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# Biological and physicochemical properties of the nests of White Stork *Ciconia ciconia* reveal soil entirely formed, modified and maintained by birds

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ABSTRACT

The physiological and behavioural activities of animals have far-reaching impacts on the characteristics and functioning of soil. This includes vertebrates, which are capable of modifying the physicochemical and biochemical properties of soil. To date, however, no species is known to be responsible for the entire process of soil formation, modification and maintenance. Large-bodied birds build nests which they then use for several years or even decades. During nest construction or renovation, birds gather and transport to the nesting site organic and mineral matter that includes tree branches of various sizes, twigs, turf, straw and hay. Over time, during subsequent breeding events, adult birds supply further loads of organic matter to the nest, such as food remains, excrement, pellets, feathers, egg shells and other materials. Taking the White Stork *Ciconia ciconia* as an example, we have shown that the materials deposited in the nests of large-bodied birds gradually produce ornithogenic soils over the years, with distinguishable layers having different physicochemical characteristics and biochemical activities. The tested nesting substrate met the criteria for ornithogenic material; the layers had appropriate thickness and phosphorus pentoxide ($P_2O_5$) content. Results of the study indicates that the material contained in White Stork nests have the characteristics of Histosols. Moreover, such nests harbour assemblages of fungi and arthropods that contain species typical of soil mycobiota and fauna, respectively. This study is the first to describe a soil that is formed, modified and maintained entirely by vertebrates and is physically isolated from the ground. Our results highlight the fact that the nests of large birds are unique structures in ecosystems and provide a habitat for a rich and diverse assemblage of organisms.



## 1. Introduction

Animals have far-reaching impacts on the properties and functioning of soil (Menta, 2012). A wide range of invertebrates contribute extensively to the decomposition of organic matter, enabling the circulation of nutrients within the soil profile and facilitating their recirculation between soil and plants (Seastedt, 1984; Blouin et al., 2013). However, vertebrates also play an important part in disturbing the soil by digging and grubbing in it or by wallowing in mud and dust (Coppedge and Shaw, 2000; Bueno et al., 2013). Moreover, the physiological and behavioural activities of vertebrates make them capable of modifying the physical, chemical and biological properties of the soil (Mallen-Cooper et al., 2019; Bedernichek et al., 2020). Vertebrates may transport some soil fractions (Davies et al., 2019), disturb topsoils (Eldridge and James, 2009), dig tunnels and chambers in the deeper parts of the soil profile (Fleming et al., 2014), and deposit biological material on the soil surface, thereby influencing the formation of soil and how it functions (Zhu et al., 2011). Examples of soils enriched with zoogenic materials include those associated with the nesting colonies and roosting sites of birds and bats (Heine and Speier, 1989; Ferreira et al., 2007; Emsile et al., 2014) and the moulting sites of marine mammals (Panagis, 1985). The impact of vertebrates on soils is quite considerable and covers a spatial range between 2 m above (the building of structures) and 6 m below the ground (Platt et al., 2016).

Organisms capable of extensively modifying the environment are referred to as ecosystem engineers (Berke, 2010). While we are familiar with the impact of animals on soil properties and functioning, we know of no species that is responsible for the entire process of soil formation, modification and maintenance. At the same time, this means that no soil has been described so far, the creation and functioning of which is entirely dependent on the presence and activity of animals. A hitherto overlooked example of structures that potentially contain soil-like material are birds' nests. Large-bodied species like birds of prey and storks build massive structures which are used for several years or even decades (del Hoyo et al., 2020). During nest construction or renovation, birds collect and transport organic and mineral matter to the nesting site, which includes tree branches of various sizes, twigs, turf, straw and hay. Then, during multiple breeding events, adult birds provide further loads of organic materials to

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the nest, such as the remains of food items, excrement, pellets, feathers, eggshells and other organic material (including dead offspring). During subsequent breeding events, birds periodically enrich the nest with additional such materials (Hansell, 2000).

All across its range, the White Stork *Ciconia ciconia* is regarded as an iconic species: it nests mainly in close proximity to human settlements in rural and suburban areas of the Western Palearctic and is easily recognized by people. It is regarded as an indicator of sustainable or close-to-nature agriculture (Tryjanowski et al., 2006). The global population of the White Stork is estimated at 224000–247000 breeding pairs (BirdLife International, 2020), and Poland is home to the largest percentage (51700–53,900 breeding pairs) (Chylarecki et al., 2018). The White Stork's nest is one of the largest and heaviest structures built by birds worldwide (Cramp and Simmons, 1998; del Hoyo et al., 2020): it can reach over 1.8 m in height, a diameter of over 2.0 m and a weight exceeding 1000 kg (Schimkat et al., 2017). White Storks are long-lived birds and have a strong nest-bond and site fidelity, so they may use their nest for many years in succession (Bocheński and Jerzak, 2005).

Since the construction of the nests by large-bodied birds is costly in the sense of time and energy expenditure, they constitute a valuable environmental resource, which can also be used for breeding by other species in subsequent years. Several large-bodied species, mainly from the orders Accipitriformes and Falconiformes, but also large species of owls (Eagle Owl *Bubo bubo*, Ural Owl *Strix uralensis*, Great Grey Owl *Strix nebulosa*), Black Stork *Ciconia nigra*, Grey Heron *Ardea cinerea*, Common Raven *Corvus corax* or Egyptian geese *Alopochen aegyptiaca*, are known to re-use the nests of other species (Creutz, 1988; Cramp and Simmons, 1998; Sumasgutner et al., 2016; del Hoyo et al., 2020). Large nest constructions are also used as nesting sites by small birds, which breed among the twigs and branches. It is common for House Sparrow *Passer domesticus*, Tree Sparrow *Passer montanus* and Starling *Sturnus vulgaris* to breed within the structure of White Stork nests. But a large group of other birds may also use such sites on occasion: for example, Great Tit *Parus major*, Redstart *Phoenicurus phoenicurus*, Pied Wagtail *Motacilla alba* and Collared Dove *Streptopelia decaocto* have all been recorded in White Stork nests (Indykiewicz, 2006; Zbyryt et al., 2017). All these secondary users may add further biological material to the nest.

Ornithogenic material is a new diagnostic criterion of soil layers that was introduced in the second edition of *World Reference Base for Soil Resources* in 2006. Such material has traces of birds (bones, feathers) and contains at least 0.25% phosphorus pentoxide ($P_2O_5$) (in a 1% citric acid extract) (WRB, 2014). The definition of the term "ornithogenic" in soil science was associated with the soils occurring in colonies of large birds in the Antarctic, on the west coast of South America and to a lesser extent in the Arctic (Ligęza, 2010). Liguang et al. (2004) reported relic ornithogenic soils morphologically described as alternating layers of relict plant-rich tundra and sediments enriched with nutrients derived from penguin droppings. However, Ligęza et al. (2020) demonstrated that soils containing ornithogenic material may be found in the temperate climate zone. This gave rise to the idea that the material in the nests of White Storks might fulfil the criteria for soil. However, in the case of nests which are built above the ground, birds are not only responsible for enriching the pre-existing soil with biological material, thereby modifying its original properties. They are actually responsible for the entire process of soil formation, modification and maintenance, and all these aspects depend closely on the behavioural and physiological activity of individuals.

To determine the properties of the studied substrate, we included a set of procedures commonly used in the soil environment studies. Besides the basic physicochemical properties, the activity of enzymes involved in the carbon (C), nitrogen (N), phosphorus (P) and sulphur (S) cycle as well as the microbial biomass C and N were determined. The enzymatic activity reflects the activity of microorganisms involved in the transformation of substrates, especially carbon ones (Błońska et al., 2017). According to Peacock et al. (2001) the quantity and quality of organic matter is one of the most important factors affecting microbial biomass, which is connected with the biomass of active bacteria and fungi. These groups of organisms play an essential role in nutrient cycle in soil as are primarily involved in the decomposition of plant material, the lignocellulosic components of which are relatively recalcitrant to bacteria (Frąc et al., 2018). Additionally, fungi play an important role in the stabilization of soil organic matter and decomposition of residues (Domsch et al., 1980). Upper layers of soil constitute an environment for numerous invertebrates (Callaham Jr. et al., 2012; Menta, 2012). As some arthropods are typical soil-dwellers, their presence could confirm the soil-like nature of the substrate deposited in White Stork nests. As adult birds renovate their nest during multiple breeding events and periodically provide additional organic materials to the nest (Hansell, 2000), we expected that nests will have layers differentiated in their decomposition rate and substrate properties.

The aim of this paper was to diagnose the physicochemical and biochemical properties of the material deposited in White Stork nests. We assumed that the nests were unique structures characterized by a diversity of physicochemical properties similar to ornithogenic soil, but that would also contain assemblages of organisms typical of a soil environment. For the latter purpose, we identified the assemblages of fungi and macroarthropods in the nests. We predicted that large birds' nests would: 1) bear a resemblance to ornithogenic soil, 2) have distinguishable layers with different physicochemical properties and biochemical activity, and 3) contain macroarthropods and fungi with high affinities to the soil environment.

## 2. Material and methods

### *2.1. Study area and materials studied*

The study was carried out in north-eastern Poland, where an active conservation project of the White Stork population was implemented in 2016–2020 (LIFE15 NAT/PL/000728). It involved the relocation of nests from buildings to solitary poles with platforms, the restoration of old nests, and the reduction of nest mass (for detailed information about the LIFEciconiaPL project, see: http://www.ptop.org.pl/ ). All these activities made it possible to collect nest material. Given the restrictions of the formal project plan and for conservation reasons (the White Stork is a legally protected species in the vast majority of its range, including Poland), the number of samples was limited and differed, depending on the study protocols (the numbers of analyzed nests and collected samples are provided at the descriptions of each protocol).

### *2.2. Determination of physicochemical and biochemical parameters of nest material*

To describe the physicochemical and biochemical properties, five intact nests of unknown age were selected. Each nest was cut lengthwise through the middle to visualize the characteristics of the internal materials. Since such procedure leads to total destruction of the nest, sample size was limited due to conservation reasons (legal restric-

tions). The horizontal layers were distinguished and described on the basis of differences in morphology and material structure (Fig. 1). The depths of the nests ranged from 60 to 110 cm and the diameter from 100 to 120 cm (Fig. 1). Three separate layers were distinguishable between depths of 15 and 50 cm (Fig. 1). To determine the properties of the substrate, samples were taken from all the separated layers. In spring 2019, a 1 kg sample of material was collected from central part of each layer of each nest, placed in a plastic container, immediately transported to the laboratory and stored in the dark at 4 °C. Tree branches, twigs or any anthropogenic materials (plastics, fabrics) were removed from samples and material was sieved (mesh size = 2 mm). Prior to the analyses, samples from each nest were divided into two homogenized subsamples for determining (1) basic soil properties and (2) microbiological parameters.

The pH of the samples was measured potentiometrically in distilled water or KCl with substrate to solution ratio 1:5. Total N, organic C and S contents were measured using a LECO CNS True Mac Analyzer (Leco, St. Joseph, MI, USA). Additionally, the C/N ratio was calculated, which was used to determine the degree of organic matter decomposition in accordance with the Classification of Polish Forest Soils (2000). Base cations (BC = $Ca^{2+}$, $Mg^{2+}$, $K^{+}$, $Na^{+}$) were determined by inductively coupled plasma – optical emission spectrometry (ICP-OES) (iCAP 6500 DUO, Thermo Fisher Scientific, Cambridge, UK). The $P_2O_5$ content was determined in a 1% citric acid extract (Van Reeuwijk, 2002). The spectrophotometric analysis of the lignin content was carried out using 25% acetyl bromide dissolved in glacial acetic acid and 70% perchloric acid (Rodrigues et al., 1999; Antczak et al., 2013). Moisture (Mw expressed as % of weight), capillary water capacity (CWCw expressed as % of weight), and bulk density (Bd) were determined using 250 $cm^3$ Kopecky cylinders (Ostrowska et al., 1991). Samples of material with an intact structure (presence of branches and twigs) were collected using a hammering head.

To determine microbial biomass carbon (MBC) and nitrogen (MBN), 5 g of nest material was weighed and fumigated with $CHCl_3$ in an exsiccator for 24 h at 25 °C. The fumigated and non-fumigated samples were extracted with 0.5 M $K_2SO_4$, then passed through Whatman filters (Vance et al., 1987). The amounts of organic C and N in nest material were determined quantitatively using a Shimadzu Total Organic Carbon analyzer (Shimadzu, Japan) (Jenkinson and Powlson, 1976). Enzyme activities were determined using fluorogenically labelled substrates (Pritsch et al., 2004; Sanaullah et al., 2016). Six fluorogenic enzyme substrates based on 4-methylumbelliferone (MUB) were used: MUB-β-D-cellobioside for β-D-cellobiosidase (CB), MUB-β-D-xylopyranoside for xylanase (XYL), MUB-*N*-acetyl-β-D-glucosaminide for *N*-acetyl-β-D-glucosaminidase (NAG), MUB-β-D-glucopyranoside for β-glucosidase (BG), MUB-phosphate for phosphatase (pH) and MUB-sulphate potassium salt for arylsulphatase (SP) (Turner, 2010). We mixed 2.75 g of soil with 92 mL universal buffer (pH 6.0). The soil suspension was then pipetted into wells on a microwell plate containing the substrate and modified universal buffer. Fluorescence was measured by incubations of the soil suspension (for 1.5 h at 35 °C) in 96-well microplates (Puregrade, Germany) and the fluorescence determined immediately on a multidetection plate reader (SpectroMax), with excitation at 355 nm and emission at 460 nm wavelength.




**Fig. 1.** External view of a nest with a breeding pair of White Storks *Ciconia ciconia*; nest profiles showing the various layers (nest IDs are from 1 to 5, read from left to right; see Tables 1 and 2).

*2.3. Isolation and fungal identification*

To describe the abundance and diversity of fungal assemblages, two subsamples of nesting material were collected with sterile, disposable plastic gloves from the centre and margin (the layer, where there is a clear change in the density of the nesting material between the looser outer part and the strongly compressed inner part) of each nest (50 g per sample) at a depth of about 0.3–0.4 m. The samples were stored in sterile plastic containers for transportation to the laboratory and stored at 5 °C for 2 days until the fungal isolations were performed. A total of 22 subsamples from 11 nests were collected. Since the procedure of material collection leads to minor interference with the nest structure, we were able to increase the number of studied nests comparing to soil analyses described in the item 2.2. For the fungal isolations, both subsamples from each nest were mixed and homogenized under laboratory conditions and a 10 g sample from each nest was taken for further procedures.

The culturable fungi were isolated by soil dilutions. The soil sample was suspended in 90 ml of sterile distilled water and stirred vigorously for three minutes ($10^{-1}$ suspension), after which $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ serial dilutions were prepared. 0.1 ml portions of each dilution were added to the sterile plates (five replicates) containing Rose Bengal Agar medium (Martin, 1950) and 2% MEA medium [20 g malt extract (Biocorp Polska Sp. z o.o.), 20 g agar (Biocorp Polska Sp. z o.o.), 1 L sterile water and 50 mg $L^{-1}$ tetracycline (Polfa)]. The plates were then incubated at 22 °C for 1–4 weeks and observed daily for fungal growth. When necessary, the cultures were purified by transferring mycelium or spore masses from individual colonies to fresh 2% MEA medium. The purified cultures were grouped according to culture morphology using an Eclipse 50i microscope (Nikon) equipped with an Invenio 5S digital camera (DeltaPix) to capture photographic images and the COOLVIEW v. 1.6.0 software (Precoptic) that enabled taxonomically relevant structures to be measured.

Depending on the size of the morphological group, from one to nine isolates from each group were chosen for molecular identification. Morphological identification was confirmed by sequencing the internal transcribed spacers 1 and 2 (ITS1–5.8S-ITS2). For penicillium- and aspergillus-like fungi as well as some other relevant fungal species, sequences of the beta tubulin (TUB*2*) and the elongation factor 1-α (TEF*1-α*) gene regions were determined to enable more accurate identification (Supplementary materials, Table S1). Altogether, 132 isolates were selected for molecular identification, and these were deposited in the culture collection of the Department of Forest Ecosystems Protection, University of Agriculture, Kraków, Poland (Supplementary materials, Table S1).

DNA was extracted using the Genomic Mini AX Plant Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. The primers used were ITS 1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) for ITS1–5.8S-ITS2, Bt2a and Bt2b (Glass and Donaldson, 1995) for TUB*2*, and EF1 and EF2 (O'Donnell et al. 1998) or EF1–728 (Carbone and Kohn, 1999) and TEF1rev (Kullnig-Gradinger et al., 2002) for TEF*1-α*.

Gene fragments were amplified in a 25 μL reaction mixture containing 0.25 μL of Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland), 5 μL Phusion HF buffer (5×), 0.5 μL dNTPs (10 mM), 0.75 μL DMSO (100%) and 0.5 μL of each primer (25 μM). The gene regions were amplified under the following conditions: a denaturation step at 98 °C for 30 s followed by 35 cycles of 5 s at 98 °C, 10 s at 52–60 °C (depending on the optimal Tm of the primers and fungal species), 30 s at 72 °C and a final chain elongation step at 72 °C for 8 min in a LabCycler thermocycler (**SensoQuest Biomedical Electronics** GmbH, Germany). The amplified products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA) at the DNA Research Centre (Poznań, Poland) using the same primers as those used for the PCR. The sequences were deposited in the NCBI GenBank (Supplementary materials, Table S1) and compared with those in GenBank using the BLASTn algorithm. Only a 99–100% match with a reliable source (ex-type sequences, taxonomic studies) was accepted as proof of identification. Sequences with similarity ≥99.6% to the ITS1–5.8S-ITS2 region (400–500 bp) were considered as belonging to identical species and were included in the alignment. The respective taxonomic thresholds for fungal identification at the genus, family, order and class levels were 94.3%, 88.5%, 81.2% and 80.9% based on ITS1–5.8S-ITS2 sequences (Vu et al., 2019). For each nest sample, the number of colony-forming units (CFU) per gram of soil was determined based on $10^{-3}$ dilution isolation results. The frequency of an individual species was defined as the percentage of isolates in the total number of isolates.

*2.4. Laboratory rearing and identification of macroarthropods*

To describe the macroarthropod assemblages, eight nests were selected at random, from which a 10 $dm^3$ sample of nest material was collected in March 2019. Since the procedure of material collection leads to moderate interference with the nest structure, we were able to increase the number of studied nests comparing to soil analyses described in the chapter 2.2. Because the density of the nest material was unevenly distributed within the nest (it is the largest in the interior and the smallest at the edges), the samples were taken at half the height of each nest at the point between the highly decomposed internal part and weakly decomposed organic matter at the margins. If present in a sample, tree branches, twigs or any anthropogenic materials (plastics, fabrics) were not removed. The samples were placed in plastic containers, covered with geotextile to ensure oxygen exchange but to prevent arthropods from escaping or entering, and transported to the laboratory.

The arthropods were reared in 10 $dm^3$ plastic containers filled with nest material. The containers were sealed with covers having a 10 cm radii circular entrance covered with fine-grained mesh. This prevented the reared individuals from escaping (or other organisms from entering the substrate) and guaranteed adequate ventilation. The cultures were kept in the laboratory at room temperature for five months. The substrate was periodically moistened to prevent the host material from drying out. The cultures were inspected one month after their establishment: this involved searching the entire substrate for arthropod larvae, pupae and imagines. The second inspection, at the end of the rearing in August 2019, involved shredding and sieving the entire substrate in the search for macroarthropods only (Callaham et al. 2010). The pre-imaginal stages found were reared to obtain imagines. The reared specimens were preserved in alcohol, counted and identified to the lowest possible systematic level.

*2.5. Statistical analysis*

Due to potential relationships between the biochemical activity and the physicochemical properties of the material from the various layers of the nests, Spearman's rank correlation coefficients between the physicochemical and biochemical properties were calculated. Differences with $p < 0.05$ were considered statistically significant. On the basis of Ward's method (Everrit, 1980), agglomeration of the nest layers differing in the selected physicochemical properties was con-

ducted. The content of $P_2O_5$, which is a diagnostic criterion for ornithogenic material, and the C/N ratio, which reflects the decomposition rate of organic material, were used in the agglomeration procedure. The classification and regression tree approach was applied to estimate the influence of the selected physicochemical and biochemical properties on the microbial biomass. The MBN that reflects the activity of the microorganisms involved in the nutrient cycle was used as dependent variable. NAG and C/N ratio that are related to the N mineralization and the residues decomposition processes were used an explanatory variables. All the statistical analyses were performed with Statistica 13.3 software.

## 3. Results

### *3.1. Physicochemical properties*

The pH in $H_2O$ of nest material varied from 3.48 to 5.47, while pH in KCl varied from 3.17 to 5.31 (Table 1). The N and C contents in this material were high, ranging from 1.79 to 3.47% and 18.90 to 31.10%, respectively, depending on the layer (Table 1). The C/N ratio varied from 7.86 to 12.92. The S content was 0.25–0.84%. The maximum and minimum base cation contents were 59.47 and 16.16 cmol $(+)\cdot kg^{-1}$, respectively. Contents of $P_2O_5$ were lower in the upper nest layers (Table 1), the lowest value at these levels being 0.9%, while the highest $P_2O_5$ contents (1.87%) were recorded in the deeper layers of the nests (Table 1). The lignin content of the nests varied from 27.58 to 116.19 $mg\cdot cm^{-3}$ (Table 1). The nest material was characterized by low density (0.13–0.52 $g\cdot cm^{-3}$), high humidity (37.2–79.0%) and high water capacity (29.4–64.5%) (Table 1).

### *3.2. Biochemical parameters*

The enzymatic activity was diversified: the activities of BG, NAG and PH were high, but that of SP was low (Table 2). The MBC and MBN differed remarkably between distinguished nests and layers (Table 2).

The biochemical activity of the nest material expressed by enzyme activity and microbial biomass was correlated with selected physicochemical properties (Table 3). There was a significant and positive relationship between the S content and the SP activity (Table 3). The SP activity was significantly and negatively correlated with the C/N ratio and positively with lignin content (Table 3). The NAG activity was significantly correlated with the capillary water capacity (Table 3).

The results of the agglomeration analysis showed that the chemical properties of the various nest layers differed (Fig. 2), i.e. the surface (most recently constructed) layers have a different $P_2O_5$ content and C/N ratio from the deeper layers.

Classification and regression tree charts were drawn to identify the properties that primarily determine MBN. They are the C/N ratio, followed by the activity of NAG (Fig. 3). The highest MBN was found at a C/N ratio < 10.1 and the lowest at C/*N* > 10.1, with NAG activity <2963 nmol MUB $g^{-1}$ d.s.$\cdot h^{-1}$.

### *3.3. Fungal assemblage*

The 2726 fungal isolates obtained included 82 taxa that were assigned to three phyla and 17 orders (Table 4). They included 6 isolates of Basidiomycota, 2523 of Ascomycotina and 197 of Mucoromycotina (Table 4). 41 taxa were identified to species level, while the remaining taxa were identified to genus (28), family or higher levels (13) (Table 4).

Members of the orders Trichosporonales and Tremellomycetes were isolated from the phylum Basidiomycota, and members of the orders Mortierellales and Mucorales from the Mucoromycotina. The large majority of fungal taxa isolated from the nests were from the Ascomycota (93%). They were distributed among 13 orders, with Eurotiales being the dominant one: it comprised 56% of the isolates (Table 4). The genus *Penicillium* was the most abundant in both total abundance (52%) and species richness (12 species). A total of 197 isolates (7%) were classified in the Mucoromycotina, mainly in the Mortierellales (6% of the isolates). The basidiomyceteous fungi were isolated from only 9% of the nests and were sparsely represented (Table 4).

The most dominant species was *Penicillium* sp. 1 (28.3% of the total number of fungal isolates), which was isolated from 82% of the nests (Table 4). *Leuconeurospora* sp., *Penicillium koreense*, *Penicillium* sp. 5, *Phialophora intermedia*, *Pseudogymnoascus* sp. and *Mortierella* sp. were also frequently isolated from the nests and comprised 4.4–11.3% of the total isolates. These species were found in 36, 55, 55, 82, 18 and 45% of the nests, respectively (Table 4). The other species were rarely isolated from the nests, although *Trichoderma* sp. was found in 73% of them (Table 4).

### *3.4. Macroarthropod assemblage*

A total of 22 macroarthropod taxa were identified (Table 5). The majority were insects belonging to four orders, mainly beetles, with three dominant species: *Carcinops pumilio*, *Tenebrio molitor* and *Trox scaber* (Table 5). Six further insect taxa belonged to the dipterans (four), true bugs and butterflies (both with one species). Among the remaining macroarthropod taxa, we found 5 myriapod, one isopod and one pseudoscorpion species (Table 5). Eurytopic forms, living in various epigeic habitats with accumulated decaying organic matter, e.g. leaf litter, droppings or wood debris, were prevalent, constituting 82% of all the recorded taxa (Table 5). Taxa preferring those habitats included myriapods of the genus *Lithobius*, all the dipterans, and some of the beetles (5 taxa), of which *Carcinops pumilio*, *Dendrophilus punctatus* and *Trox scaber* are known to occur occasionally in the nests of various bird species. Typical upper soil layer dwellers were the eurytopic isopod *Trichoniscus pusillus*, the eurytopic fly *Minettia lupulina* and the stenotopic pseudoscorpion *Allochernes peregrinus* (Table 5).

## 4. Discussion

This is the first ever comprehensive survey of the physicochemical and biochemical properties of the material contained in White Stork nests. The properties of the *nesting substrate have provided* evidence that the nests of large-bodied birds form biological systems resembling natural soil habitats. According to WRB (2014), ornithogenic material must have a layer at least 15 cm thick in which there are constituents indicating the life activity of birds. Furthermore, the minimum content of $P_20_5$ (0.25%) is an important criterion for the "ornithic" material. In the case of the White Stork nests described here, the above requirements were met at most levels. The $P_20_5$ content requirement was not met, however, in the top nest layers, which appears to be related to the fact that they are formed by material added to the nest in recent years. According to the criteria presented in the WRB (2014) the discussed material collected from the nests can be classified as Lignic Histosol Ornithic. The Lignic qualifier refers to the presence of undecomposed fragments of branches.

One of the characteristic features of soil formation is the occurrence of levels differing in morphological, physical, chemical and bi-

**Table 1**
Basic physicochemical properties of the materials collected from nests of White Stork *Ciconia ciconia*; Depth – range of the layers (cm), N – nitrogen content (%), C – carbon content (%), S – sulphur content (%), $P_2O_5$ – phosphorus pentoxide content (%), BC – base cation content (Ca, K, Mg, Na) (cmol(+)·kg$^{-1}$), Lignin – lignin content (mg·cm$^{-3}$), Bd – bulk density (g·cm$^{-3}$), Mw – moisture content (% of weight) and CWCw – capillary water capacity (% of weight).

| Nest ID | Depth | Description of layers | pH in $H_2O$ | pH in KCl | N | C | C/N | S | $P_2O_5$ | Ca | K | Mg | Na | BC | Lignin | Bd | Mw | CWCw |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0–15 | light brown, piece-fibre structure, remains of insects | 5.2 | 4.48 | 2.62 | 30.71 | 11.74 | 0.33 | 0.10 | 22.54 | 1.45 | 5.31 | 0.42 | 29.73 | 27.58 | 0.24 | 297.1 | 64.5 |
| | 15–40 | dark brown, amorphous structure, remains of insects | 3.65 | 3.38 | 2.55 | 29.02 | 11.39 | 0.34 | 0.25 | 22.32 | 3.23 | 3.98 | 1.37 | 30.90 | 58.40 | 0.13 | 294.5 | 40.8 |
| | 40–70 | brown, piece structure, small branches (30%), remains of insects | 3.48 | 3.17 | 2.11 | 24.77 | 11.73 | 0.32 | 0.18 | 16.20 | 2.24 | 2.55 | 0.90 | 21.88 | 38.21 | 0.21 | 273.2 | 58.9 |
| 2 | 0–17 | light brown, piece structure | 4.95 | 4.34 | 1.79 | 23.09 | 12.92 | 0.25 | 0.08 | 16.34 | 0.94 | 2.05 | 0.23 | 19.56 | 33.06 | 0.35 | 201.5 | 51.7 |
| | 17–37 | dark brown, piece-fibre structure, a large admixture of sand | 3.74 | 3.39 | 1.85 | 18.90 | 10.22 | 0.28 | 0.12 | 11.02 | 2.18 | 2.27 | 0.68 | 16.16 | 63.33 | 0.52 | 122.1 | 29.4 |
| | 37–60 | brown, piece structure, small branches (40%) | 3.63 | 3.38 | 2.25 | 23.52 | 10.46 | 0.30 | 0.18 | 16.05 | 3.39 | 1.98 | 0.84 | 22.26 | 74.53 | 0.44 | 149.2 | 30.9 |
| 3 | 0–25 | brown, piece-fibre structure | 4.16 | 3.87 | 2.37 | 25.12 | 10.60 | 0.34 | 0.12 | 17.96 | 3.46 | 3.41 | 1.03 | 25.86 | 72.14 | 0.34 | 223.2 | 60.8 |
| | 25–45 | light brown, piece structure, small branches (50%) | 4.38 | 4.14 | 2.08 | 22.85 | 11.00 | 0.36 | 0.29 | 33.24 | 4.92 | 3.05 | 1.53 | 42.75 | 116.19 | 0.31 | 253.4 | 62.9 |
| | 45–80 | dark brown, amorphous structure, fragments of wood | 4.02 | 3.76 | 2.74 | 21.55 | 7.86 | 0.41 | 0.44 | 22.71 | 4.34 | 2.57 | 1.41 | 31.03 | 45.86 | 0.28 | 212.5 | 57.7 |
| 4 | 0–18 | brown, piece-amorphous structure | 4.42 | 3.96 | 2.99 | 31.10 | 10.40 | 0.36 | 0.09 | 20.99 | 2.10 | 1.56 | 0.77 | 25.42 | 42.30 | 0.25 | 235.5 | 29.7 |
| | 18–50 | brown, piece-amorphous structure, small branches (60%) | 3.58 | 3.26 | 3.47 | 34.70 | 10.00 | 0.48 | 0.20 | 18.31 | 1.71 | 1.55 | 0.62 | 22.20 | 96.75 | 0.17 | 265.5 | 31.3 |
| | 50–80 | light brown, piece structure, small branches (60%) | 4.87 | 4.92 | 3.23 | 25.43 | 7.88 | 0.84 | 1.87 | 42.00 | 7.03 | 6.38 | 4.06 | 59.47 | 92.07 | 0.25 | 297.7 | 67.9 |
| 5 | 0–28 | brown, piece structure, small branches (50%) | 4.9 | 4.58 | 1.93 | 20.04 | 10.39 | 0.34 | 0.34 | 40.75 | 7.37 | 7.24 | 3.91 | 59.27 | 52.77 | 0.31 | 237.4 | 59.2 |
| | 28–60 | dark brown, piece-amorphous structure, small branches (30%) | 5.47 | 5.31 | 2.53 | 27.49 | 10.86 | 0.46 | 0.43 | 26.83 | 7.98 | 5.46 | 1.72 | 41.98 | 54.71 | 0.29 | 251.1 | 63.4 |
| | 60–110 | brown, piece-fibre structure, admixture of sand | 3.95 | 3.71 | 2.49 | 22.10 | 8.88 | 0.41 | 0.17 | 16.50 | 11.18 | 3.03 | 1.84 | 32.55 | 107.52 | 0.33 | 224.0 | 55.0 |

**Table 2**
Biochemical properties of the materials from nests of White Stork *Ciconia ciconia*; Depth – range of the layers (cm), MBC – microbial biomass carbon (μg·g$^{-1}$), MBN – microbial biomass nitrogen (μg·g$^{-1}$); enzyme activities (nmol MUB·g$^{-1}$ d.s.·h$^{-1}$): CB – β-D-cellobiosidase, BG – β-glucosidase, NAG – *N*-acetyl-β-D-glucosaminidase, XYL – xylanase, SP – arylsulphatase and PH – phosphatase.

| Nest ID | Depth | MBC | MBN | Enzyme activities | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | CB | BG | NAG | XYL | SP | PH |
| 1 | 0–15 | 365.1 | 87.3 | 1103.7 | 4128.5 | 5283.1 | 453.5 | 0.0 | 5244.5 |
| | 15–40 | 459.3 | 13.1 | 442.7 | 2782.7 | 2851.6 | 478.9 | 0.0 | 2763.9 |
| | 40–70 | 761.0 | 335.9 | 1158.5 | 3560.7 | 3650.6 | 652.5 | 0.0 | 4884.8 |
| 2 | 0–17 | 948.3 | 132.6 | 446.7 | 1532.3 | 1125.9 | 243.5 | 0.0 | 2248.2 |
| | 17–37 | 695.8 | 121.3 | 993.4 | 2363.0 | 2136.6 | 374.4 | 25.6 | 2633.3 |
| | 37–60 | 837.7 | 3.7 | 1412.4 | 2742.1 | 1839.1 | 432.0 | 5.3 | 2812.2 |
| 3 | 0–25 | 1.02 | 8.7 | 2150.4 | 4423.8 | 2808.0 | 502.6 | 0.0 | 4114.7 |
| | 25–45 | 4.08 | 176.9 | 3220.3 | 4619.1 | 3073.6 | 1122.2 | 34.3 | 4256.6 |
| | 45–80 | 97.4 | 4.7 | 4719.4 | 4215.1 | 3313.2 | 904.4 | 32.2 | 2219.0 |
| 4 | 0–18 | 1254.5 | 134.7 | 937.3 | 2117.7 | 1181.5 | 428.0 | 26.1 | 2867.4 |
| | 18–50 | 1267.4 | 216.8 | 374.6 | 1154.3 | 1969.1 | 130.7 | 66.6 | 820.3 |
| | 50–80 | 993.5 | 451.8 | 282.3 | 977.3 | 2497.1 | 25.6 | 54.1 | 712.8 |
| 5 | 0–28 | 245.1 | 26.9 | 460.0 | 1532.2 | 1885.4 | 228.7 | 0.0 | 2303.7 |
| | 28–60 | 238.2 | 126.1 | 760.9 | 2099.7 | 4090.5 | 305.5 | 69.1 | 1483.1 |
| | 60–110 | 75.6 | 334.5 | 2835.8 | 4562.4 | 4408.6 | 683.1 | 116.3 | 4331.9 |

**Table 3**
Spearman's rank correlation coefficients between the chemical and biochemical properties (microbial activity) of the materials collected from nests of White Stork *Ciconia ciconia*; N – nitrogen content (%), C – carbon content (%), S – sulphur content (%), $P_2O_5$ – phosphorus pentoxide content (%), BC – base cation content (Ca, K, Mg, Na) (cmol(+)·kg$^{-1}$), Lignin – lignin content (mg·cm$^{-3}$), Bd – bulk density (g·cm$^{-3}$), Mw – moisture content (% of weight) and CWCw – capillary water capacity (% of weight), MBC – microbial biomass carbon (μg·g$^{-1}$), MBN – microbial biomass nitrogen (μg·g$^{-1}$); enzyme activities (nmol MUB·g$^{-1}$ d.s.·h$^{-1}$): CB – β-D-cellobiosidase, BG – β-glucosidase, NAG – *N*-acetyl-β-D-glucosaminidase, XYL – xylanase, SP – arylsulphatase and PH – phosphatase. Significant relationships with $p<0.05$ are shown in bold.

| | pH $H_2O$ | pH KCl | N | C | C/N | S | $P_2O_5$ | BC | BD | Mw | CWC | Lignin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MBC | −0.19 | −0.21 | 0.29 | 0.45 | −0.03 | −0.05 | −0.19 | −0.45 | −0.29 | 0.14 | −0.44 | −0.19 |
| MBN | −0.03 | 0.04 | 0.16 | 0.19 | −0.08 | 0.38 | 0.03 | 0.08 | −0.30 | 0.46 | 0.19 | 0.22 |
| CB | −0.17 | −0.19 | −0.24 | −0.46 | −0.05 | −0.14 | −0.09 | 0.02 | 0.37 | −0.41 | 0.04 | 0.10 |
| BG | −0.20 | −0.26 | −0.18 | −0.26 | 0.17 | −0.15 | −0.16 | 0.05 | 0.14 | −0.14 | 0.11 | 0.14 |
| NAG | 0.05 | 0.05 | 0.19 | 0.03 | 0.07 | 0.28 | 0.25 | 0.35 | −0.31 | 0.42 | **0.56** | 0.02 |
| XYL | −0.29 | −0.34 | −0.14 | −0.26 | 0.14 | −0.11 | −0.06 | 0.05 | 0.03 | −0.11 | 0.05 | 0.08 |
| SP | 0.00 | 0.11 | 0.43 | 0.04 | **−0.62** | **0.77** | 0.40 | 0.35 | 0.00 | 0.02 | 0.04 | **0.61** |
| PH | −0.15 | −0.26 | −0.27 | −0.03 | 0.46 | −0.44 | **−0.52** | −0.16 | 0.03 | 0.05 | 0.04 | −0.14 |

ological properties. Arising during pedogenesis, they are associated with the impact of climatic factors, living organisms and time. Typically, the longer the exposure time for these factors, the deeper the profile and the more diverse the genetic levels (Hillel, 2008; Osman, 2013). In the White Stork nests, we noted that the material deposited into levels differing in physicochemical and biochemical properties was highly differentiated. The pooling analysis confirms the stratification of the material deposited in the nests. Cluster analysis confirmed the distinctiveness of the surface layers of nests in terms of decomposition stage and $P_2O_5$ content. The organic and mineral material accumulated by birds is transformed and becomes compacted over time as the organic material slowly decomposes and binds to mineral substances. We found organic-mineral compounds in the deep levels of the nests; this is reflected in the relatively advanced degree of organic matter decomposition, and the large amounts of P, exchangeable cations and mineral substances.

A typical feature required of soils is their biochemical activity (Hillel, 2008). The material collected from our nests revealed high activities of enzymes involved in nutrient cycling. Equally high enzyme activities have been recorded in eutrophic soils overgrown with deciduous stands and in organic histosol horizons (Błońska, 2010; Błońska et al., 2017). The high MBC and MBN also indicate a high level of biochemical activity within the White Stork nest material: this is due to the presence of large amounts of diverse organic substrates that provide a source of energy for the microorganisms involved in its transformation (Cleveland et al., 2002; Zhao et al., 2018). Whereas biochemical activity is limited by the availability of C, N and P, the nest material offers a very rich supply of nutrients – hence the high level of biochemical activity. The nest material contained large amounts of C and N: the C content was >20% in most of the nest layers. The statistical analysis points to a relationship between the enzymatic activity, MBN and the $P_2O_5$ content on the one hand, and the degree of decomposition of organic matter expressed as the C/N ratio on the other. The ratio of percentage of C to N in the soil is an indicator of the degree to which N contained in remains is available to microorganisms and has long been known as a parameter to assess the degree of organic matter decomposition (Cools et al., 2014; Ostrowska and Porębska, 2015). The C/N ratio indicates that the organic matter in the material from the various levels of the nests was in a good state of decomposition.

The diversity and activity of fungi is regulated by wide range of biotic factors, such as the presence of plants and other organisms, as well as abiotic ones like soil pH, moisture, salinity, structure and temperature (Frąc et al., 2018). Birds' nests accumulate all manner of organic debris, so they constitute a unique environment for microbial activity. Depending on the nest type, different species of fungi are a characteristic component of this specific microhabitat playing important roles in nutrient cycling (Apinis 1967; Pugh and Evans, 1970; Hubálek et al., 1973; Hubálek and Balat, 1974, 1976; Korniłłowicz-Kowalska and Kitowski, 2009, 2013, 2017; Korniłłowicz-Kowalska et al., 2010, 2011, 2018; Jankowiak et al., 2019). The present study revealed 82 fungal taxa from White Stork nests, including 42 exhibiting no similarity to species level with any known fungal sequences in the GenBank database. The fungal community was dominated by Ascomycota, followed by Mucoromycotina: this was consistent with previous studies of the fungal diversity of nest environments (Pugh,

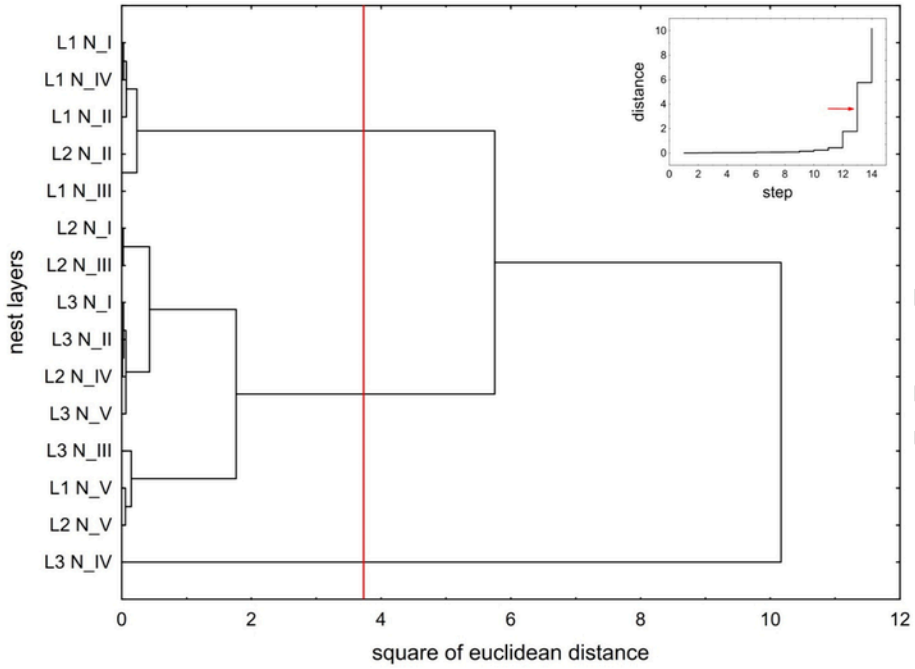


**Fig. 2.** Cluster analysis grouping the various layers in nests of White Stork *Ciconia ciconia*; chemical properties (phosphorus pentoxide ($P_2O_5$) content and the rate of decomposition carbon/nitrogen (C/N) ratio) were used in the design of the diagram; N_I-V – nest ID; L1–3 – nest layers (see Tables 1 and 2 for the details of the nests and layers). The vertical red line indicates the distance cut corresponding to the between-step change of the inter-cluster distances (red mark at the inset figure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

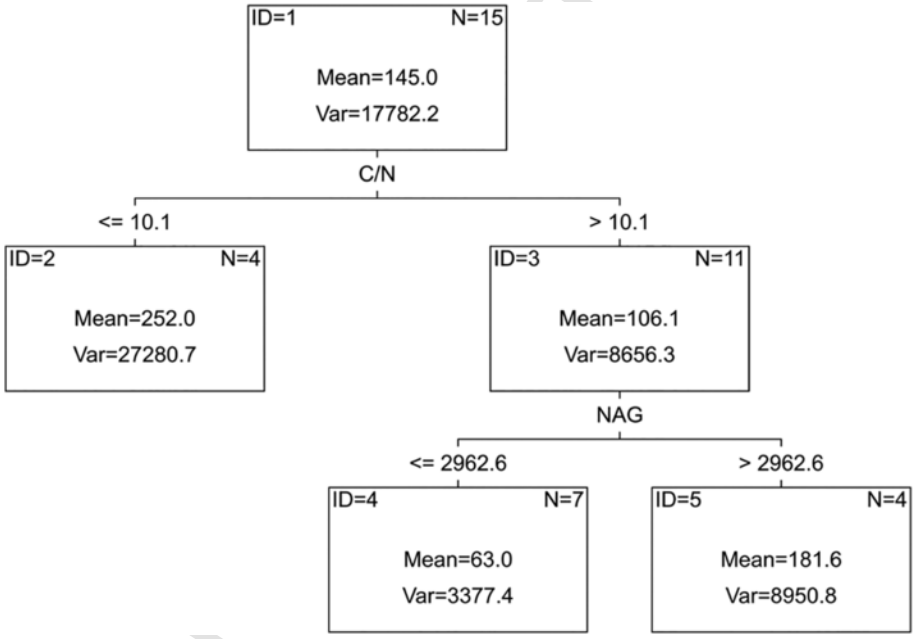


**Fig. 3.** Regression tree for microbial biomass nitrogen in the layers of White Stork *Ciconia ciconia* nests based on the rate of decomposition carbon/nitrogen ratio (C/N) and the activity of *N*-acetyl-β-D-glucosaminidase (NAG) (see Tables 1 and 2 for the details of the nests and layers).

1965, 1966; Apinis and Pugh, 1967; Otčenašek et al., 1967; Pugh and Evans, 1970; Korniłłowicz-Kowalska and Kitowski, 2009, 2017; Korniłłowicz-Kowalska et al., 2010, 2011, 2018; Jankowiak et al., 2019). The following 12 *genera* were the most prominent in these studies: *Alternaria*, *Aphanoascus*, *Arthroderma*, *Aspergillus*, *Chaetomium*, *Chrysosporium*, *Fusarium*, *Mucor*, *Penicillium*, *Petriella*, *Scopulariopsis* and *Trichoderma*. Eight of them (*Alternaria*, *Aspergillus*, *Chaetomium*, *Fusarium*, *Mucor*, *Penicillium*, *Scopulariopsis* and *Trichoderma*) were present in our White Stork nests.

Birds use a wide variety of plant materials, animal remains (hairs, feathers) and anthropogenic materials (textiles, refuse) with which to construct their nests (Hansell, 2000), which is a situation favouring the development of diverse ecological and physiological groups of

**Table 4**
Fungal taxa identified in nests of White Stork *Ciconia ciconia* and their frequencies (%) of occurrence (number of nests = 11).

| Taxon | Order | No. (%) | % of nests with the taxon |
|---|---|---|---|
| **Basidiomycota** | | | |
| *Cutaneotrichosporon guehoae* | Trichosporonales | 12(0.4) | 9 |
| *Tausonia pullulans* | Tremellomycetes | 4(0.1) | 9 |
| **Ascomycota** | | | |
| *Acaulium* sp. | Microascales | 21(0.8) | 36 |
| *Acaulium acremonium* | Microascales | 4(0.1) | 9 |
| *Alternaria abundans* | Pleosporales | 13(0.5) | 9 |
| *Alternaria rosae* | Pleosporales | 1(0) | 9 |
| *Alternaria* sp. | Pleosporales | 1(0) | 9 |
| *Aspergillus puulaauensis* | Eurotiales | 5(0.2) | 9 |
| *Aspergillus* sp. 1 | Eurotiales | 2(0.1) | 9 |
| *Aspergillus* sp. 2 | Eurotiales | 1(0) | 9 |
| *Aspergillus* sp. 3 | Eurotiales | 2(0.1) | 9 |
| Aspergillaceae sp. 1 | Eurotiales | 39(1.4) | 27 |
| Aspergillaceae sp. 2 | Eurotiales | 21(0.8) | 9 |
| Aspergillaceae sp. 3 | Eurotiales | 2(0.1) | 18 |
| Aspergillaceae sp. 4 | Eurotiales | 17(0.6) | 27 |
| Aspergillaceae sp. 5 | Eurotiales | 25(0.9) | 36 |
| *Botryotrichum piluliferum* | Sordaraiales | 3(0.1) | 27 |
| *Botryotrichum* sp. | Sordariales | 45(1.7) | 9 |
| *Byssochlamys nivea* | Eurotiales | 1(0) | 9 |
| *Chaetomium rectangulare* | Sordariales | 25(0.9) | 27 |
| *Chaetomium subaffine* | Sordariales | 3(0.1) | 9 |
| *Chaetomium* sp. 1 | Sordariales | 29(1.1) | 18 |
| *Chaetomium* sp. 2 | Sordariales | 9(0.3) | 9 |
| Cephalothecaceae sp. | Sordariales | 1(0) | 9 |
| Clavicipitaceae sp. | Hypocreales | 2(0.1) | 9 |
| *Clonostachys rosea* | Hypocreales | 3(0.1) | 9 |
| Coniochaetaceae sp. | Coniochaetales | 2(0.1) | 27 |
| *Dinemasporium japonicum* | Chaetospaeriales | 16(0.6) | 18 |
| *Fusarium culmorum* | Hypocreales | 3(0.1) | 9 |
| *Fusarium oxysporum* | Hypocreales | 1(0) | 9 |
| *Fusarium sporotrichioides* | Hypocreales | 15(0.6) | 9 |
| *Fusarium* sp. | Hypocreales | 7(0.3) | 9 |
| *Gamsia columbina* | Microascales | 1(0) | 9 |
| Hypocreales sp. 1 | Hypocreales | 1(0) | 9 |
| Hypocreales sp. 2 | Hypocreales | 1(0) | 9 |
| *Leuconeurospora* sp. | Leotiomycetes incertae sedis | 119(4.4) | 36 |
| *Lophotrichus* sp. | Microascales | 2(0.1) | 18 |
| Myxotrichaceae sp. 1 | Leotiomycetes inserte sedis | 5(0.2) | 27 |
| Myxotrichaceae sp. 2 | Leotiomycetes inserte sedis | 1(0) | 9 |
| *Neocosmospora rubicola* | Hypocreales | 1(0) | 9 |
| *Neogymnomyces* sp. | Onygenales | 2(0.1) | 9 |
| *Neosetophoma* sp. | Pleosporales | 22(0.8) | 27 |
| *Oidiodendron* sp. | Leotiomycetes incertae sedis | 2(0.1) | 18 |
| Ophiostomataceae sp. | Ophiostomatales | 15(0.6) | 9 |
| *Parafenestella* sp. | Pleosporales | 1(0) | 9 |
| *Penicillium brevicompactum* | Eurotiales | 3(0.1) | 18 |
| *Penicillium griseofulvum* | Eurotiales | 1(0) | 9 |
| *Penicillium koreense* | Eurotiales | 119(4.4) | 55 |
| *Penicillium paneum* | Eurotiales | 1(0) | 9 |
| *Penicillium roqueforti* | Eurotiales | 1(0) | 9 |
| *Penicillium solitum* | Eurotiales | 4(0.1) | 36 |
| *Penicillium* sp. 1 | Eurotiales | 772(28.3) | 82 |
| *Penicillium* sp. 2 | Eurotiales | 1(0) | 9 |
| *Penicillium* sp. 3 | Eurotiales | 116(4.3) | 45 |
| *Penicillium* sp. 4 | Eurotiales | 11(0.4) | 18 |
| *Penicillium* sp. 5 | Eurotiales | 308(11.3) | 55 |
| *Penicillium* sp. 6 | Eurotiales | 74(2.7) | 45 |
| *Phialophora intermedia* | Chaetothyriales | 278(10.2) | 82 |
| *Phialemonium inflatum* | Sordariales | 1(0) | 9 |
| *Phialemonium* sp. | Sordariales | 1(0) | 9 |
| *Phialocephala humicola* | Helotiales | 3(0.1) | 9 |
| *Pseudeurotium bakeri* | Leotiomycetes incertae sedis | 1(0) | 9 |
| *Pseudocosmospora* sp. | Hypocreales | 2(0.1) | 18 |
| *Pseudogymnoascus* sp. | Leotiomycetes incertae sedis | 146(5.4) | 18 |
| *Purpureocillium lilacinum* | Hypocreales | 1(0) | 9 |
| *Sagenomella oligospora* | Eurotiales | 1(0) | 9 |
| *Sarocladium strictum* | Hypocreales | 10(0.4) | 18 |
| *Scedosporium apiospermum* | Microascales | 1(0) | 9 |
| *Scopulariopsis brevicaulis* | Microascales | 38(1.4) | 9 |
| *Simplicillium aogashimaense* | Hypocreales | 2(0.1) | 9 |
| *Sporothrix schenckii* | Ophiostomatales | 24(0.9) | 9 |
| *Venustampulla parva* | Leotiomycetes incertae sedis | 5(0.2) | 18 |
| *Venustampulla* sp. | Leotiomycetes incertae sedis | 1(0) | 9 |
| *Thyridium* sp. | Sordariomycetes incertae sedis | 1(0) | 9 |
| *Trichoderma atroviride* | Hypocreales | 7(0.3) | 27 |
| *Trichoderma harzianum* | Hypocreales | 5(0.2) | 9 |
| *Trichoderma hamatum* | Hypocreales | 9(0.3) | 27 |
| *Trichoderma* sp. | Hypocreales | 73(2.7) | 73 |
| **Mucoromycotina** | | | |
| *Mortierella alpina* | Mortierellales | 16(0.6) | 9 |
| *Mortierella polycephala* | Mortierellales | 1(0) | 9 |
| *Mortierella* sp. | Mortierellales | 159(5.8) | 45 |
| *Mucor circinelloides* | Mucorales | 21(0.8) | 45 |
| Total No. of fungal isolates | | 2726 | |
| Species richness | | 82 | |

fungi (Korniłłowicz-Kowalska et al., 2010, 2018), among which toxigenic, cellulolytic, keratinolytic, thermotolerant and homeothermic fungi are prominent (Korniłłowicz-Kowalska et al., 2018). Birds' nests are also refuges of potentially phytopathogenic and zoopathogenic fungi (Korniłłowicz-Kowalska and Kitowski, 2013; Korniłłowicz-Kowalska and Kitowski, 2017; Jankowiak et al., 2019). However, information on the mycobiota of large nests, used over a period of many years, is extremely sparse, and there is none whatsoever on fungi occurring in the nests of White Storks and other large-bodied birds.

The most prevalent Ascomycetous genera recovered in this study were *Penicillium* species: this is in agreement with previous reports that this is one of the most common genera in birds' nests (Apinis and Pugh, 1967; Hubálek et al., 1973; Korniłłowicz-Kowalska and Kitowski, 2013). The considerable amounts of cellulolytic Penicillia can be attributed *to* the *high content* of endoglucanase and exoglucanase activity (cellobiohydrolase and β-glucosidase) in the nest ma-

**Table 5**
Arthropods identified in the materials collected from nests of White Stork *Ciconia ciconia* (number of nests = 8); E – eurytopic species, ST – stenotopic species, ZOO – zoophagous species, SAP – saprophagous species.

| Order | Taxon | Number of individuals | % of nests with the taxon | Ecological characteristics |
|---|---|---|---|---|
| Isopoda | *Trichoniscus pusillus* Brandt | 1 | 12.5 | E, SAP, epigeic, humicolous |
| Pseudoscorpiones | *Allochernes peregrinus* Lohm. | 37 | 37.5 | ST, ZOO, epigeic, humicolous |
| Diplopoda | *Proteroiulus fuscus* (Am Stein) | 21 | 50.0 | E, SAP, xylophilous |
| Chilopoda | *Lithobius erythrocephalus* Koch | 3 | 12.5 | E, ZOO, xerophilous |
| | *Lithobius forficatus* (L.) | 5 | 12.5 | E, ZOO |
| | *Lithobius schuleri* Verh. | 4 | 12.5 | E, ZOO, silvicolous, montane |
| | *Lithobius tenebrosus* Meinert | 27 | 62.5 | E, ZOO, silvicolous, hygrophilous |
| Hemiptera | *Lyctocoris campestris* (Fabr.) | 1 | 12.5 | E, ZOO, corticolous |
| Coleoptera | *Anostirus castaneus* (L.) (Elateridae) | 1 | 12.5 | ST, ZOO, xylophilous |
| | *Carcinops pumilio* (Erichs.) (Histeridae) | 39 | 50.0 | E, ZOO, saprophilous, also nidicolous |
| | *Dendrophilus punctatus* (Hbst.) (Histeridae) | 3 | 25.0 | E, ZOO, silvicolous, also nidicolous |
| | *Euplectus karstenii* (Reichb.) (Staphylinidae) | 1 | 12.5 | E, ZOO, phytodetriticolous |
| | *Gnathoncus rotundatus* (Kug.) (Histeridae) | 1 | 12.5 | E, ZOO, also nidicolous |
| | *Quedius* sp. (Staphylinidae) | 1 (larva) | 12.5 | E (presumed), ZOO, humicolous or phytosaprophilous |
| | *Scydmaenus rufus* Müll. et Kun. (Staphylinidae) | 7 | 50.0 | E, ZOO, phytodetriticolous |
| | *Tenebrio molitor* L. (Tenebrionidae) | 32 (30 larvae) | 50.0 | ST, SAP, synanthropic, also xylodetriticolous |
| | *Trox scaber* (L.) (Trogidae) | 32 (19 larvae) | 50.0 | E, necrophage, also nidicolous |
| Diptera | Sciaridae (♀♀ - undetermined species) | 3 | 12.5 | E, SAP |
| | *Fannia* sp. (Fanniidae) | 1 | 12.5 | E, SAP |
| | *Minettia lupulina* (Fabr.) (Lauxaniidae) | 1 | 12.5 | E, SAP, humicolous, also nidicolous |
| | *Tephrochlamys rufiventris* (Meig.) (Heleomyzidae) | 1 | 12.5 | E, SAP, detriticolous, also stercoricolous |
| Lepidoptera | *Niditinea striolella* (Matsum.) (Tineidae) | 9 | 25.0 | ST, SAP, nidicolous |

terial. *Penicillium* fungi are known to be good producers of cellulolytic enzymes (Fang and Ou, 2018). In this study, the high cellulose content in White Stork nests also provided a suitable substrate for the growth of other cellulolytic fungi producing extracellular cellulolytic enzymes, such as *Aspergillu*s, *Chaetomium*, *Fusarium*, *Mucor* and *Trichoderma*. These saprotrophic fungi are generally associated with the decomposition of organic matter present in different types of *soil (*Domsch et al., 1980*;* Frąc et al., 2018). Other fungi detected in White Stork nests also display strong affinities to the soil environment: they included *Acaulium acremonium*, *Botryotrichum piluliferum*, *Byssochlamys nivea*, *Clonostachys rosea*, *Mortierella* spp., *Neocosmospora rubicola*, *Neosetophoma* sp., *Neogymnomyces* sp., *Oidiodendron* sp., *Pseudogymnoascus* sp., *Purpureocillium lilacinum*, *Sagenomella oligospora*, *Sarocladium strictum*, *Scedosporium apiospermum*, *Scopulariopsis brevicaulis*, *Sporothrix schenckii* and *Venustampulla parva* (Domsch et al., 1980). The large number of edaphic fungi in the nests is probably the effect of conditions in them being similar to those in soil (pH, organic matter content, air-water conditions).

Other fungi from the White Stork nests include wood-inhabiting fungi (*Parafenestella* sp., *Phialophora intermedia*), mycoparasites (*Clonostachys rosea*), phytopathogens (*Fusarium* spp., *Alternaria* spp., *Dinemasporium japonicum*, *Sarocladium strictum*), and to a greater extent potential zoopathogens (*Acaulium acremonium*, *Cutaneotrichosporon guehoae*, *Phialocephala humicola*, *Pseudogymnoascus* sp., *Scedosporium apiospermum*, *Scopulariopsis brevicaulis*, *Sporothrix schenckii*). These findings suggest that White Stork nests provide a favourable habitat for these fungi. In this study, we discovered a large number of unknown fungal species, which accounted for 50% of the total taxa, indicating that the environments within birds' nests may harbour a taxonomically diversified fungal community. However, future investigations should look into the ecological roles of fungi in this unique habitat and attempt to gain an understanding of the links between the members of the fungal communities.

Most of the arthropods we found were eurytopic, saprophagous or predatory, exhibiting a distinct preference for biotopes with decomposing organic matter, mainly plants, less often of animal origin. Two of the seven saprophages – the eurytopic isopod *Trichoniscus pusillus* and the eurytopic fly *Minettia lupulina* – are regarded as typical upper soil layer (leaf litter) dwellers, and thus as soil habitat indicators (Miller and Foote, 1976; Gregory, 2009). The other five saprophagous taxa do not display any particular environmental preferences, although the millipede *Proteroiulus fuscus* is a saproxylic species, and the necrophagous *Trox scaber* is known to occur occasionally in the nests of various bird species (Blower, 1985; Koch, 1989; Oosterbroek, 2006; Kimsey et al., 2018). The zoophagous taxa were largely eurytopic: most of the 11 taxa inhabit a broad spectrum of environments with accumulated detritus, in which they hunt for tiny saprophagous organisms. Characteristic of these taxa are three nidicolous species of clown beetles Histeridae, associated facultatively with the nests of larger birds (Mazur, 1981; Koch, 1989; Saulich and Musolin, 2009; Zapparoli, 2003; Voigtländer, 2005).

In view of their specific habitat requirements, the four stenotopic species found (two saprophages and two predators), are indicators of certain features of the White Stork nest habitat. The larvae of the click beetle *Anostirus castaneus* develop in woodland biotopes, specifically in the decomposing roots of wind-throws and the superficial layers of soil coating them, where they forage on the preimaginal

stages of various insects (Tarnawski, 2000). The mealworm beetle *Tenebrio molitor* is a synanthropic pest of food products stored in dark, damp conditions; under natural conditions, it is an omnivorous hygrophilous saprophage inhabiting woodland leaf litter, decaying wood, animal burrows and birds' nests (Koch, 1989; Stebnicka, 1991). *Niditinea striolella* is a saprophagous, nidicolous tineid moth, the caterpillars of which live in the nests of various birds (Boyes and Lewis, 2019). Of particular interest was the finding of the stenotopic pseudoscorpion *Allochernes peregrinus*: before now, this rare species was caught almost exclusively in leaf litter and the superficial soil layers in fertile woodlands, and only occasionally in decaying wood (Krajčovičová et al., 2012). This species was fairly abundant in our White Stork nests, having been passively transported there along with nest-building material or as a result of zoophoresy on harvestmen (Opiliones) or flies (Diptera) (Krajčovičová et al., 2012). This example raises the question of the origin of the invertebrate fauna inhabiting our White Stork nests. Since the imagines of more than half of the invertebrates we found are active fliers, they would have freely colonized the incipient substrate with properties suitable for their development, whereas the apterous forms would have hitched lifts to the nest on the storks themselves along with material for building, repairing or utilizing the nest. Although the White Stork is associated mainly with the farming landscape, it has been known to forage in woodland, especially in eastern Poland (Tryjanowski et al., 2018), so it is perfectly possible for woodland organisms to be transferred to nests standing beyond woods and forests. The complexity of the processes taking place during the formation and utilization of nests, i.e. the deposition and decay of organic matter, enhanced microbiological activity and the appearance of ecologically diversified fungi, may imply that the age of a nest is of key significance for the appearance of humicolous arthropods. In summary, it should be stated that all the arthropod taxa found in our White Stork nests are associated with decomposing organic matter, either as direct consumers or as predators of these saprophage-consumers. A small, though characteristic, part of this fauna was typically associated with soil and/or leaf-litter, which is indicative of the soil-like nature of the nest substrate.

According to Dokuchaev (1883), soil is a natural independent body which, like any other natural body or organism, has a specific origin, history of development, and external appearance. How we define soil depends on what we know and what insight we have about its use, features and distribution. However, the definition of soil has evolved. Hartemink (2016) noted that the modern definition of soil includes the following key elements: “the soil is a living, four-dimensional natural entity containing solids, water (or ice) and air; a soil can have any colour, be of any age, be very shallow or deep, and consists mostly of a structured mixture of sand, silt and clay (inorganics), rocks and organic material (dead and alive); the soil has one or more genetic horizons, is an intrinsic part of the landscape and changes over time; soils store and transform energy and matter. Soil is an integral part of the natural world interacting with the climate, lithosphere and hydrosphere”. In the Polish Soil Classification (SGP6), soil is defined as the surface part of the lithosphere or the accumulation of mineral and organic materials permanently connected to the lithosphere by buildings or permanent constructions, derived from weathering or accumulation processes, originating naturally or anthropogenically, subject to transformation under the influence of soil-forming factors, and able to supply the living organisms it contains with water and nutrients (Kabała et al., 2019). Taking into account the above, the material in the nests of White Stork fulfils the definition of soil. Our research has confirmed that the nests have the characteristics of soils: i) they contain layers with different morphological and physicochemical properties; ii) the biogeochemical and physical processes within the nests are continuous; iii) the system of water, air and thermal conditions permits the development of living organisms; iv) they contain organic material in various stages of degradation and biochemical transformation.

Taking into account the abundance of the breeding population in Poland (Chylarecki et al., 2018) and the mean nest weight (Zbyryt et al. submitted), the total mass of material accumulated in White Stork nests is 19,543–20,374 tons distributed in agricultural landscapes nationwide. Although the size of a nest depends on its age (Schimkat et al., 2017), regional differences in nest volume are relatively slight throughout the species' range (A. Zbyryt – unpublished information). Therefore, the global mass of nest materials accumulated by White Storks is potentially around 100,000 tons.

There are certain limitations to our results that should be taken into account when interpreting them. The description of the nest layers and the physicochemical and biochemical analyses were based on just a few nests. Although our results revealed a consistent pattern in the layers and their properties, a larger sample of nests would obviously have provided a more varied selection of nest material. Nests can differ in age (number of years in use), location in specific environmental conditions and reflect both a bird's reproductive output (the number of offspring produced in its lifetime) and an extended phenotype, as expressed by variation in the use of different types of material for nest construction, including anthropogenic material. As all these factors may influence the properties of the nest layers, the link between the use, age, location, bird quality and nest properties requires further study on a larger number of nests. However, one needs to bear in mind that in order to describe the nest soil and to perform the relevant analyses, a nest has to be destroyed. Since the White Stork is legally protected throughout its range of occurrence, permission to conduct such studies on a larger number of nests is formally limited for conservation reasons.

The results of our study are restricted to macroarthropods and fungi obtained by rearing and cultivation methods, respectively. Application of further methods could reveal the occurrence of microarthropods, other invertebrates, fungi and prokaryotic organisms. Next generation sequencing of environmental DNA (NGS) is currently used for analyses of a diversity of organisms (Pfrender et al., 2010; Shokralla et al., 2012). However, a serious limitation of NGS is that the list of organisms thereby produced could reveal species which do not use nests as a habitat but were only transported and deposited there, e.g. as food remnants. As White Stork feeds on a wide variety of prey and uses different types of materials to construct the nest (Tryjanowski et al., 2006), numerous organisms or their parts must be transferred to the nest over the years. Although NGS could reveal biological traces of such organisms, rearing or cultivation methods can identify species which live, develop or forage in nests. The identification of species for which such nests are a living habitat is crucial to understanding the biological system that is functioning within these nests. Nevertheless, the collection of material for biological analyses must disrupt the nest structure, so future research may be also limited for legal reasons.

## 5. Conclusions

In conclusion, on the basis of the physicochemical and biochemical properties of the material deposited in White Stork nests and revealed assemblages of fungi and macroarthropods, we have shown that the materials deposited in the nests of large-bodied birds lead to the formation of ornithogenic soils over the years, with distinguishable layers displaying different physicochemical characteristics and biochemical activities. Our study has revealed rich and diverse as-

semblages of fungi and macroarthropods containing taxa typical of the soil environment. This work is the first to describe a soil that is formed, modified and maintained entirely by vertebrates, and is physically isolated from the ground. The material deposited in White Stork nests can be classified as Lignic Histosol Ornithic.

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### Ethical statement

The study was performed in accordance with Polish law.

### Uncited reference

Dokuchaev, 1952

## CRediT authorship contribution statement

**Ewa Błońska:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization. **Jarosław Lasota:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization. **Robert Jankowiak:** Conceptualization, Methodology, Investigation, Resources, Writing - original draft. **Jakub Michalcewicz:** Conceptualization, Methodology, Investigation, Resources, Writing - original draft. **Tadeusz Wojas:** Investigation, Writing - original draft. **Adam Zbyryt:** Resources, Writing - original draft. **Michał Ciach:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization, Supervision.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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