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Protocol for the isolation of processed animal proteins from insects in feed and their identification by microscopy

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Abstract

Insect processed animal proteins (PAPs) constitute a new alternative source of proteins in feed. In 2017, a closed list of insect species was authorized on the European market for use in aquafeed production. Authenticity and contamination controls will have to be set up by authorities and feed actors and supported by adequate detection methods, which are lacking. The present paper presents an original isolation and detection protocol for insect material. The protocol, based on sedimentation by a mixture of petroleum ether and tetrachloroethylene to concentrate insect particles, was developed and tested on a series of ten different aquafeeds fortified at 1 % w/w with four different commercially available insect meals (from *H. illucens*, *T. molitor*, *G. assimilis* and *A. diaperinus*). The results showed that this sedimentation protocol combined with light microscopic observation was adequate for insect detection and more efficient than the current official method. Morphological key features for reliable characterization of insect PAPs were also investigated. Structural details of cuticular fragments, such as sensilla and tracheolar structures, combined with patterns of muscle

fibers, were found to constitute robust identification keys to establish the insect origin of particles. The prospective use of these markers for lower taxonomic ranking, at order level, was also addressed. Finally, the value of the markers proposed was discussed in terms of their ability to distinguish insect PAP from other types of invertebrate meal, such as that produced from marine arthropods, but also within the global framework of controls for the enforcement of the legal feed ban.

Highlights:

- Isolation of insect fragments from a complex ingredient matrix by a double sedimentation
- Morphological features useful for insect identification by light microscopy
- Possibility of taxonomic sorting of edible insects is discussed for authenticity
- Amendment of European regulation for official control and enforcement of feed ban

Key words:

Edible insect, animal nutrition, feed, control, detection, microscopy

1. Introduction

The use of insects as a source of food is as old as humanity and about two billion people traditionally consume them. They are a rich source of proteins, fats, vitamins and minerals. In recent years their potential use in animal feed has been investigated, largely in response to the urgent quest for proteins as well as to the high price of fishmeal and soya used in aquaculture and elsewhere (van Huis et al., 2013). In addition, insects constitute valuable alternatives for feeding fish and poultry since they are part of their natural diet. In 2015, the European Food Safety Authority analyzed the risk profile for the production and use of insects in food and feed (EFSA, 2015). Based on EFSA's recommendation, the European authorities agreed to introduce the use of insects for feeding aquaculture animals (farmed fish) as of July 2017 (EU, 2017). A closed list of seven insect species authorized to be reared and used in aquaculture was established: black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*).

This introduction of farmed insects and their processed animal proteins (PAPs) raises questions about the methods to be used for quality control as well as contamination and fraud detection. At the time

of writing, the only authorized methods for PAP detection in feed are light microscopy and polymerase chain reaction (PCR) (EU, 2013). For aquaculture feed, the combination of both methods is required under some conditions, but priority is usually given to light microscopy. This method is perfectly adequate for the detection of particles of fish and terrestrial (grouping all other vertebrates) PAPs. It relies principally on the categorization of the bone fragments into those two groups, since other types of animal remains are minor. Bone observation is facilitated by a sedimentation process with tetrachloroethylene (TCE), by which all material of a density higher than 1.62 $g.cm^{-3}$, including bones, is concentrated. Recently, Ottoboni et al. (2017) concluded that light microscopy could be used for the identification of insect fragments as opposed to marine arthropod PAP fragments. However, the authors limited their investigations to pure insect material produced at lab scale under experimental conditions, as against PAPs from marine arthropods. The proper detection of insect PAPs incorporated into a feed is still to be investigated, and whether or not light microscopy would be suitable for this is regarded by them as questionable. Among the principal reasons, also raised by Ottoboni et al. (2017), insects, which lack bones, have exoskeletal cuticular fragments of lighter densities. Hence insect fragments may not be concentrated by the current official sedimentation process. Therefore, the first objective of this study was to develop an alternative protocol which could be used to concentrate insect particles more effectively, and to test this new method on a representative set of aquafeeds fortified with different industrial insect PAPs on the basis of the established list of species. The study also tried to characterize the type of particles that can be expected to be microscopically recognized and to establish morphological criteria. Finally, the new protocol was compared with the current official one.

2. Material and methods

2.1. Insect PAPs

Insect meals were collected from the industry, and corresponded to PAPs produced on a large scale. The assortment of meals originated from four different species; no other species were available from the European market. The first two species are currently regarded as the most economically interesting (International Platform of Insects for Food and Feed, pers. comm.): black soldier fly (*H. illucens*) and mealworm (*T. molitor*). The other species were lesser mealworm (*A. diaperinus*) and field cricket (*G. assimilis*).

2.2. Fortified aquafeeds

Ten compound feeds for fish were used in order to cover a variety of formulations. All aquafeeds were ready-to-use commercial products. They were two complete feeds for Atlantic salmon (Sa1,

Sa2), two complete feeds for salmonid fry (Sf1, Sf2), two complete feeds for trout (Tr1,Tr2), a feed for tilapia (Ti), a complete feed for parent stock (Pa), a compound feed for trout juvenile (Trj) and finally a complete feed for sturgeon (St). None of the aquafeeds contained insect-derived ingredients. The compound feed for parent stock (Pa) contained krill meal, which was the sole ingredient which could interfere with insect PAPs.

All aquafeeds were spiked with each insect PAP at the level of 1 % (w/w) to obtain 40 samples. After spiking, the fortified aquafeeds were ground at 2 mm by a rotor mill (Retsch ZM 200) to ensure homogeneity and optimal size reduction for microscopic observations.

2.3. Preliminary study

A preliminary study was conducted to define which solvent mixture (i.e. different ratios of petroleum ether bp 40-60 °C / tetrachloroethylene (or PE/TCE)), according to its density, would best concentrate or isolate particles of insect origin from those of other origins (fish, minerals, plants, etc.) present in aquafeeds. The density of the various solvent mixtures was calculated by recording the weight (in mg) of an exact volume of 100 ml and expressed in $g.cm^{-3}$. This was repeated in triplicate for each solvent mixture in order to obtain a mean value and a standard deviation (SD). This preliminary experiment series was performed on two pure insect PAPs (from *H. illucens* and *T. molitor*) and on one aquafeed (Sf1). Proportions of floating fractions against sediment fractions were visually estimated for different ratios of PE/TCE.

2.4. Double sedimentation and isolation of fractions

Double serial sedimentation was used for this study, as illustrated in figure 1. All steps were realized in a closed sedimentation funnel of 250 ml. Using 10 g of sample material, a first sedimentation was performed with 100 ml of tetrachloroethylene (TCE, with a density of 1.62 $g.cm^{-3}$) as per Annex VI of EU/152/2009 (EU, 2009). This legally mandatory step allowed a first sediment to be recovered. This sediment was collected on a filter paper placed on a funnel. The volume of TCE drained was calculated at 30 ml. Once this volume had been achieved, the stopcock was closed and an additional volume of 30 ml of petroleum ether bp 40-60 °C (with a density of 0.65 $g.cm^{-3}$) was added into the sedimentation funnel. The 30 % PE/ 70 % TCE mixture obtained (with a density of approx. 1.26 $g.cm^{-3}$) was thoroughly mixed and the material allowed to settle down for 10 min. Two new fractions segregated: a second sediment and a final flotate. This second sediment was recovered in a petri dish. The sedimentation funnel was reversed, its wall was rinced with PE and the flotate with the remaining liquid was recovered on a filter paper placed on a funnel. After air drying of the three fractions, they were collected and weighed separately.

< FIGURE 1>

The first sediment concentrated bones, fishbones, scales and minerals as well as all fragments with a density higher than 1.62 g.cm^{-3}. The second sediment contained the fraction with all material with a density ranging from 1.62 to 1.26 g.cm^{-3}. The last fraction was the flotate, concentrating insect fragments and other fragments with a density lower than 1.26 g.cm^{-3}.

All fortified aquafeed samples were submitted to this double PE/TCE sedimentation as well as to the official one-step TCE sedimentation for comparison, as illustrated in figure 2. The experiment was carried out in triplicate.

< FIGURE 2 >

2.5. Microscopic observations

Permanent slides were prepared according to Veys & Baeten (2010) from the flotates obtained from both sedimentation protocols: current official TCE sedimentation and double PE/TCE sedimentation. Only one slide (fig. 2) was prepared for each repetition (3 repetitions for both sedimentation protocols as per the minimal legal requirement imposed by the current method). No staining reagent was used. Observations were made on a Carl Zeiss Axio Imager A1 (Zeiss, Germany) under conventional transmitted bright-field (BF), polarized (POL) and differential interference contrast (DIC) light microscopy. Observations were made at several magnifications. Per slide the number of identifiable fragments of insect origin was counted and reported. Micrographs, all taken from flotates of spiked aquafeeds submitted to the double PE/TCE sedimentation, were recorded with a Carl Zeiss AxioCam MRc (Zeiss, Germany) coupled with a 0.63 port.

2.6. Data treatment

Means of the number of insect PAP fragments identified from both TCE and PE/TCE sedimentation protocols were compared by *t*-test.

3. Results

3.1. Preliminary study for the optimal solvent mixture

The two insect PAPs used, from *H. illucens* and *T. molitor*, behaved in the same way in the different PE/TCE mixtures used in the preliminary study. For mixtures with less than 20 % PE (with densities ≥ 1.37 g.cm^{-3}), 100 % of insect fragments floated and no sedimentation occurred. When the concentration was increased to 30 % PE (reaching a density of 1.26 g.cm^{-3}), about half of the insect material floated and half settled. At concentrations of 40 % PE (with a density of 1.14 g.cm^{-3}) the

share of floating insect fragments was approx. 10 %. At densities ≤ 1.06 g.cm^{-3} all insect materials precipitated. In comparison, the aquafeed almost entirely floated at densities ≥ 1.37 g.cm^{-3}, at a density of 1.26 g.cm^{-3} its floating fraction accounted for approx. 10 % and at lower densities it settled entirely. These results are summarized in figure 3.

< FIGURE 3 >

From this experiment it was estimated that the optimal PE/TCE mixture for segregating a majority of insect particles from an aquafeed matrix was 30 % PE/ 70 % TCE with a density of 1.26 ± 0.07 g.cm^{-3}. With this solvent mixture, the proportion of floating insect material was calculated by recovering the fraction and weighing it after drying. This was done for all insect PAPs. The proportion ranged from 40 % for *H. illucens* to 69 % for *T. molitor* (with intermediate values of 57 % for *A. diaperinus* and 60 % for *G. assimilis*). Great variability around a mean value of 57% floating insect fraction was revealed according to the species of origin of the PAPs.

3.2. Detection of insect particles based on the double sedimentation protocol

The 40 aquafeeds adulterated with the insect PAPs at 1 % w/w were sedimented both by TCE only and by the double serial sedimentation protocol. The mean numbers of insect particles identified per slide are summarized in figure 4.

< FIGURE 4 >

Results showed that for 37 samples out of 40, more insect particles were isolated and identified by the double PE/TCE sedimentation protocol than by the simple TCE protocol, whatever the type of aquafeed and insect PAP used. These increases in number of particles ranged from a factor of 1.22 (*T. molitor* in Sa1) up to 12.90 (*A. diaperinus* in Sa2) with a large variability. A slight diminution in the mean number of insect particles was observed on just three occasions. Two concerned *H. illucens,* in the aquafeeds for tilapia Ti and sturgeon St, and the third related to *A. diaperinus,* in a salmon feed Sa1. *H. illucens* also presented the lowest mean numbers of particles per slide for both sedimentation protocols, with values ranging from 0 to 2.67 for the TCE sedimentation protocol and from 0.67 to 7.00 for the PE/TCE sedimentation protocol. These values are below those observed for the PAPs from other insect species as illustrated in fig 4. Of the 37 cases in which the mean number of particles obtained by the PE/TCE sedimentation protocol was higher, t-tests found only 15 cases in which the means were significantly (at $p < 0.05$) or highly significantly (at $p < 0.01$) higher. The variability in the mean number of insect particles counted over 3 slides as illustrated by fig. 5 from the counting data for *G. assimilis* is quite high (see especially the results for Sf2 feed) and explains the lack of statistical differences.

< FIGURE 5 >

The double PE/TCE sedimentation protocol also diminished the amount of floating material from the adulterated aquafeeds. Whereas TCE sedimentation produced an average 98 % of floating aquafeed material (ranging from 95.1 % for Ti to 99.8 % for Sf2), the same fraction resulting from the PCE/TCE double sedimentation protocol averaged 3.6 % (ranging from 0.2 % for Sf1 to 13.2 % for Ti). The reduction of weight percentage from the aquafeed matrix combined with a high share of insect particles in the fraction <1.26 g.cm^{-3} results in the concentrating effect of this double sedimentation protocol for insect PAPs.

3.3. Identification of morphological markers of insect fragments

Several potential morphological markers for insect particle recognition were also investigated during this study. All insect species were submitted to this investigation. Particles of insect origin were of two major types: fragments from the exoskeleton (illustrated in figs. 6, 7 and 8) and fibers from the locomotor system (illustrated in figs. 9 and 10).

< FIGURE 6 >

< FIGURE 7 >

Insect exoskeletal – or cuticular – fragments (fig. 6a-f and fig. 7a-c) were recognizable from other structures collected in the fraction <1.26 g.cm^{-3} by their general shape and the presence of sensory appendices. Cuticular fragments appear as irregularly shaped scales or plates with cell-like casts, regular (e.g. honeycombed) (fig. 6c) or irregular (fig. 6a, 6f). Some cuticular fragments lack these casts (fig. 6e). This is also true for fragments originating from arthrodial areas. Cuticular fragments could be almost transparent or deeply colored (fig. 6d), depending on the tanning level of the exocuticle. Fragments from arthrodial areas, lacking exocuticle, therefore appear as translucent. By contrast, fragments of appendices are more sclerotinized and appear brownish (fig. 6d) to opaque and hence black (fig. 7c). More differentiated short segmented fragments from antennae or leg parts were also present in *G. assimilis,* which was not the case for PAPs produced from other insect species. A common characteristic found in all insect PAPs was hair-like structures. Such structures (fig. 6a-b, 7a) correspond to setae – or trichoid sensilla (Simpson & Douglas, 2013). These setae are widely distributed and attached to the cuticle (fig. 6a-b) or separated from it (fig. 7a-c). They vary in size from 50 µm to several hundred micrometers. They could appear as single cellular structures (fig. 7b) or as more complex ones with tiny hairs on their surface (fig. 7c). Setae were entirely or nearly unpolarized (fig. 7b), allowing them to be distinguished from the plant trichomes (fig. 7d) which can be found in aquafeeds (e.g. from wheat bran and gluten) and are highly polarized. Traces of sockets

of setae were recognizable on etched cuticular fragments: they appear as small reniform or round punctuations (fig. 6e-f) occasionally surrounded by a lighter area (fig. 6f) corresponding to the thin cuticular joint membrane within the cuticle. Occasionally circular structures of campaniform sensilla could also be observed (fig. 6a). Spines or denticles were also observed for PAPs produced from diptera larvae (e.g. *H. illucens*) (fig. 6b). These denticles, belonging to the spinose bands of the segments, are usually in small linearly organized groups and pointing in the same direction. Finally, some fragments of the exoskeleton presented elements of the insect respiratory system: portions of the intricate anastomotic network of tracheae (fig. 8). Such structures are specific to insects. They were frequently observed on larger fragments from the cuticle, as illustrated in fig. 8. In this study, their occurrence was mainly restricted to the PAP produced from *G. assimilis*.

< FIGURE 8 >

Muscle fibers were also abundant in the fraction < 1.26 $g.cm^{-3}$. Their pattern is that of quadratic or rectangular translucent structures. Most of these muscle fibers were partly attached to conglomerates of cuticular and other undefined fragments. When such fibers were isolated, the closure of the condenser diaphragm made it possible to visualize the striated aspect of the sarcomers (e.g. fig. 9a). The insect origin of such muscle fibers can be confirmed by the observation of the tracheal system ensuring gaseous exchanges in insects. Both tracheae and tracheoles may be visible within muscle fibers. The use of DIC improved the visualization of the branching pattern of this network compared to bright field illumination (fig. 9b vs fig. 9a). At higher magnification, DIC revealed the transverse spiral lining of the taenidia from the tracheoles (fig. 9c). Some insect muscle fibers also differ from other muscle fibers which can be found in terrestrial or fish PAPs by the typical zigzag striation pattern of the sarcomeres (fig. 10a vs fig. 10b). PAPs prepared from *G. assimilis* were also characterized by the occurrence of long fibers which were weakly polarized. Such fibers could be as long as 1 mm (fig. 9d). At higher magnification, their rectangular pattern as well as a faint cross-striation show them from muscular origin to (fig. 9e). However, DIC observations revealed a different striation pattern from the zigzag one (fig. 9f). This latter type of muscle fiber is not observed in terrestrial or fish PAPs.

< FIGURE 9 >

< FIGURE 10 >

4. Discussion and conclusion

Isolation of particles from insect PAPs by double sedimentation using a PE/TCE solvent mixture was found to be fit for purpose in many aspects. First, the obtained results demonstrated the possibility

of successfully concentrating and isolating insect fragments from a wide variety of aquafeed matrices, regardless of the species of insect. The proposed 30 % PE/70 % TCE (at a density of ca. 1.26 $g.cm^{-3}$) is efficient for segregating insect particles from other particles of feed matrices. Effectively, the preliminary study showed that such segregation does not occur at either higher or lower densities of solvent mixture. The share of insect material recovered in the flotate ranged from 40 % to 69 %. From the data of the present study, the average concentration of insect particles was estimated to be around 25 times higher than the current legal sample sedimentation based on TCE with a density of 1.62 $g.\ cm^{-3}$. However, this concentration varies according to the properties of the feed matrices as well as the insect PAP (cf. below). A second advantage of the developed protocol is strictly practical, in the sense that it complements the legal implemented method. No other lab equipment or specific knowledge is required to perform the double sedimentation, which may even be separated in time from the official TCE sample preparation for animal protein detection. This means that the protocol can be applied only when insects need to be scrutinized and on the fraction originating from the legal sample preparation as described in Annex VI of EU/152/2009 (EU, 2009). Light microscopic observations of a single slide prepared from the obtained flotate showed better insect detection than that achievable by the TCE sedimentation treatment. The increase in mean number of insect particles with the new protocol fully supports a qualitative detection (i.e. presence or absence) but not a quantitative estimation of insect PAP. Because of the variability in the counting of insect particles any reliable quantitative assessment has to be excluded. The conditions of the study, limited to a concentration of 1 % insect PAP in fishfeeds, did not make it possible to fix a limit of detection (LOD) for the proposed protocol. On the other hand, such a parameter is not essential considering that it deals with legalized sources of proteins and not with prohibited material as is the case for other PAPs in the framework of the feed ban (e.g. ruminant PAP) imposed to prevent TSE spreading. Nonetheless, in order to estimate the potential sensitivity which could be reached, a simple additional experiment was performed. A batch of poultry feed accidentally contaminated by *Trilobium castaneum* at 0.015 % (calculated w/w) was sampled, ground and submitted to double PE/TCE sedimentation followed by a single slide observation. This was repeated twice. Two insect fragments were found in the first repetition and four in the second. Fragments were identified as remains of elytra. The method is thus very sensitive, although this sensitivity may be impacted by several factors:

- The composition of the feed matrix, which may affect the theoretical increase in concentration factor resulting from double sedimentation: a matrix containing high percentages of feed materials with lighter densities (e.g. wheat bran, bulk density of 192 $kg.m^{-3}$) will generate a flotate with a higher proportion of particles of non-insect origin and

hence a lower share of insect particles. Likewise, matrices with ingredients such as molasses or modified starch, acting as aggregating compounds and forming conglomerates of particles which will not be disaggregated due to the hydrophobic solvent mixture, may also influence the expected concentrating effect on insect fragments.

- The species of insect used for the production of PAP: as mentioned, the share of flotate of pure insect meals varies. The lowest share was recorded for *H. illucens,* which also showed the lowest mean number of insect particles per slide as clearly shown in fig. 4.
- The ability of the microscopist to distinguish insect from non-insect particles, and hereof also influencing on some specificity issues. An example of this is reported by van Raamsdonk et al. (2017), who commented on the general failure of insect identification from a proficiency test organized by the RIKILT based on the official TCE sedimentation: 90 % of the participants (out of 52) were unable to identify insect fragments at a comparable level of content of 1 %. These authors notified some specificity issues with krill fragments and muscle fibers which, according to the authors, cannot be ranked into a defined taxonomic category within the animal kingdom. This underlines the need for unambiguous morphological criteria for accurate identification of insect particles; this was the second objective of this research.

The light microscopic observations performed in this study made it possible to fix some morphological landmarks for the reliable identification of fragments from insect PAPs, even without any staining. The presence of cuticle fragments with setae at the surface is a first relevant indication. However, crustacean cuticles may have, at first sight, a confusingly similar structure, especially shrimp or krill which have a soft chitinous exoskeleton. In shrimp and krill cuticle fragments, the presence of chromatophores as well as calcareous deposits organized in an irradiating polarized rosette shape (Makowski et al., 2011) prevents them from being confused with insect fragments. Moreover, krill and shrimp particles are often naturally colored by the presence of carotenoids. Insects are unable to synthetize carotenoids (Klowden, 2013; Simpson & Douglas, 2013) and do not present a pinkish-orange color. Other commercially used crustacean species such as crabs have a much more biomineralized exocuticle or shell: deposits of calcium carbonates and calcium phosphates are so dense that most of their exocuticular particles will not float, but will be observed in the first sediment, concentrating the bones and minerals. By contrast, all insect PAP fragments of exocuticular origin have a variable sclerotization level, with color range from dark brown to almost translucent, but no trace of mineralization was ever observed. This is because mineralization in insects is not frequent and often limited to mandibular cuticles (Simpson & Douglas, 2013). Although high amounts of Ca are recorded in some species, notably *H. illucens* (Arango Gutierrez et al., 2004), this presence is not linked to any visible calcification and is limited to flies in the last larval instar

(Willis, 1999). This is in line with the absence of any staining reaction in insect cuticular particles by Alizarin red reported by Ottoboni et al. (2017), who prudently proposed this staining as a potential tool for distinguishing between insects and marine crustaceans. However, this would only be helpful if large quantities of poorly differentiated arthropod fragments were observed. Such a situation was not encountered in this study. Finally, in unclear situations regarding exoskeletal remains, looking for the presence of tracheal structures is recommended. Although not very frequent in insect PAPs, except PAP from *G. assimilis*, their presence is sufficient to ascertain a taxonomic ranking in the insect class against all other invertebrates. In addition, tracheal structures are not restricted to cuticle fragments; they are also present in muscle fibers, as illustrated in fig. 9a-c. This also permits a confirmed taxonomic ranking of insect muscle fibers against other animal ones. Another morphological feature of insect muscle fibers is the commonly observed typical zigzag striation pattern of the sarcomeres. It is well known that insects have a wide range of specialized muscles with structural and functional differentiation; for a general review, see Klowden (2013) and Simpson & Douglas (2013). For instance, the lattice ratio of myosin/actin is of 6/1, with variations in regularity inside skeletal muscle fibers other than flight muscles, where this ratio is of 3/1 and invariably regular (Klowden, 2013). Therefore the relative widths of I-, A- and H-bands may differ even inside a muscle fiber when observed by light microscopy. This influences the lengths of the sarcomeres and generates the waving or zigzag pattern. Histological atlases (Rothschild et al., 1986) exhibit this structural variability of insect sarcomeres. Even the long birefringent muscle fibers observed in *G. assimilis* may be explained by the structural differences among insect muscles. According to Simpson & Douglas (2013), they probably correspond to asynchronous flight muscles, which may be very long and composed of only a few fibers, with much reduced I-bands and a predominance of A-bands (A standing for anisotropic or birefringent). This is supported by the fact that such fibers were only visualized in *G. assimilis*, because this species is the only hemimetabolous one in which the nymphs have wings: all other species are holometabolous, with less differentiated larvae lacking such flight muscles. Such taxonomic attribution of muscles had previously been considered impossible, as still recently argued by van Raamsdonk et al. (2017), who therefore recommended the use of other methods such as DNA-based methods to overcome this problem. This is not required for insect muscle identification because of their proper morphological characteristics.

Morphological data collected from the present study prove that light microscopy is adequate for detecting insect PAPs in feed. However, further distinction relating to the species of insect may be necessary in the future. Currently, European legislation (EU, 2017) only authorizes the use of a closed list of insect species. Insect PAPs produced from other species are thus prohibited for the time being. Authenticity issues and potential fraud detection need to be addressed, although the number of

other candidate species for insect PAP production is limited mainly due to rearing conditions and other technical issues (International Platform of Insects for Food and Feed, pers. comm.). Accurate identification of all insect species relying exclusively on light microscopy is unrealistic, but a sorting at a higher taxonomic level (e.g. order level down to family level) is nevertheless conceivable. The wide diversity of the insect class and its subdividing orders offers enough proper characteristics to investigate this possibility. First indications in this respect, obtained from the analyses of a reduced number of PAP samples, are provided by this study. The four insect PAPs that were used represent the three legally authorized orders: Diptera (*H. illucens*), Coleoptera (*T. molitor* and *A. diaperinus*) and Orthoptera (*G. assimilis*). Initial parameters for sorting the three orders may be summarized as follows:

- Diptera meal is composed of several larval instars and pre-pupae. These developmental stages of a complete metamorphosis are poorly differentiated. As a direct consequence of this, a lower number of particles were recognized as of insect origin. However, the presence of a majority of unsclerotinized cuticle fragments as well as the presence of denticles, organized in spinose bands, combined with long setae are good criteria for Diptera larvae. The identification of fly larvae is abundantly documented, not in the context of feed science but because of its medico-legal importance. This identification is based particularly on the shape and organization of the denticles as well as other morphological markers (Szpila, 2009). Recent publications provide keys for species identification in taxa that were previously poorly studied such as Muscidae (Grzywacs et al., 2017). Today such keys are lacking in the context of feed authentication and control, but would certainly support proper identification of PAPs from the two authorized flies against other non-authorized Diptera meals.
- Coleoptera meals are composed of different larval instars. Although they also go through a complete metamorphosis, their larvae are more differentiated (with mouthparts and legs) and have a more sclerotinized cuticle than the observed Diptera ones. Although the number of larval instars varies before pupation, they do not vary significantly in their morphology after the second instar (Park et al., 2014); this means that sclerotinized fragments are much abundant and thus detected without difficulty. This may also account for the higher mean number of particles recorded for the two species used. The larvae of the two species were lacking denticles as observed in *H. illucens*. No distinction between fragments of *T. molitor* and *A. diaperinus* larvae could be observed.
- Orthoptera meal is prepared from nymphs or imagos. Species for this order are hemimetabolous with nymphal stages that are morphologically more differentiated than larvae. Nymphs are closely similar in appearance to imagos: they have legs, a head (with

eyes, mouthparts and antennae), and even wings present in the last instars. There is thus a more complex diversity of cuticular fragments with appendages for the later species. This differentiation level is also reflected by the presence of long birefringent muscle fibers as discussed.

In conclusion, an improvement of Annex VI of EU/152/2009 (EU, 2009) by the introduction of double PE/TCE sedimentation together with a third category of animal material in addition to terrestrial and fish, would make up for the absence of a dedicated method for the detection of insects. This new category should be referred to as “terrestrial invertebrates” as opposed to “terrestrial vertebrates” (i.e. a minor change of reference to the terrestrial category). Marine arthropods would thus be excluded from this new category. This is justified because material from the latter group, like that from other aquatic invertebrates, is legally considered as fishmeal as per Commission Regulation EU/142/2011 (EU, 2011). Such a modification of the legal framework should nevertheless be further monitored, and may need to be refined if it is not reinforced by complementary methods. This is because the identification of insect fragments from PAPs will interfere with the detection of natural insect contamination, in cereals for instance. Four options for monitoring insects in feed were recently proposed by van Raamsdonk et al. (2017) before the legal introduction of insect in aquafeed (EU, 2017). As a result of this introduction only one of the four proposed options, based on a limited list of authorized species, turned out to be realistic. These authors considered light microscopy to be usable as a first control level. The present protocol and morphological criteria obtained not only confirm this but go further, especially in case of contamination. In the natural environment the synchronicity of insect developmental stages is lacking, by contrast with a PAP production context in which individuals collected are in almost identical instars, either larval or nymphal. Looking at the genera of insect pests, a large majority belongs to the order Coleoptera (e.g. *Cryptolestes sp*., *Tribolium sp*., *Oryzaephilus sp*., *Rhyzopertha sp*., *Sitophilus sp*., *Trogoderma sp.*) and the presence of imagos is usually predominant. Even after introduction and processing in a feed matrix, remains of coleopteran elytra, membranous hindwings, compound eyes, antennae fragments and other adult fragments will certainly be distinguishable from fragments originating from insect PAPs as it was noted from the slides observation of the contaminated poultry feed. At this point, however, the absence of descriptive literature and image libraries related to the insect species used for PAP production in feed and food limits further microscopic discrimination between authorized species and undesired ones. Hence the use of other methods such as DNA targeting of authorized species would offer additional information. Recently, candidate target sequences for *T. molitor* (Debode et al., 2017) or *H. illucens* (Marien et al., 2018; Zagon et al., 2018) were found fit for purpose, at least to certify, when insects are detected, that they belong to authorized species. But a gap will still remain.

Two pests are included in the authorized list of insects, *T. molitor* and *A. diaperinus*. The use in feed production of cereal batches contaminated by these species, constituting fraud, would be overlooked if we restricted analyses to the use of DNA identification alone. So far, such cases can only be resolved by the use of light microscopy as previously explained. Therefore, DNA-based methods, like other methods used in feed control, e.g. immunoassay, near-infrared spectrometry or mass spectrometry (Fumière et al., 2009, Veys et al., 2012; Lecrenier et al., 2016), should only be used as complements once insect fragments have been isolated and microscopically confirmed. This is a continuation of an effective strategy legally adopted for decades for the disclosure of PAPs in feed.

Acknowledgments

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Figure captions

Fig. 1: Proposed double serial sedimentation protocol specifically for concentration of insect particles. (Legend: *TCE* = tetrachloroethylene, *PE* = petroleum ether, *Sed* = sediment, *Flo* = flotate)

Fig. 2: Operational scheme for the comparison of TCE and double PE/TCE sedimentation protocols for the counting of insect particles in fortified samples. (Legend: *TCE* = tetrachloroethylene, *PE* = petroleum ether)

Fig. 3: Floating/settling behavior of insect PAPs and aquafeed in relation to different PE/TCE solvent percentages. Arrowheads pointing downwards refer to sedimentation while those pointing upwards refer to flotation. The relative positions (above/below) of the double arrows around a mean density indicate the share of floating/settling fractions. (Legend: *TCE* = tetrachloroethylene, *PE* = petroleum ether, ● = calculated mean densities [n = 3, m ± 2SD])

Fig. 4: Comparison of the mean numbers [n = 3] of insect particles detected per slide according to the species of insect PAP used to adulterate the aquafeed samples and the type of sedimentation protocol (Legend: *TCE* = tetrachloroethylene, *PE* = petroleum ether, * = significant at $p < 0.05$, ** = significant at $p < 0.01$)

Fig. 5: Detailed data on the mean numbers of *G. assimilis* particles detected per slide according to the aquafeed samples and the type of sedimentation protocol (Legend: SEM = standard error of mean, *TCE* = tetrachloroethylene, *PE* = petroleum ether, * = significant at $p < 0.05$, ** = significant at $p < 0.01$)

Fig. 6: Light microscopy morphology of insect PAP exoskeletal fragments: (a) *H. illucens* larval cuticle covered with numerous trichoid sensilla and a campaniform sensillum (arrowhead); (b) *H. illucens* larval cuticle with trichoid sensilla (double arrowhead) and denticles (arrowhead); (c) *G. assimilis* nymphal cuticle with regular honeycombed pattern; (d) *G. assimilis* sclerotinized fragment of appendage; (e) *T. molitor* larval cuticle presenting sockets of setae (arrowheads) (f) *T. molitor* larval

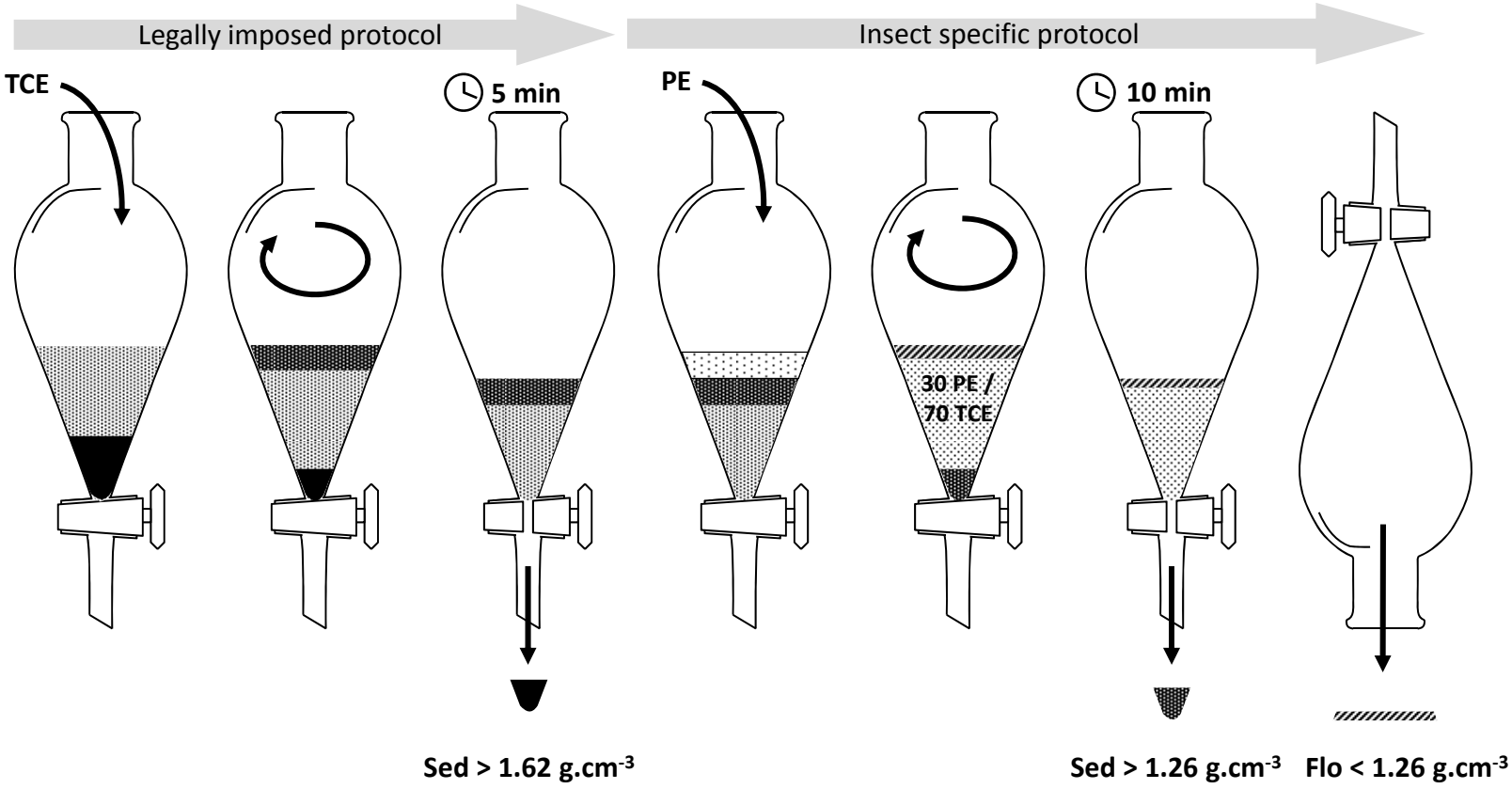
cuticle fragment with sclerotinized areas (asterisk); sockets of setae are visible (double arrowhead) with their surrounding thin unsclerotinized joint membranes (arrowhead). [a-b-c-d-e = BF, f = DIC]

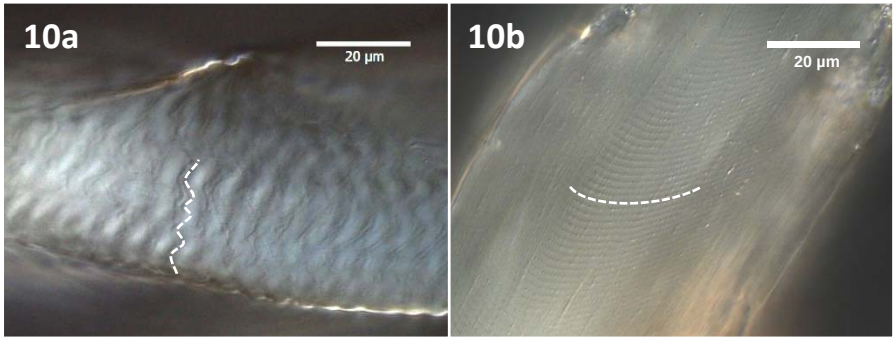
Fig. 7: Light microscopy of sensory appendices (a-c): (a) *H. illucens* isolated larval trichoid sensillum; (b) *G. assimilis* nymphal trichoid sensillum showing an absence of birefringence; (c) *H. illucens* sclerotinized hair-like seta; (d) birefringent epidermal trichome of plant origin from the aquafeed matrix. [a = DIC, b-d = POL, c = BF]

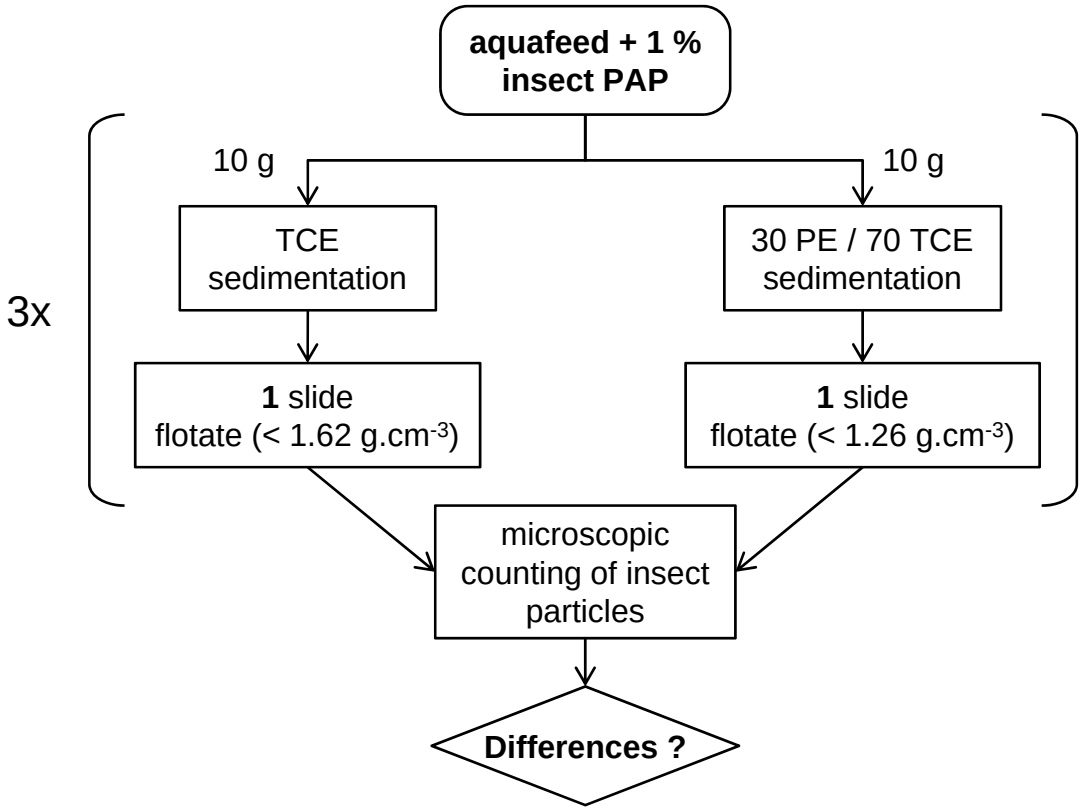
Fig. 8: *G. assimilis* nymphal exoskeletal fragment with visible tracheal network. [BF]

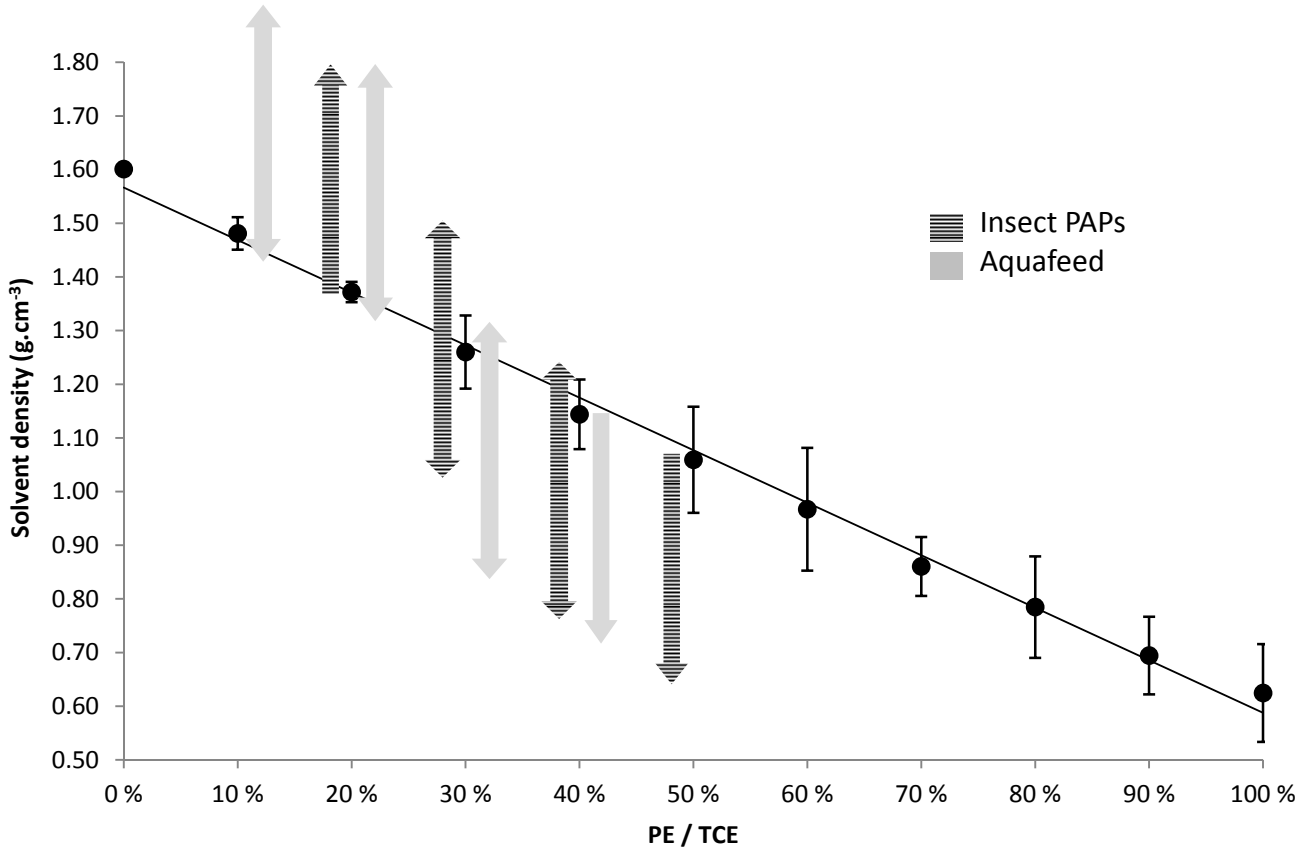
Fig. 9: Light microscopy of muscle fibers from insect PAPs: (a) muscle fiber detail from *A. diaperinus*; (b) same as fig. 9a but using DIC revealing a tracheole (arrowhead); (c) detail of tracheole inside a muscle fiber from *A. diaperinus* with annealing taenidia (arrowhead); (d) long birefringent fiber from *G. assimilis*; (e) higher magnification of a birefringent fiber from *G. assimilis* revealing its striated muscular pattern; (f) striated pattern detail of a long muscle fiber from *G. assimilis*. [a = BF, b-c-f = DIC, d-e = POL]

Fig. 10: Details of striation pattern of muscle fibers: (a) zigzag pattern of a muscle fiber from *A. diaperinus* highlighted by dotted line; (b) linear pattern of a muscle fiber from fishmeal highlighted by dotted line. [a-b = DIC]


Legally imposed protocol
Insect specific protocol
TCE
5 min
PE
30 PE /
70 TCE
10 min
Sed > 1.62 g.cm-3
Sed > 1.26 g.cm-3
Flo < 1.26 g.cm-3


10a
20 µm
10b
20 µm




Solvent density (g.cm-3)
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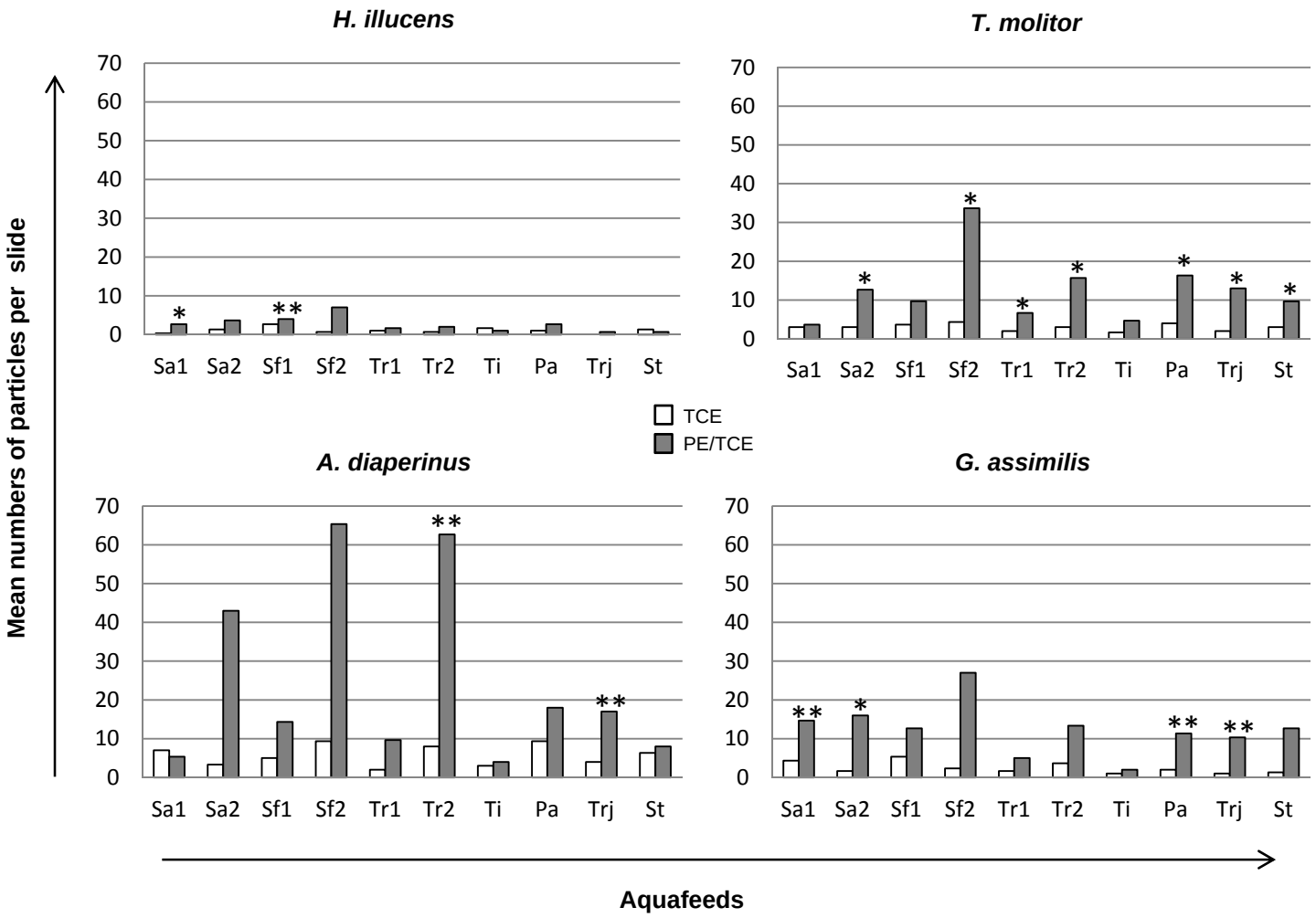
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0 %
10 %
20 %
30 %
40 %
50 %
60 %
70 %
80 %
90 %
100 %
PE / TCE
Insect PAPs
Aquafeed


H. illucens
T. molitor
A. diaperinus
G. assimilis
Mean numbers of particles per slide
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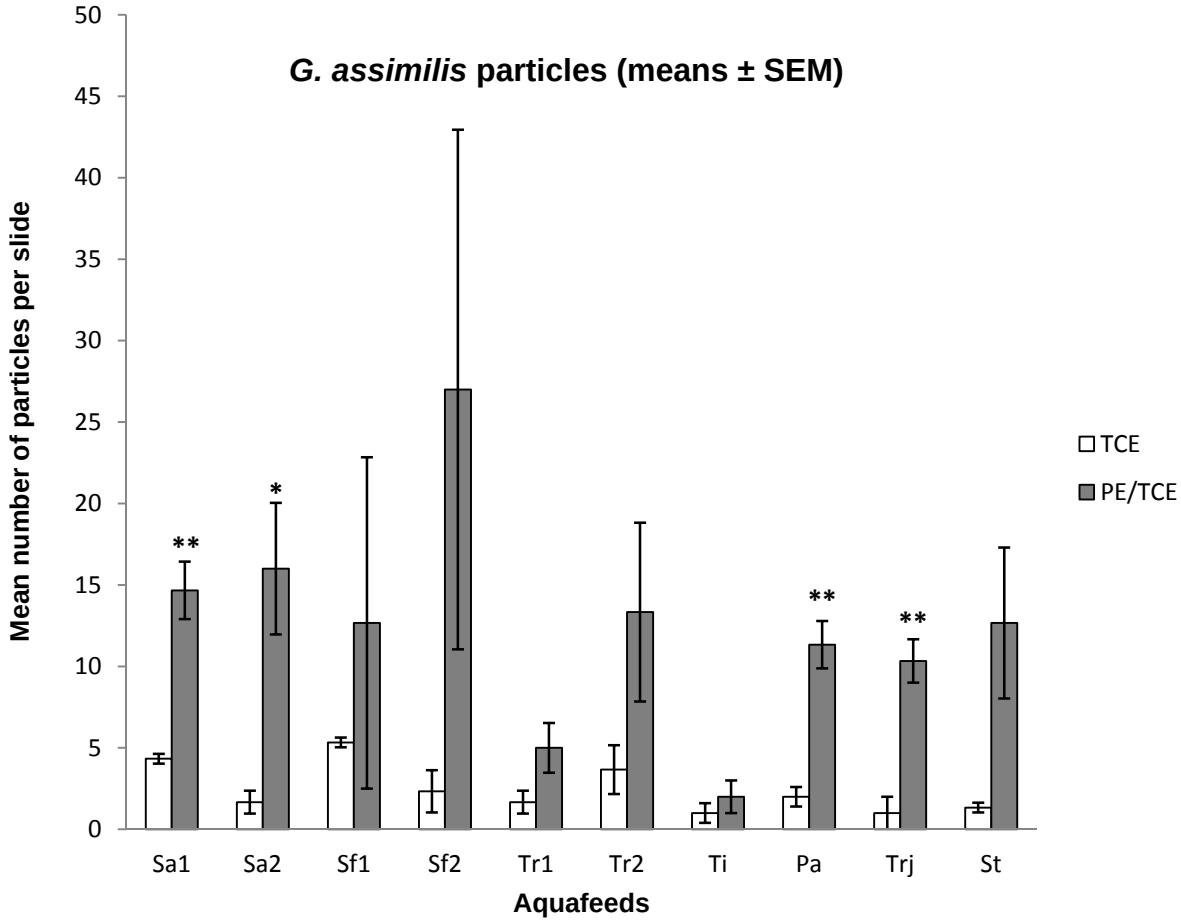
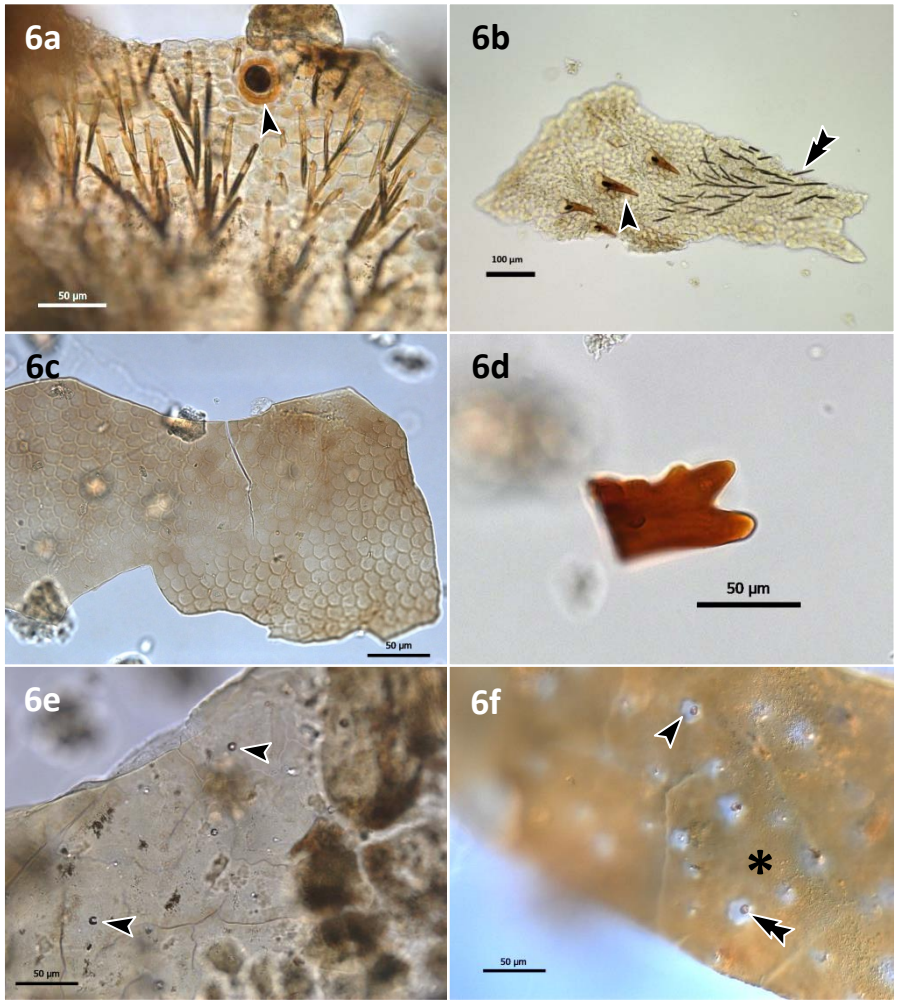
10
20
30
40
50
60
70
Sa1
Sa2
Sf1
Sf2
Tr1
Tr2
Ti
Pa
Trj
St
TCE
PE/TCE
Aquafeeds


G. assimilis particles (means ± SEM)
Mean number of particles per slide
50
45
40
35
30
25
20
15
10
5
0
**
*
**
**
Sa1
Sa2
Sf1
Sf2
Tr1
Tr2
Ti
Pa
Trj
St
Aquafeeds
TCE
PE/TCE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6b
100 µm
6c
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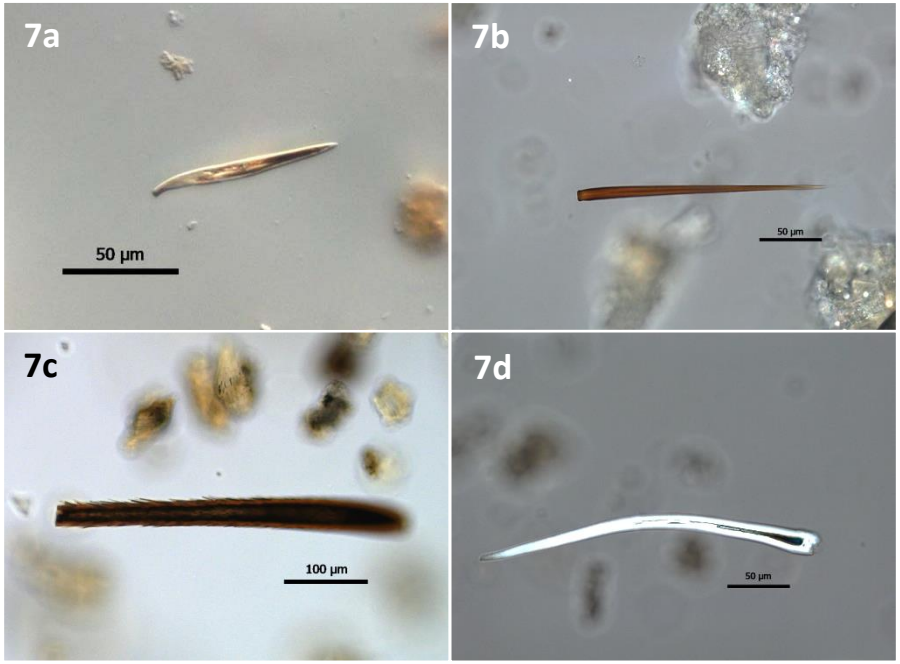
6d
50 µm
6e
50 µm
6f
50 µm


7a
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7b
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7c
100 µm
7d
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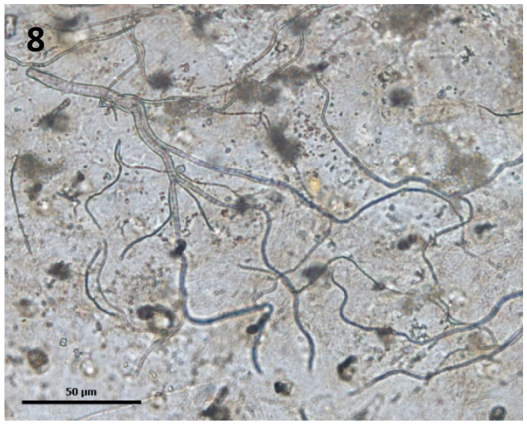
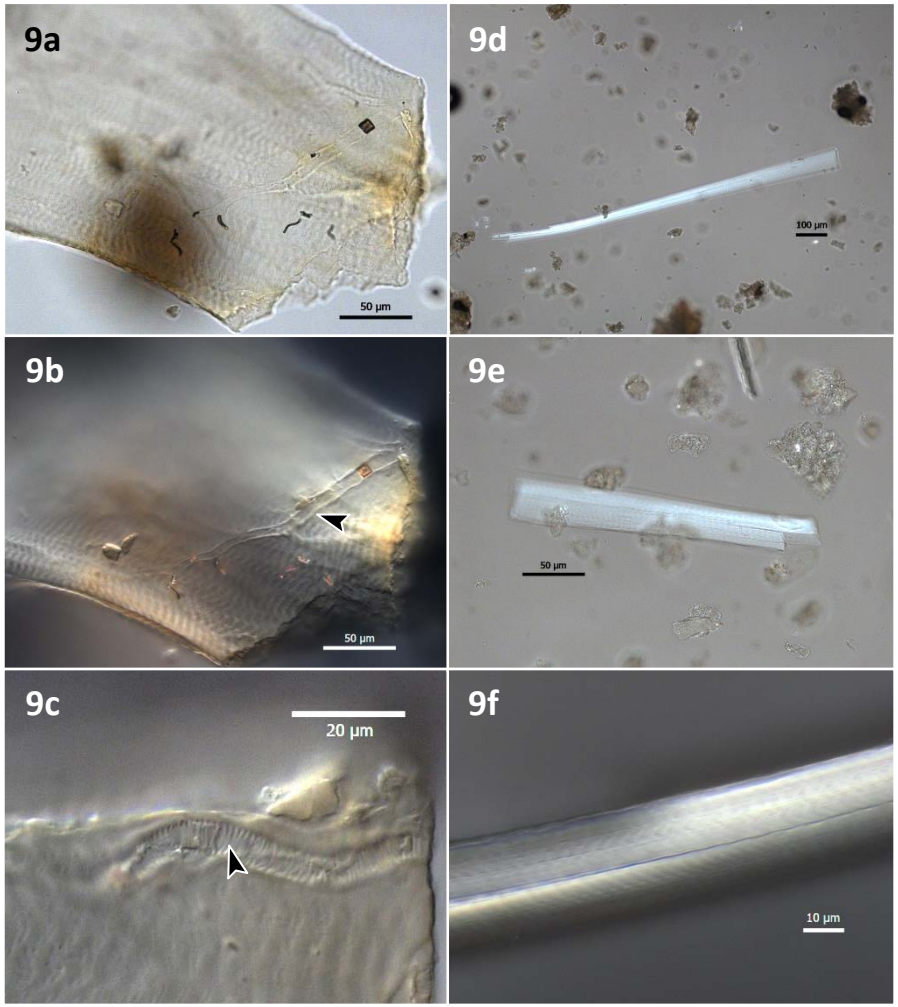

8
50 µm


9a
50 µm
9d
100 µm
9b
50 µm
9e
50 µm
9c
20 µm
9f
10 µm