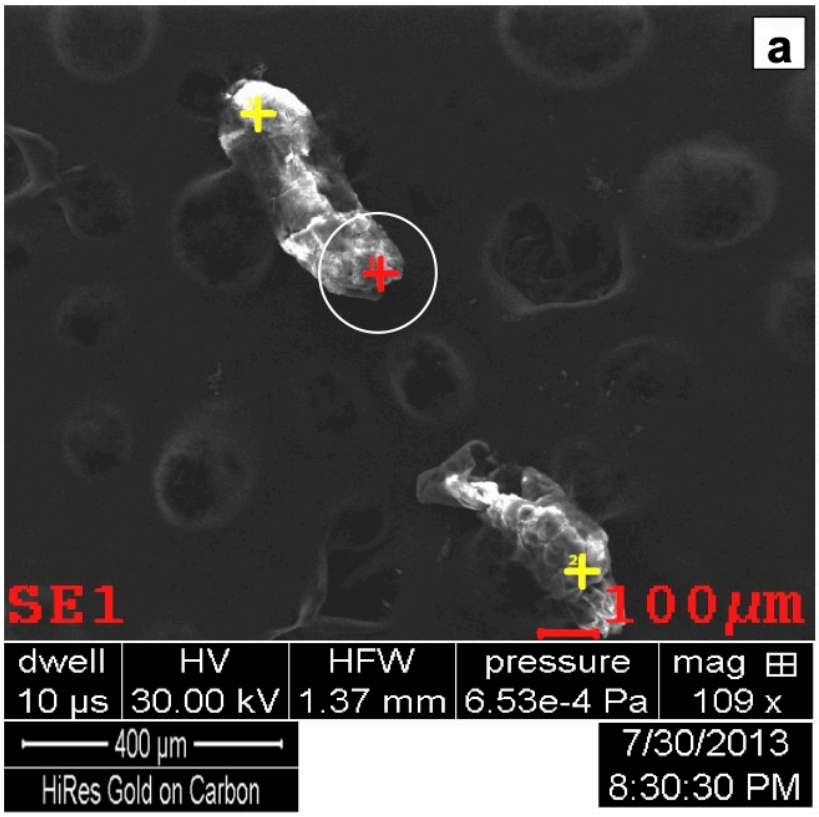

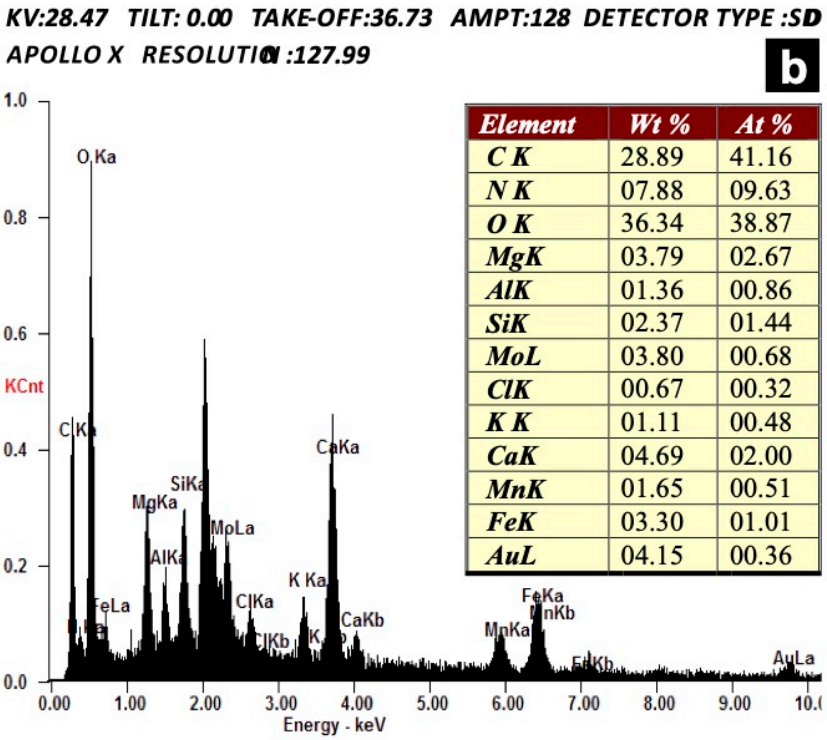

Element	Wt %	At %
C K	28.89	41.16
N K	07.88	09.63
O K	36.34	38.87
MgK	03.79	02.67
AlK	01.36	00.86
SiK	02.37	01.44
MoL	03.80	00.68
ClK	00.67	00.32
K K	01.11	00.48
CaK	04.69	02.00
MnK	01.65	00.51
FeK	03.30	01.01
AuL	04.15	00.36

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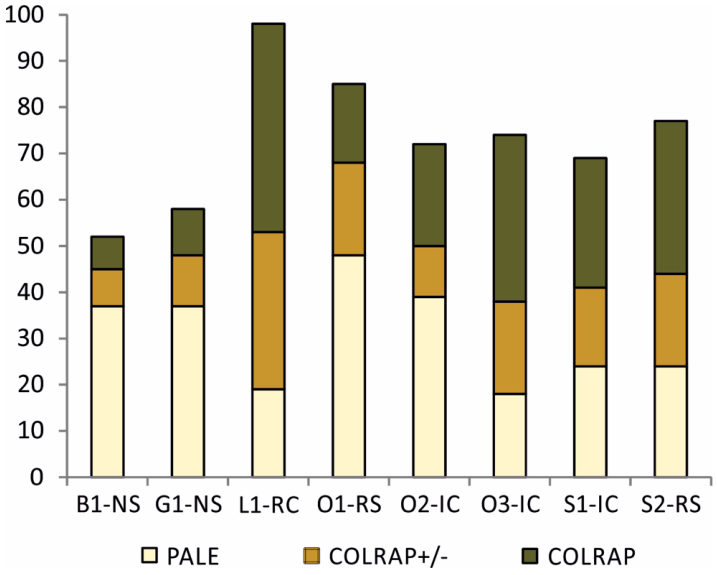

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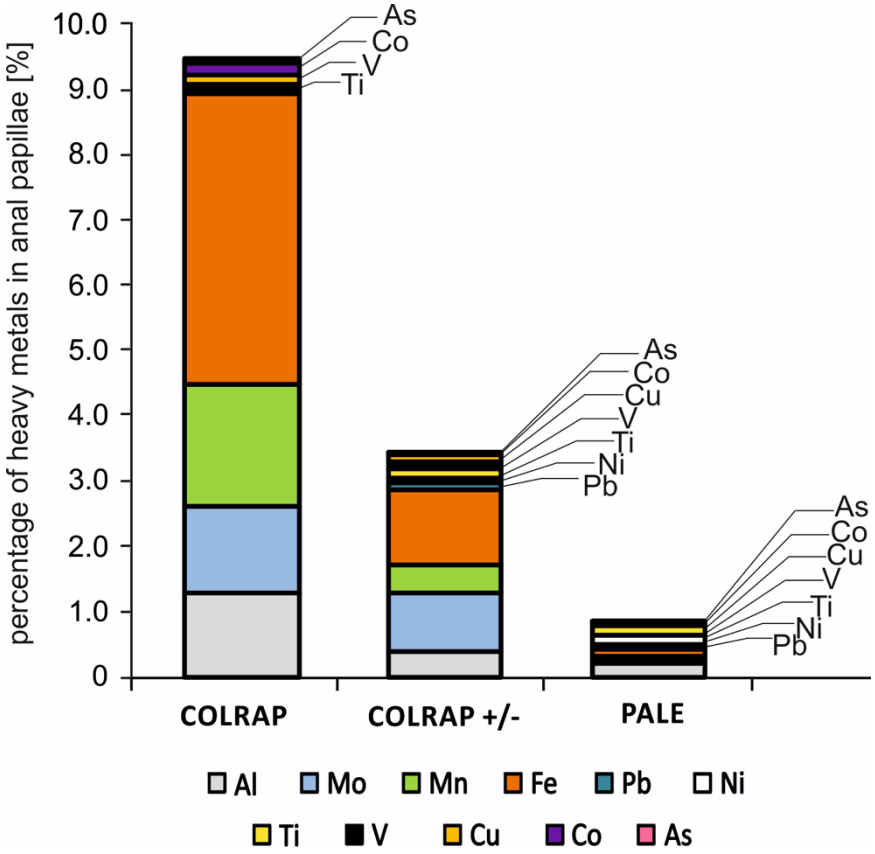

Table 1. Mean values of heavy metals accumulated in anal papillae. Mean values (min-max) of the percentage of heavy metals (*Wt* – weight ratio) in *H. angustipennis* anal papillae at the sampling sites. F values represent the results of one-way ANOVA test. Significant differences (*p*) among sampling sites were marked in bold.

sampling sites	Fe [%]	Mo [%]	Mn [%]	Al. [%]	Cu [%]	Pb [%]	Ni [%]	Co [%]	As [%]	Ti [%]	V [%]	Total [%]
B1-NS	0.06 (0-1.14)	0.17 (0-3.14)	0.14 (0-1.94)	0.09 (0-0.73)	0	0.14 (0-2.45)	0	0	0.05 (0-0.78)	0	0	0.64 (0-10.18)
G1-NS	0	0	0	0.04 (0-0.24)	0	0	0	0	0	0	0	0.04 (0-0.24)
L1-RC	3.21 (0-11.24)	2.21 (0-7.37)	2.86 (0-7.27)	1.00 (0-3.91)	0.37 (0-2.23)	0	0	0.13 (0-0.76)	0	0.04 (0-0.24)	0.03 (0-0.15)	9.84 (0-33.17)
O1-RS	0.09 (0-0.71)	0.21 (0-1.64)	0.13 (0-1.04)	0.05 (0-0.36)	0	0.06 (0-0.49)	0	0	0	0	0	0.53 (0-4.18)
O2-IC	1.92 (0-9.44)	0.30 (0-2.27)	0.04 (0-0.4)	0.78 (0-1.81)	0.70 (0-5.90)	0.36 (0-2.85)	0.51 (0-4.56)	0.04 (0-0.4)	0.04 (0-0.4)	0.04 (0-0.4)	0.04 (0-0.4)	4.79 (0-28.83)
O3-IC	1.60 (0-5.77)	4.51 (0-12.30)	0.13 (0-0.9)	0.55 (0.17-1.78)	0	0	0	0	0	0	0	6.79 (0.17-10.75)
S1-IC	6.62 (0-13.24)	0	0	1.40 (0-2.79)	0	0	0	0	0	0	0	8.01 (0-16.03)
S2-RS	0.70 (0-2.11)	0	1.11 (0-3.33)	0.16 (0-0.47)	0	0	0	0	0	0	0	1.97 (0-5.91)
F_{ANOVA}	3.3615	3.6353	6.1144	2.9982	0.8409	0.4979	0.7718	1.0660	0.2844	0.7827	0.7423	5.7109
p_{ANOVA}	**0.0050**	**0.0029**	**0.0000**	**0.0103**	0.5588	0.8316	0.6135	0.3986	0.9572	0.6048	0.6374	**0.0001**

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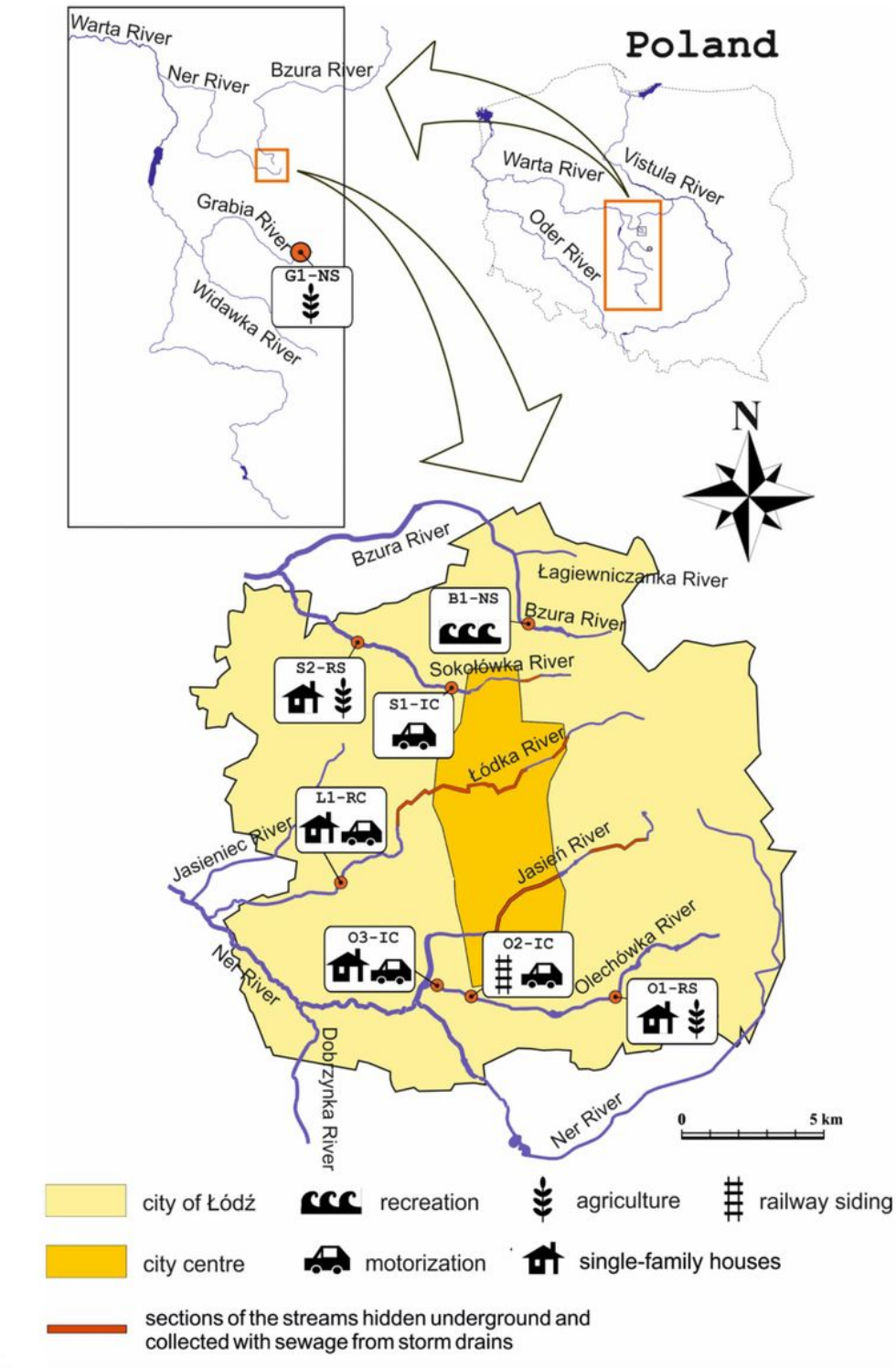
Figures



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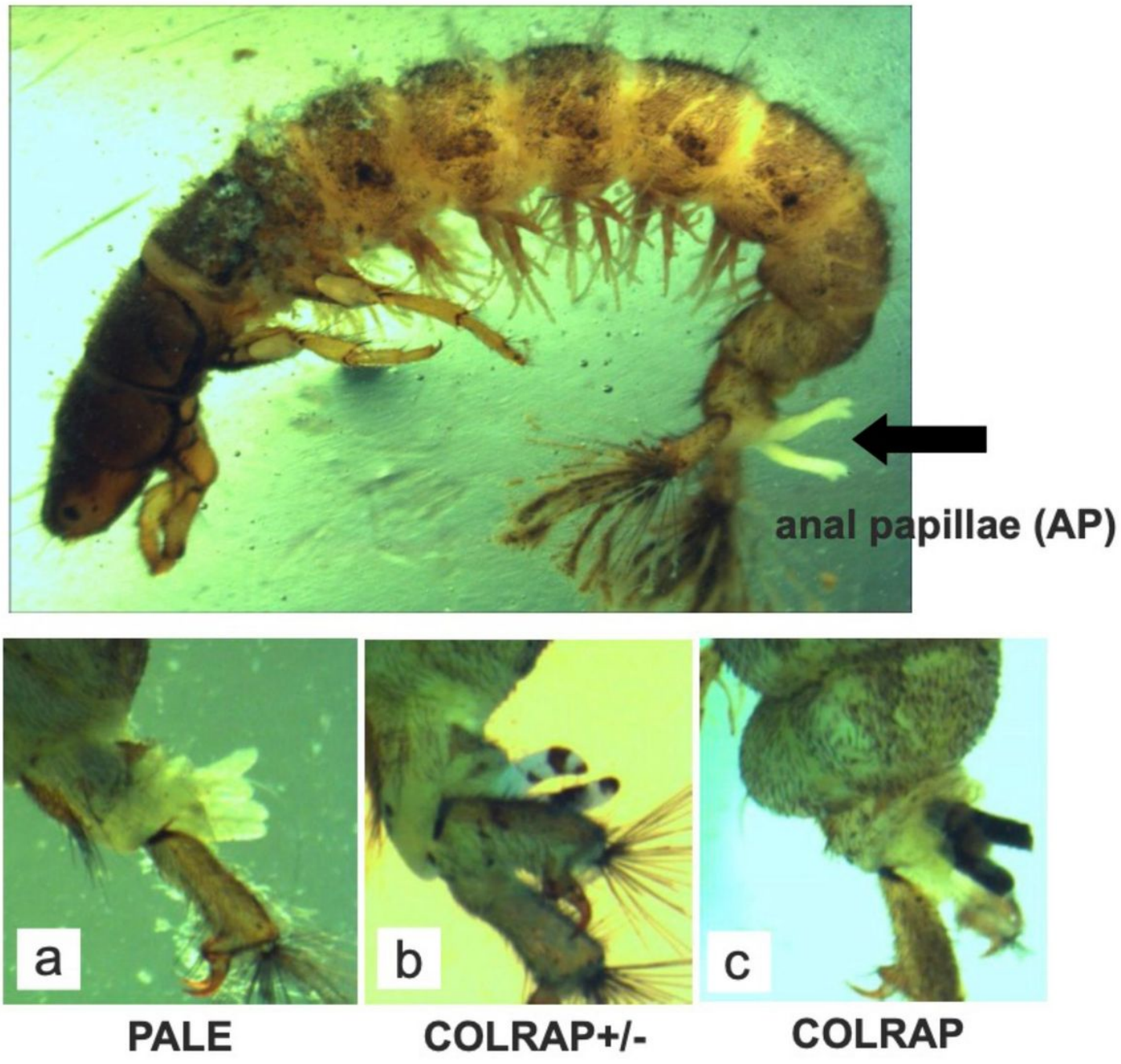

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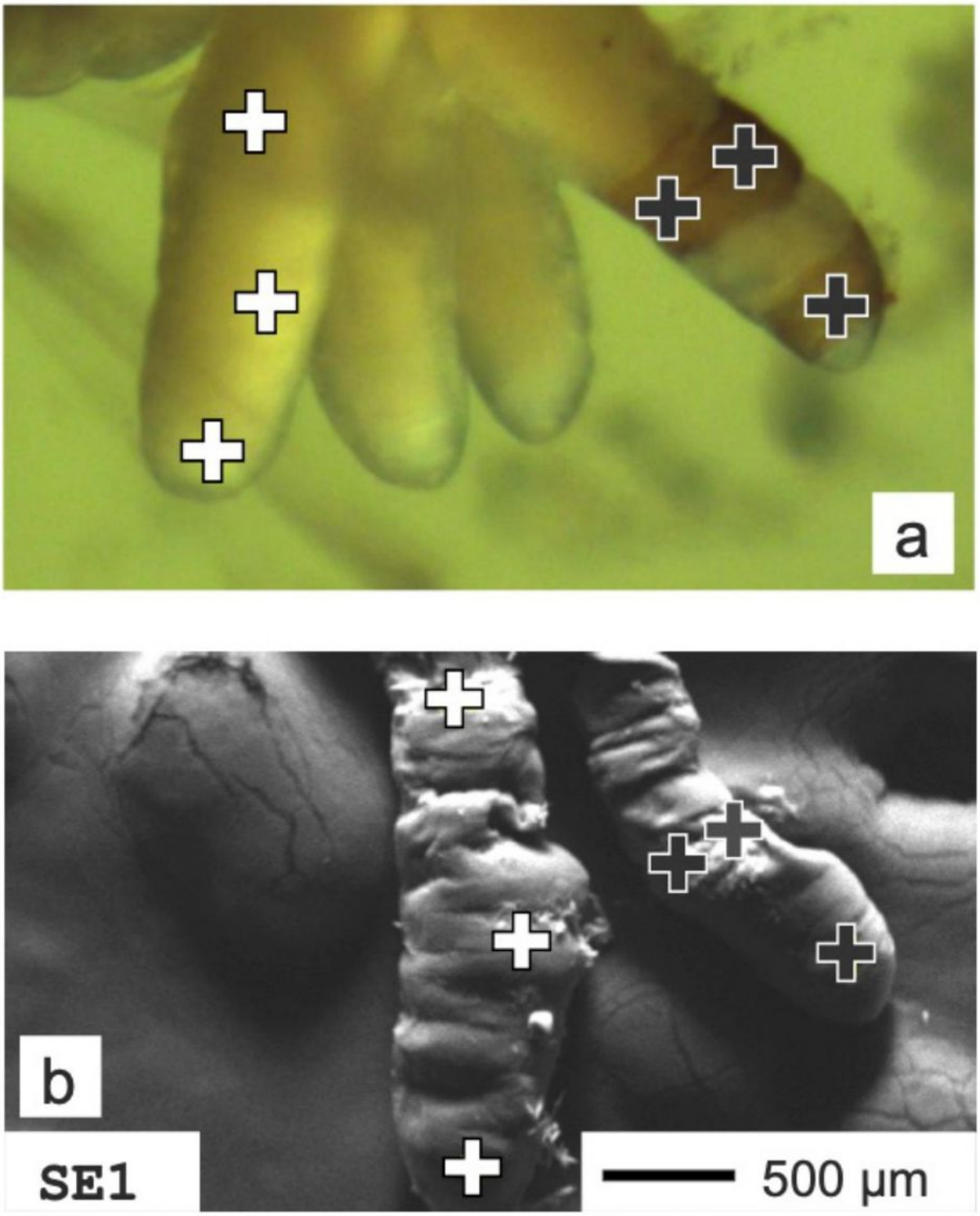

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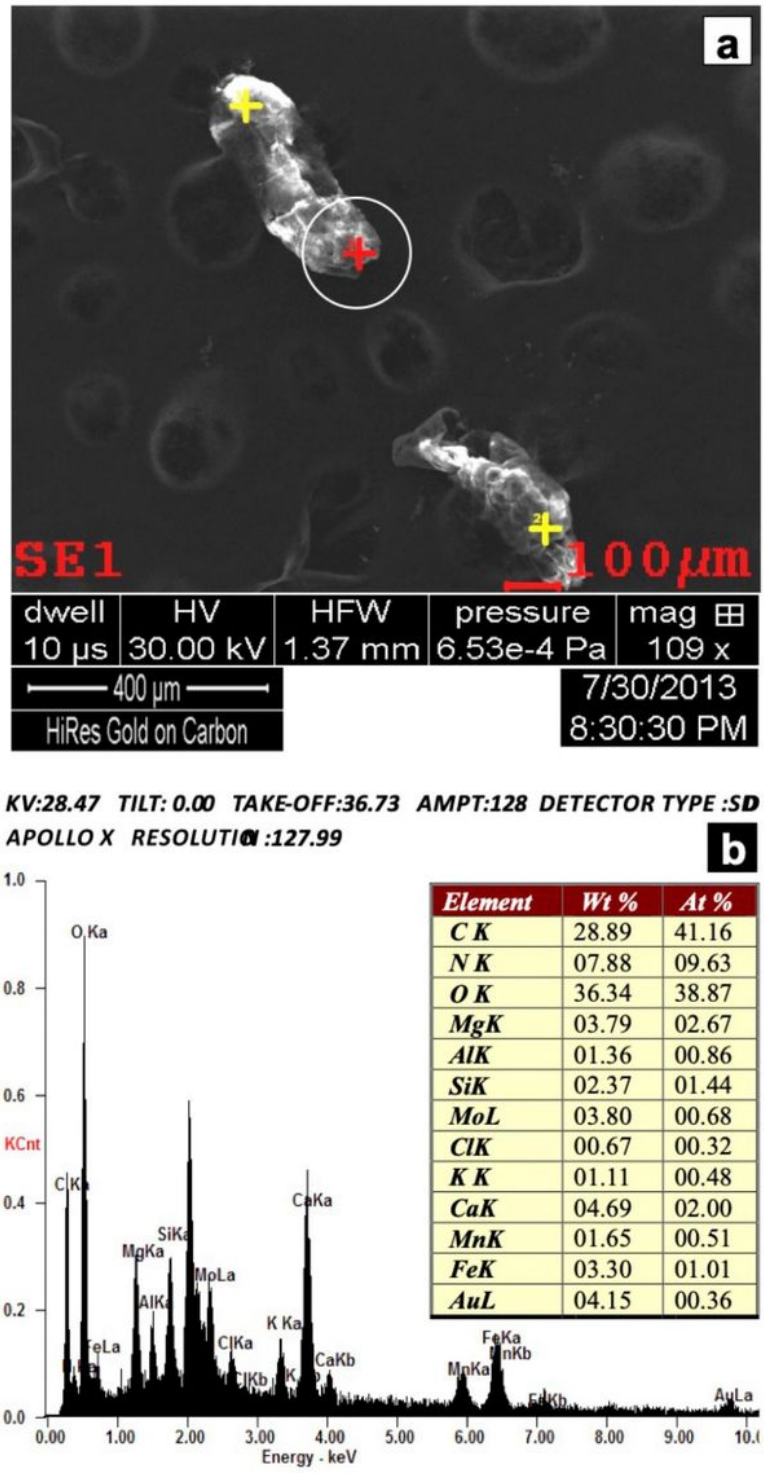

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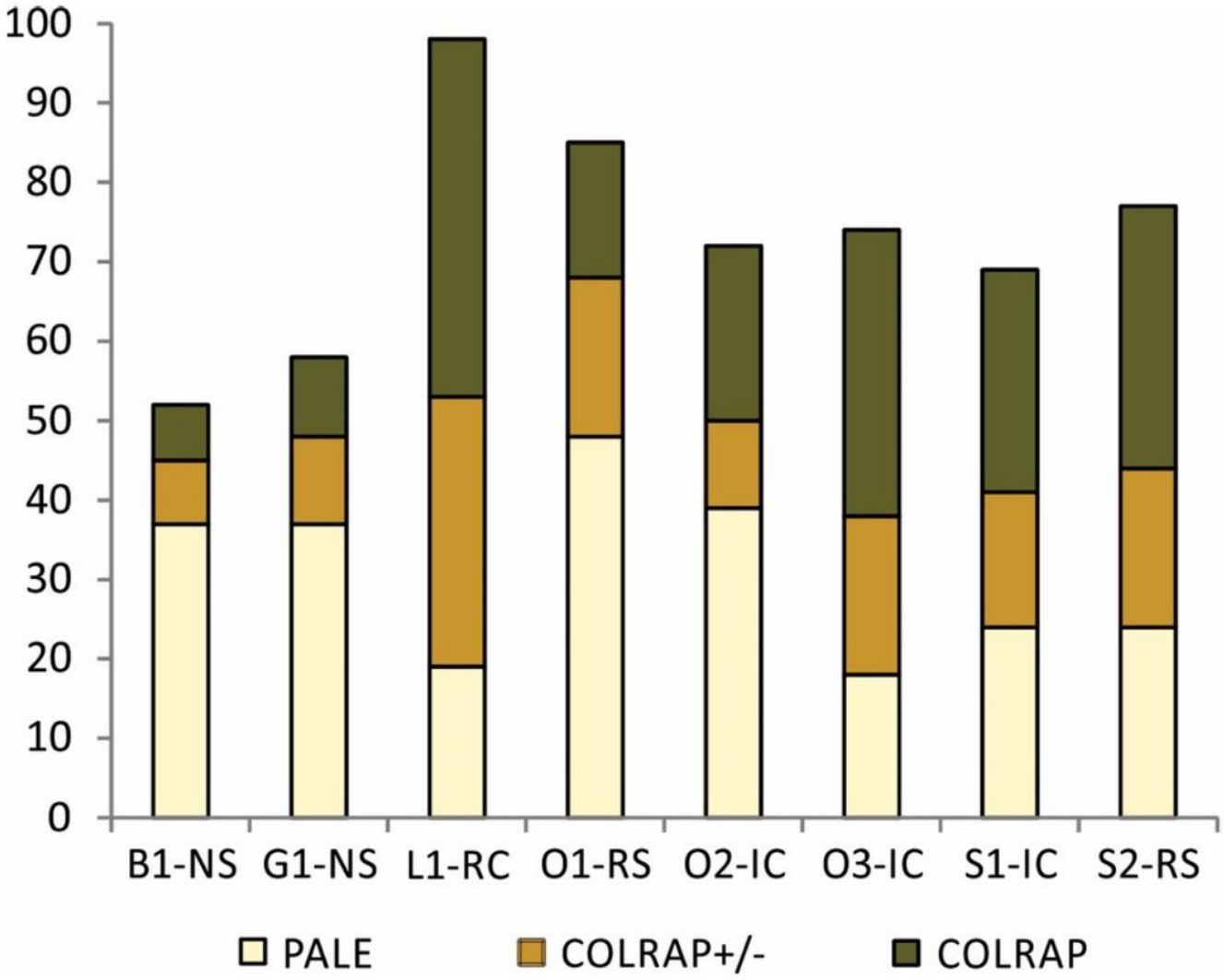

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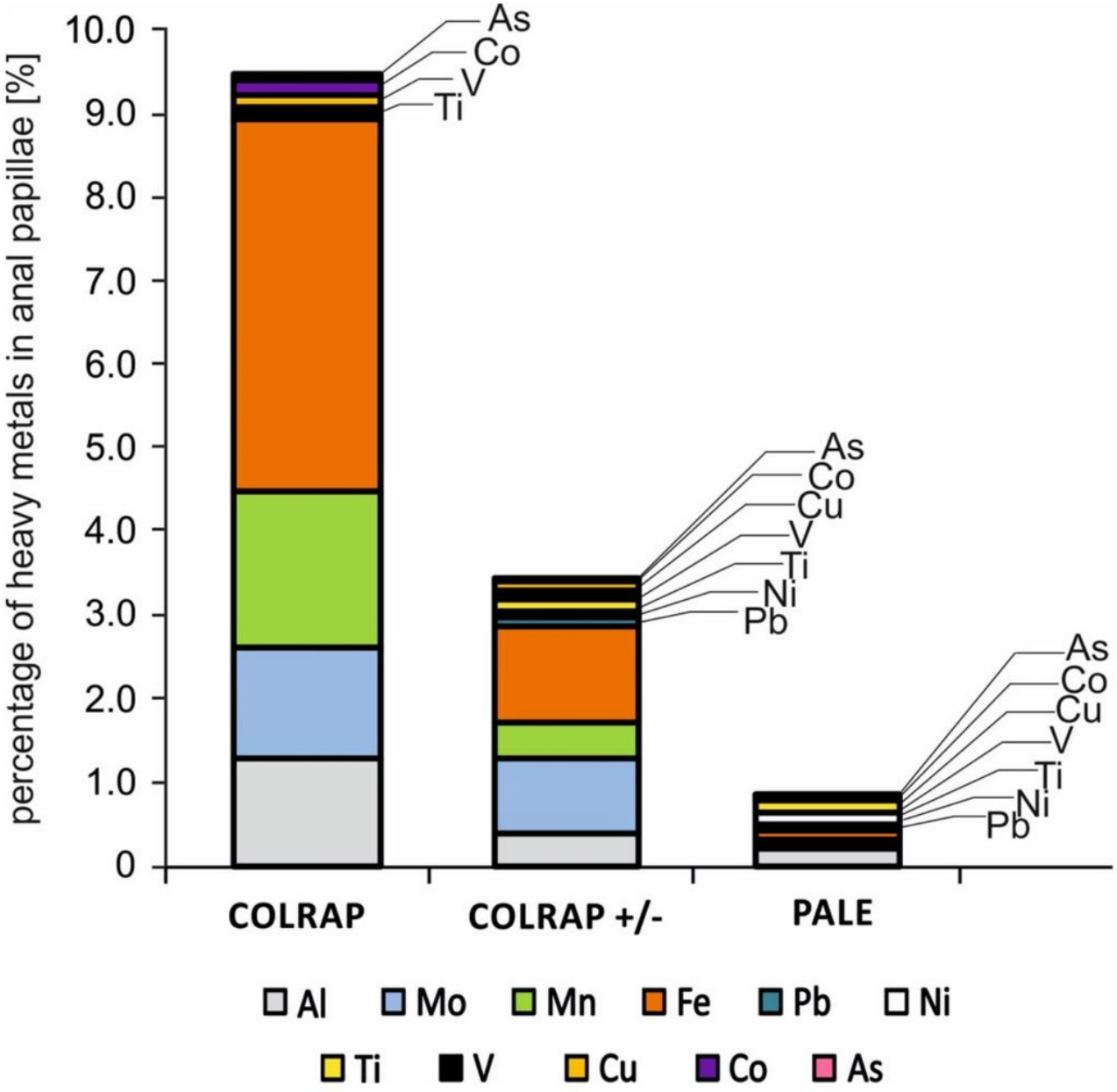

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