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Development of a Method of Analysing TNT and its Derivatives in the Trichoptera Larvae of the Genus Hydropsyche Angustipennis, Curtis 1834, Selected as a Bioaccumulation Indicator for the Detection of Aquatic Environment Pollution with Explosive Residues

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Abstract. Threats associated with the production, storage and use of warfare agents containing high explosives against the backdrop of political and social events emerging in the 21st century present new challenges for specialized laboratories involved in detecting contamination and identifying its source. A common analytical problem in studies conducted in the ecosystem is the low concentration of the test substance - below the detection limit of the instrument. Helpful in solving this dilemma are accumulation bio-markers, which appear to be an excellent tool for detecting contamination in, for example, flowing water. Such biomarkers make it possible to determine the presence of specific chemical agents at the same time they are a sensitive indicator of the ecosystem's response to contamination. The presented chromatographic method allows quantitative and qualitative determination of 2,6-diamino-4-nitrotoluene, 2,4-diamino-6- nitrotoluene, 1,3,5-trinitrobenzene, trinitrotoluene, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and tetryl in the biological matrix. The effects of accumulation in the tissue of crustaceans on TNT test solutions were studied from 1 to 24 hours. The saturation effect was observed and the concentration of TNT derivatives was measured. The observed effects confirmed the usefulness of the selected larva as a bio-indicator of TNT contaminant accumulation in the ecosystem.
Keywords: security, contamination, HPLC, TNT, bio-indicator

1. INTRODUCTION

Trinitrotoluene (TNT) is a high explosive material which, by virtue of its relatively uncomplicated and low-cost production, stability, meltability, cast moulding, and hot moulding into shells, has found many applications around the globe, both military (as an explosive charge in warheads, mines, and other munitions) and commercial (for the production of commercial blasting products, such as detonating charges and demolition explosives). TNT is manufactured by sequential nitration of toluene with a mixture of nitrous and sulphuric acids in a batch process, resulting sequentially in mononitrotoluene, dinitrotoluene, and ultimately, trinitrotoluene (it is a continuous process with a cross-flow of acids and the organic phase, toluene). The 2,4,6-TNT isomer is treated to strip asymmetric TNT isomers using sodium sulphite by production of water-soluble dinitrotoluene-sulphonic acids, which are waste byproducts [1]. The wastewater from TNT production is called ‘red water’ or ‘pink water’, as it turns pink after exposure to light at neutral to alkaline pH. The wastewater is loaded with TNT and dinitrotoluene compounds [2, 3]. Testing for the presence of TNT in the environment [4] demonstrated photolytic and redox reactions which lead to the formation of various decomposition products, as shown in Fig. 1.

During production, storage, and application of explosives such as TNT (trinitrotoluene), occupational or accidental exposure to the chemicals listed above may ensure by digestion, inhalation, or through skin contact [5-9]. Depending on the duration of exposure, the effects on the human system may result in liver damage and anaemia, irritation of the mucosa, leucocytosis or leukopenia, muscular pain, cardiac irregularities, and kidney irritation [10-12]. The metabolism of TNT is dominated by reduction paths.

The primary TNT metabolites in urine are 4-amine-2,6-dinitrotoluene and 2-amine-4,6-dinitrotoluene,[1] whereas people exposed to TNT reveal the formation of haemoglobin adducts of amine-dinitrotoluene compounds [11].

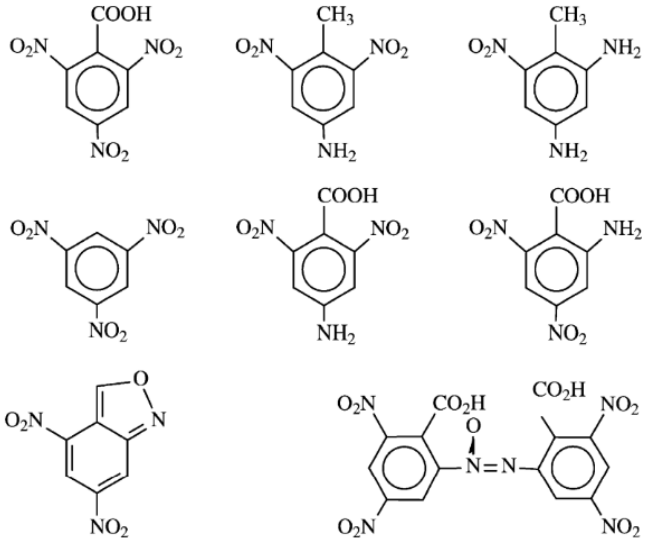

Fig. 1. TNT derivatives – degradation products [4] – in the order shown are: 2,4,6-trinitrobenzoic acid, 4-amine-2,6-dinitrotoluene, 2,4-diamine-6-nitrotoluene, 1,3,5-trinitrobenzene, 4-amine-2,6-dinitrobenzoic acid, 2-amine-4,6-dinitrobenzoic acid, 4,6-dinitro-1,2-benzoxazole, 2',4,4',6'-tetranitro-2',6-azoxydibenzoic acid

Contamination of soil and water with explosive materials could be related to poor waste disposal during production, demilitarisation operations, military training, UXO (unexploded ordinance) left on former battlefields, and disposal techniques in manufacturing processes where poorly treated wastewater is discharged to water streams. Groundwater contamination is usually highly dependent on rain and snowfall, and the solubility of the chemical contaminant and the kinetics of its solubility [13]. TNT from munitions production plants, explosive and munitions storage facilities, and demilitarised facilities certainly continues to infiltrate or may still infiltrate soil and water [14], which is currently a global problem of exposure of humans, vegetation and animals to environmental pollutants from TNT and its derivatives.

[1] Most research suggests that excretion of amine-dinitrotoluenes (4-amine-dinitrotoluene with 2-amine-dinitrotoluene) at concentrations from 1 to 10 mg L(-1) (5–50 mM) in urine is not uncommon, for example, in professionals who handle military waste disposal. TNT is mutagenic to certain strains of Salmonella typhimurium, with or without exogenous metabolic activation. Mutagenicity was found in the urine of professionals with occupational exposure to the effects of TNT.

It is imperative to understand the toxicological effects of explosives on the natural environment, and for various military conflicts around the world, it is essential to develop a method of detecting explosive compounds. To achieve this objective, indicator organisms should be selected along with a laboratory methodology for the detection of the pollutants. In the case of a routine method of testing potentially contaminated sites, the method should facilitate simultaneous determination of commonplace secondary explosives, their manufacturing contaminants and environmental transformation products; the method should use standard laboratory equipment, be resilient enough so that deviations from the standard procedure do not significantly affect the reliability of the test results, and ensure a limit of detection which is no less stringent than the criteria established for human safety and environmental protection.

2. PREVIOUS RESEARCH

The works published to date discussed research into testing TNT's toxicity to amphibians [15], aquatic invertebrates [16 - 18] and fish [5, 19] in aquatic ecosystems for risk assessment and identification of remediation objectives[2] for contaminated sites. For the bioconcentration and bioaccumulation of TNT in aqueous organisms, only several research projects were completed (such as experiments for assessing the bioaccumulative potential of TNT in benthic invertebrates and insects or for assessing the TNT exposure by digestion severity in the accumulation of TNT in fish) [13].

The test methods are usually developed to ensure worst-case scenarios of exposure and facilitate conservative estimation of the risks posed by the accumulation of TNT and its biotransformation products in living organisms (as an effect of oral administration or osmosis).

The basic assessment of the impact of the pollutants of interest on aquatic ecosystems most often involves physical and chemical testing of water quality, as well as a biomonitoring of the living organisms present in the ecosystems [20]. It is often found that various contaminants present in water become diluted or displaced (in flowing water), rendering them impossible to detect with analytical test methods due to reduced concentration (that is, below the limits of detection of test equipment). However, if living organisms accumulate in the tissues a contaminant they are in contact with, the contaminant's 'fingerprint' lasts longer. This is why, while assessing the contamination of the surface water environment, researchers now focus on monitoring bioindicator species [21, 22].

[2] Pursuant to Journal of Laws 2021.0.1973 – Polish Environmental Protection Law of 27 April 2001 – remediation is *soil, land and groundwater activities to remove or reduce the number of substances causing risk, to control them, and to reduce the spread so that the [polluted area stops posing] a risk to human health or the state of the environment, taking into account the current and, if possible, planned future use of the site; remediation may rely on self-cleaning, if it brings the greatest environmental benefits.*

Specific taxons used as universal single-species bioindicators of accumulation in the contamination of water seem to be a promising tool for detecting contamination, even some time after the direct contamination release event.

3. MATERIALS AND METHODS

3.1. Chemicals

The trinitrotoluene (TNT) used to create the test conditions during the experiment was sources from a Polish manufacturer. The analytical class standards for the TNT derivatives most prevalent in the reference literature from around the world, meaning 4-amine-2,6-dinitrotoluene (4-ADNT) and 2-amine-4,6-dinitrotoluene (2-ADNT), were sourced from Merck Sp. z o.o., whereas 2,4-diamine-6-nitrotoluene (2,4-DANT) and 2,6-diamine-4-nitrotoluene (2,6-DANT) were sourced from LGC Standards.

An aqueous stock solution of TNT was prepared by dissolving pure TNT, with holding at heat and stirring for 24 hours, followed by dilution to the target concentration of TNT. The test environment set up was a 5 mg/L aqueous TNT solution.

3.2. Biological material

For this research, Trichoptera larvae of the genus *Hydropsyche angustipennis* were chosen in their fifth (and most advanced) larval stage. To estimate the TNT accumulation, a Trichoptera larvae sampling stand on the Mroga river was selected, near the town of Bogdanka, close to Koluszki, Poland. The Trichoptera larvae sourced from the natural environment were conditioned to achieve laboratory conditions. A refrigerated display was used and set to the temperature of water from which the larvae were sampled. A standard conditioning run of 3 full days was performed, during which the larvae eliminated the previously digested food to ensure their excrements would not affect the transformation/adsorption of the test contaminant.

3.3. Exposure conditions and experimental setup

The experiment was preceded by pilot tests to assess how the larvae of the aquatic insect species of interest would behave in contact with TNT. It was determined that the optimum duration of exposure to TNT would be 2 full days, as, for the selected bioindicator organism to be applied in the role of a potential environmental contamination detector, it must react promptly to exposure to the contaminant of interest.

The Trichoptera larvae (each approximately 2 cm long and with an approximate body weight of 30 mg) were held in 400 ml plastic containers, each with 10 specimens, and exposed to the effects of the 5 mg/L aqueous TNT solution which provided the test environment. Three test iterations were performed for each exposure duration. A control was adopted with a test environment free of the stressor, and subsequent containers with the specimens held for the duration of exposure to the stressor. The exposure solutions made with distilled water were not replenished or aerated during the exposure. Each container was held in the refrigerated display (holding temperature 11 ± 1 °C, no access to light). The environmental conditions (steady holding temperature and limited effects of UV radiation) were close to those encountered during the sampling of the Trichoptera larvae from the Mroga river, which made it possible to gauge any potential stress and thermal shock of the tested specimens during cultivation which could trigger a deviation from the individual behaviour and affect the accumulation physiology of the test contaminant.

In the sampling experiments (at control times of t_t = 1/3/6/10/24/48 h) the duration of exposure to TNT was up to 48 h. After 1 h and 24 h, the control specimens (held in pure water without TNT) were sampled. In the self-cleansing experiments, some of the larvae (at control times of t_t = 1/3/6/10/24 h) were processed for 24 h by depuration in distilled water. Next, the larvae were sampled from the test environment (both for the exposure and self-cleansing experiments) and kept frozen until laboratory analysis of their tissues. All 30 larvae from each control time of this experiment were collected for tissue analysis (none of the specimens died during the experiment).

3.4. Chemical analysis

Stock solutions were made with the standard chemicals, 1,3,5-trinitrotoluene, 2-amine-4,6-dinitrotoluene, 4-amine-2,6-dinitrotoluene, 2,4-diamine-6-nitrotoluene, and 2,6-diamine-4-nitrotoluene. Analysis was run with a selection of conditions to provide optimum separation of the standards. For each test sample, the frozen larvae were divided into the chitin-clad part (the head and the thorax with the legs) and the soft tissue part (the abdomen). The dividing cut was made directly behind the third section of the carapax that covered the thorax with the legs. Each of the tissue parts was weighed (the chitin clad part was ca. 0.100 g, the soft tissue part was ca. 0.250 g), deep frozen, and mechanically ground, followed by dissolution in acetylonitrile and filtering through FilterBio® NY 0.45 μm pore size Nylon syringe filters. The test specimens thus obtained were analysed by High Performance Liquid Chromatography (HPLC), with an Intersil C8 5 μm 4.6 × 150 mm column that features an 18:82 (v/v) mobile phase of 2-propanol to water, using an isocratic method (230 nm UV detector). A blind test of this method was also performed.

4. RESULTS

4.1. Absorption and metabolism over time – an example comparison of SEI and MEI (1/24 h of exposure)

The levels of TNT and its metabolised derivatives in the specimens of the chitin-clad parts and soft tissue parts were compared to one another for the control times of 1 h and 12 h (exposure duration) to determine the effect (attenuation) of the detection signals by the biological matrix. The chromatograph produced (see Fig. 1) for the comparison of the chitin-clad part (designated 'SEI') to the soft tissue part (designated 'MEI') of the test organisms at the control times of 1 h and 24 h suggested that the soft tissue part exhibited significantly higher absorption of TNT, coupled with an equally significant increase in the concentration of two amine derivatives of TNT (2-amine-4,6-dinitrotoluene and 4-amine-2,6-dinitrotoluene), which were formed by metabolism.

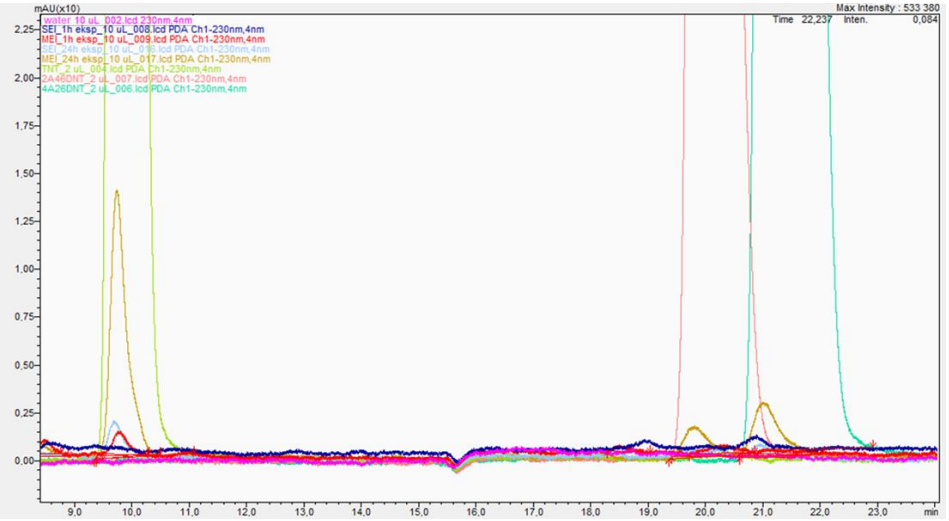

Fig.1. Comparative chromatograph of the levels of TNT and its metabolised derivatives in the specimens of the chitin-clad parts and soft tissue for the control times of 1 h and 12 h (exposure duration) to determine the effect (attenuation), with superimposed standard plots

A graphical comparison was made for the determined levels of TNT, and its derivatives metabolised in the specimens of the chitin-clad parts and soft tissue parts were compared to one another for the control times of 1 h and 12 h (exposure duration) to determine the effect (attenuation). The result for the 24 h exposure to the stressor, when compared to the 1 h exposure result, was already satisfactory and boded very well for the feasibility of the selected living organisms as bioindicators (see Fig. 2).

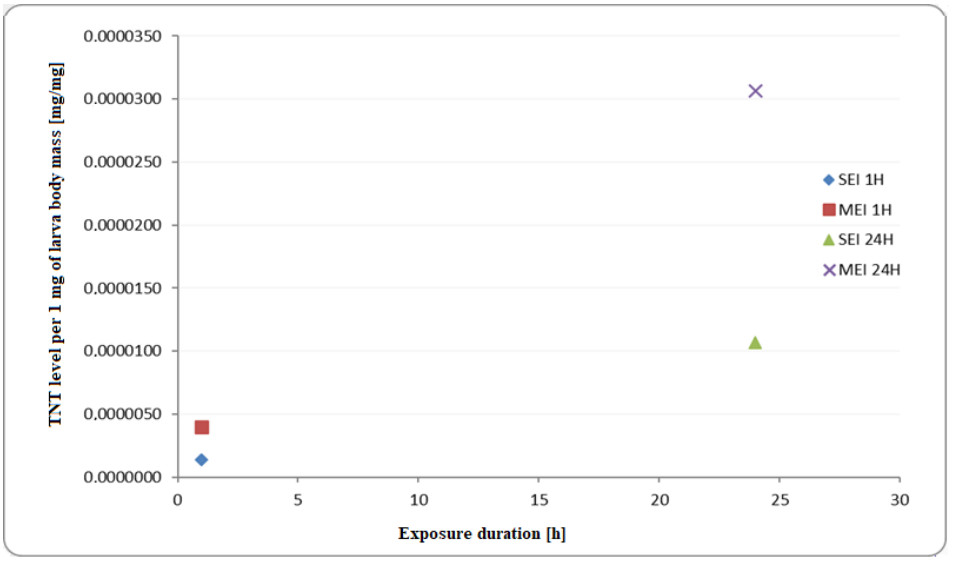

Fig. 2. Comparison of the levels of TNT in the specimens of the chitin-clad parts and soft tissue for the control times of 1 h and 12 h (exposure duration) – the chitin-clad part is detailed

The significant difference in the levels of determined TNT values between the chitin-clad part and the soft tissue part, shown in Fig. 2 above, suggested that the chitin-clad part absorbed less of the stressor and that the analysis is loaded with a higher bias of determination of the analytes of interest.

Figure 3 provides another overview of a detailed chromatograph produced from HPLC of the chitin-clad part (SEI) and the soft tissue part (MEI) at the control time of 24 h, inclusive of the exposure and the self-cleansing process. The results obtained for the chitin-clad and soft tissue parts of the Trichoptera were partial to the proper metabolism of the environmental factor by the specimens' bodies. The differences in the TNTH and derivative detection signals between the chitin-clad part, shown in green (low, broad peaks without separation down to the baseline) versus the soft tissue part, shown in blue (high, slender peaks with good separation) confirmed the higher absorption of the soft tissues and their feasibility as potential bioidentifiers for aquatic contamination with TNT.

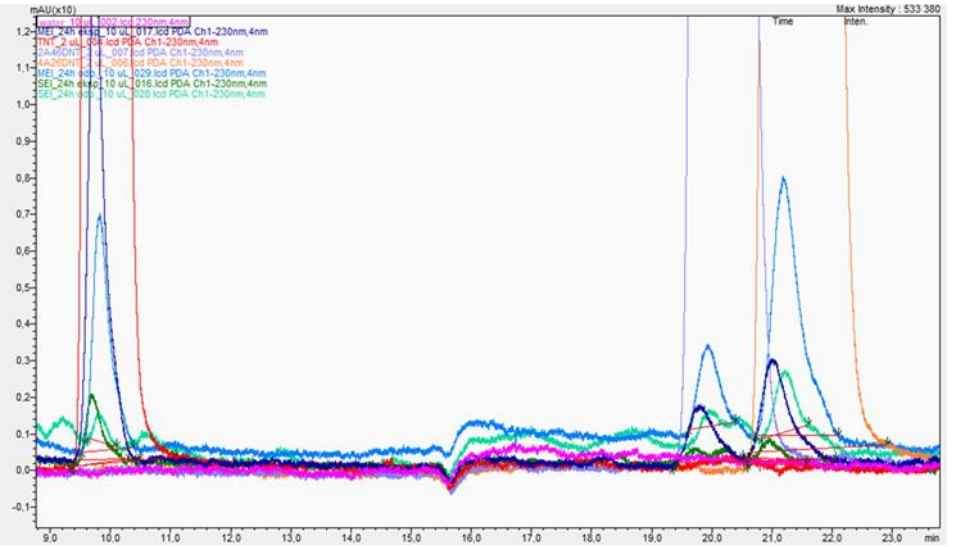

Fig. 3. Comparative chromatograph of the levels of TNT in the specimens of the chitin-clad parts and soft tissue for the control time of 12 h (exposure – self-cleansing) – the chitin-clad part is detailed

The distinct difference in the TNT levels between the exposed specimens and the self-cleansed specimens, as well as the determined higher contents of the derivatives (2-amine-4,6-dinitrotoluene and 4-amine-2,6-dinitrotoluene) in the soft tissue parts of the specimens made it possible to decide on the significance of the results produced from the soft tissue parts. A major contributing factor of the decision was that, for the chitin-clad parts, the levels of the substances of interest at the initial control times were below the limit of detection. Moreover, the biological matrix of the chitin-clad part increased the noise (meaning lower SNR), which translated into a higher measurement uncertainty.

4.2. Accumulation and metabolism over time

Chart 4 shows a summary chromatograph of the analyte level change trends in the test organisms, exemplified by the soft tissue parts (MEI) in successive test samples (at control times t_t = 1/3/6/10/24 h). The analytical results produced explicitly pointed to a relationship between the TNT level in the organic specimens and the levels of two TNT amine derivatives (2-amine-4,6-dinitrotoluene, 4-amine-2,6-dinitrotoluene) versus the exposure duration. No diamine derivatives of TNT were detected.

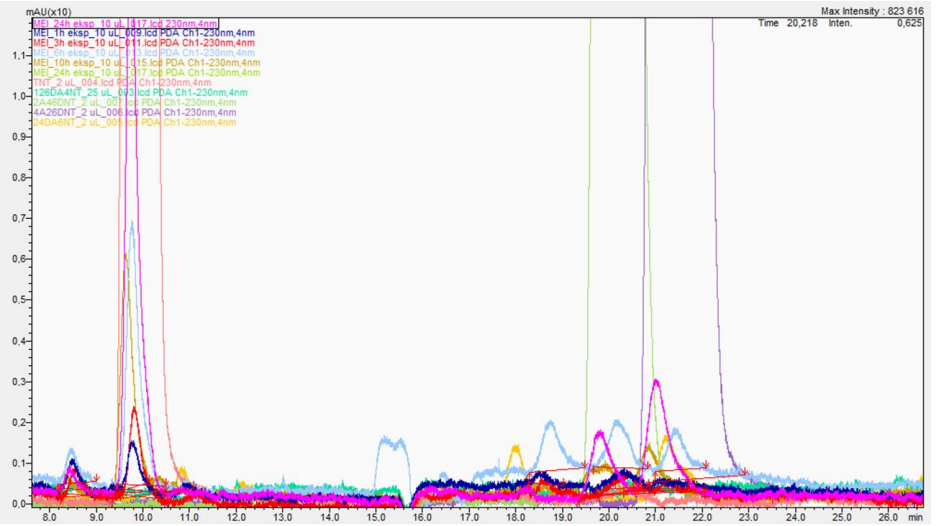

Fig. 4. Summary chromatograph for the soft tissue parts (MEI) at successive control times (t_t = 1/3/6/10/24 h) of residence in the text environment (exposure) – analyte elution range

A correct relationship between the change in the levels of TNT and its two amine derivatives versus the control times and absorption of TNT by the organisms in the successive control points, with increasing levels of metabolites, 2-amine-4,6-dinitrotoluene and 4-amine-2,6-dinitrotoluene, in the organic specimens.

The changes in time shown in the chromatographs were subtle but satisfactory when translated into the relationship between the quantities of the tested chemicals and the exposure duration. The relationship of the produced TNT derivatives during exposure and absorption of TNT vs. the duration (the selected control times) of exposure to the stressor is shown in Fig. 5.

The saturation of the test organisms in time was observed (and confirmed in the pilot tests), with a distinct relationship between the increase of two amine derivatives of TNT in the samples versus the exposure elapsed and the level of TNT in the test environment at successive control points. At the control time of 24 h, the detected signal was clear and unambiguous, both for TNT and its derivatives; hence the conclusion that the organisms responded to the contaminant quickly enough to be considered as feasible bioidentifiers.

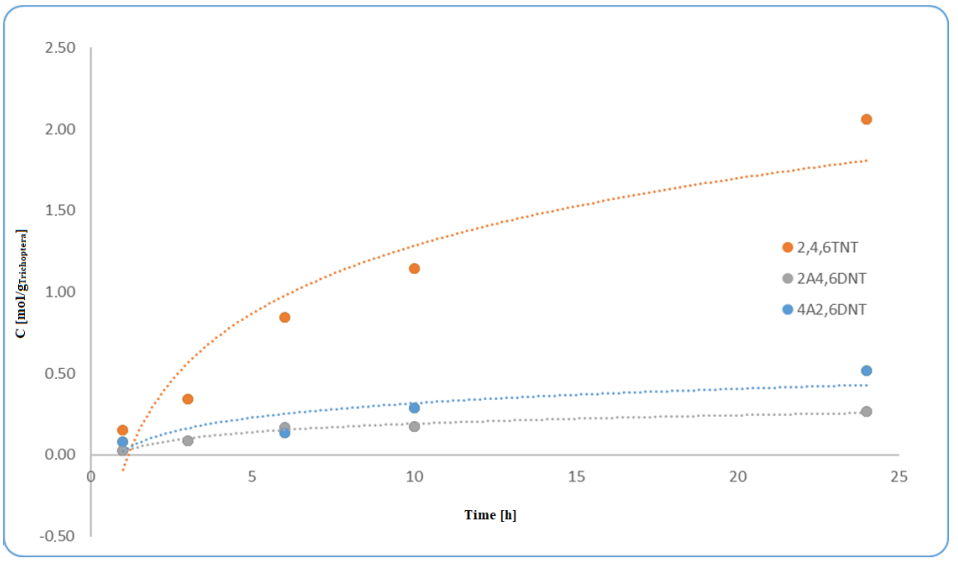

Fig. 5. TNT absorption and metabolism over time on the example of MEI

4.3. Self-cleansing of the organisms of TNT and its metabolites

Another test group of the Trichoptera larvae were organisms which, after a suitable duration of exposure to TNT in the test environment, were processed by depuration, or cleansing. The changes of analyte levels in the organisms exemplified by the soft tissue parts at the successive control times (t_t = 1/3/6/10/24 h) that were processed by depuration are shown in chromatographs, see Figs. 5 and 6. The differences in the TNT residue levels were slight at control times of 1 h, 3 h, 6 h and 10 h after depuration, followed by a significant reduction in the TNT residue levels in the 24 h sample. The analytical results explicitly suggested than the TNT concentration after 24 hours of exposure, followed by depuration (during which TNT was metabolised into its amine derivatives) was the lowest, but still determinable, and with the highest levels of the amine derivatives of TNT (2-amine-4,6-dinitrotoluene and 4-amine-2,6-dinitrotoluene). No diamine derivatives of TNT were detected.

A considerable increase in the amine metabolite levels, being the TNT derivatives, was found for the test organisms with 24 h of exposure in comparison to the test organisms after shorter durations of exposure. The differences between the 3 h, 6 h and 10 h exposure samples, post-depuration, were slight. The changes in the TNT residual levels of the test organism after the depuration are shown in Fig. 6. The increasing levels of the metabolites (2-amine-4,6-dinitrotoluene and 4-amine-2,6-dinitrotoluene) in the samples are shown in Fig. 7.

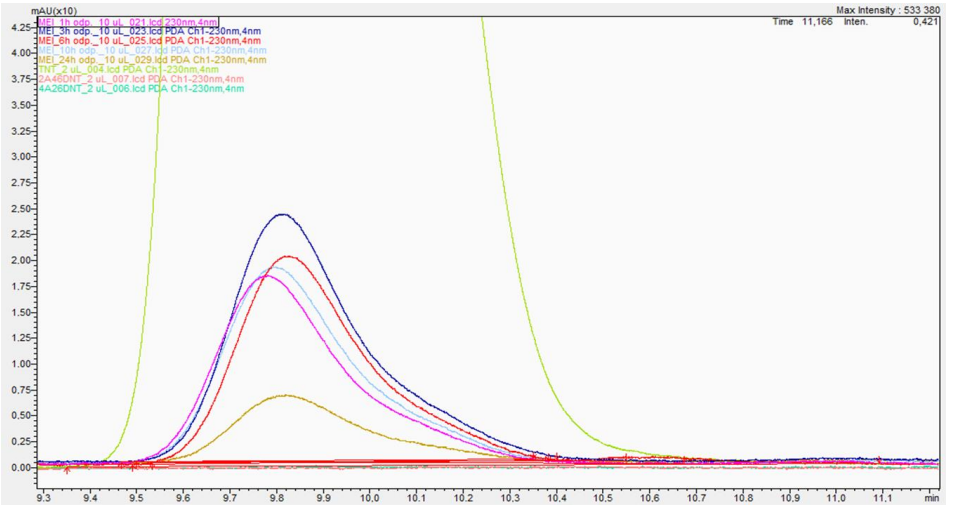

Fig. 6. Summary chromatograph of TNT levels in the soft tissue parts (MEI) processed by depuration at successive control times (at t_t = 1/3/6/10/24/48 h) – TNT elution range

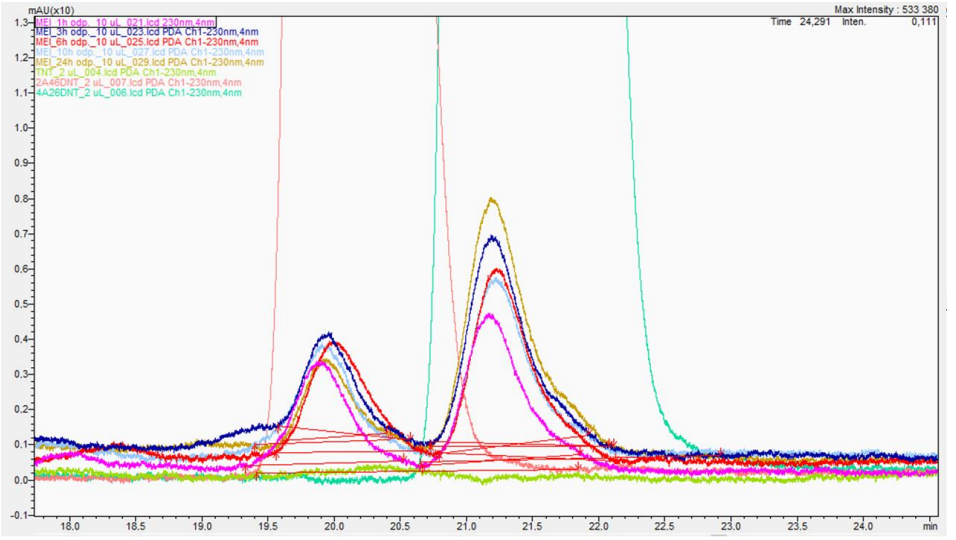

Fig. 7. Summary chromatograph of TNT levels in the soft tissue parts (MEI) processed by depuration in the subsequent test samples (at t_t = 1/3/6/10/24/48 h) – TNT derivative elution range

The relationship between the test compounds and exposure duration was also satisfactory. The levels of TNT and its derivatives determined at selected control times in the soft tissue parts (MEI) of the organisms processed by depuration are shown in Fig. 8.

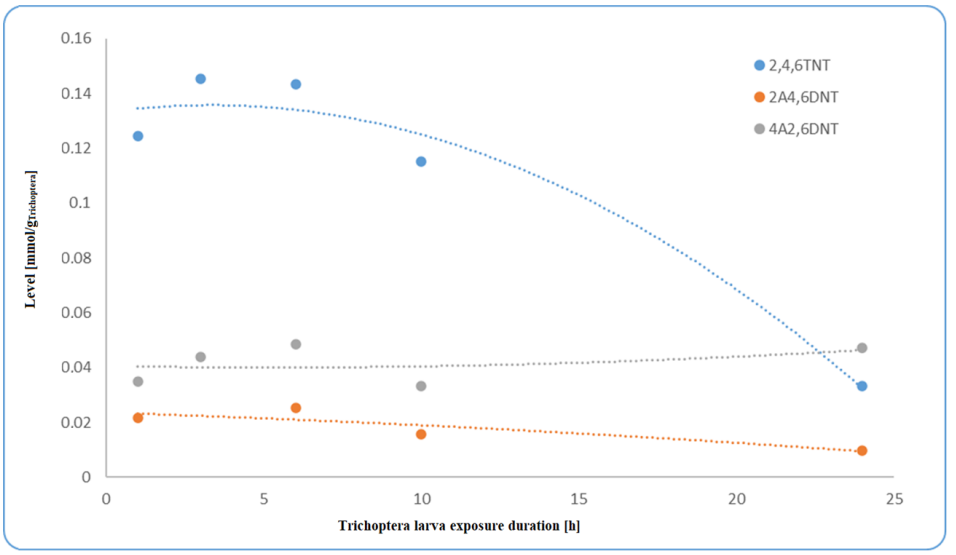

Fig. 8. Levels of TNT and its derivatives determined at selected control times in the soft tissue parts (MEI) of the test organisms processed by depuration

5. SUMMARY

A comparison between the tests of the chitin-clad parts and the soft parts of the test organisms at control times of 1 h and 24 h to determine the effect of the tested tissues/body parts on the intensity of accumulation of the test contaminant indicated that the soft tissue part, being the abdomen of the Trichoptera larvae, would absorb significantly more TNT, with an equally significant increase of two metabolites, which are amine derivatives of TNT (2-A-4,6-DNT and 4-A-2,6-DNT). The chitin-clad part, comprising the epicranium and thorax, would absorb less of the stressor and the analysis of this larva part was encumbered by a higher uncertainty in the determination of the analytes of interest (the TNT and its derivatives). The results produced after 24 hours of exposure to the stressor permit the use of the insect organisms selected for this work as quick bioindicators of TNT contamination in aqueous environments.

The relationship between the tested compounds and the exposure duration of the test organisms exposed to the stressor and post-depuration is shown in Fig. 9, which provides a graphical summary of all relationships that occurred during the experiment: absorption, metabolism, and cleansing.

The determined levels of TNT and its metabolised derivatives, as shown in Fig. 9 and varying in time, at the successive control points on the example of the soft tissue part (MEI) of the test organisms exposed to TNT and depurated, confirmed that Trichoptera larvae are suitable for biomonitoring of water ecosystem contamination.

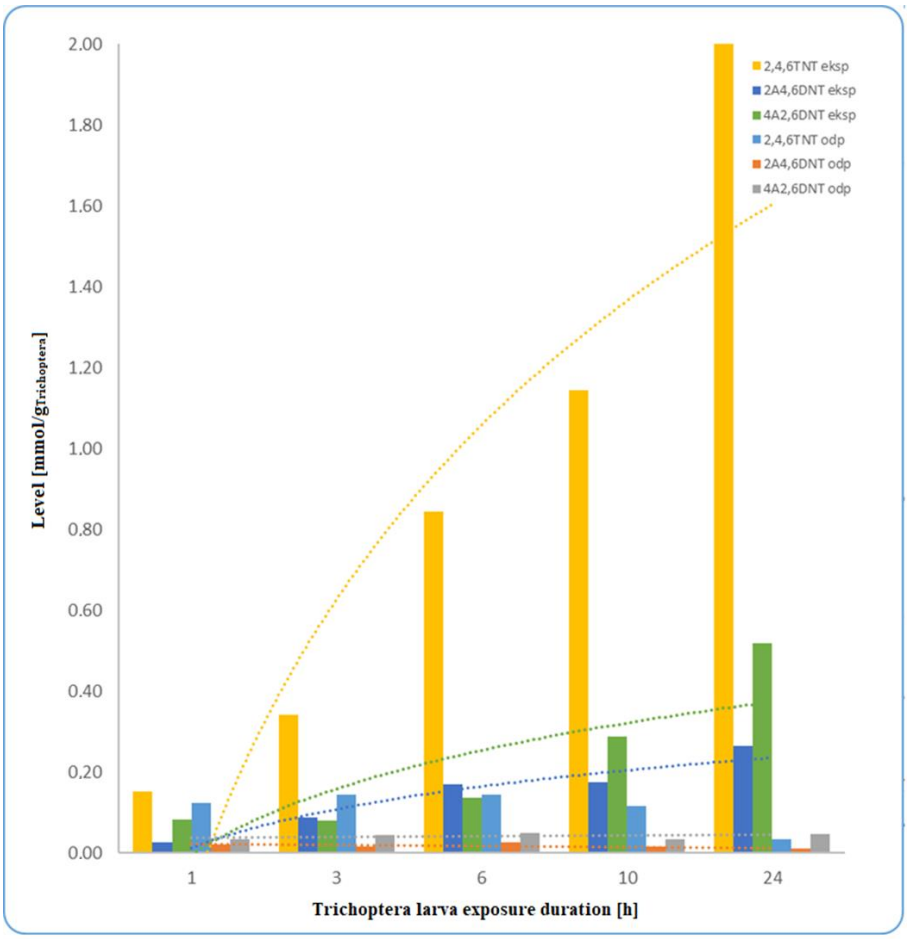

Fig. 9. Summary of the levels of TNT and its derivatives determined at selected control times in the soft tissue parts (MEI) of the test organisms exposed to TNT and processed by depuration

6. CONCLUSIONS

The results of the pilot tests and the initial research lead to the conclusion that the developed method for determining the levels of TNT and its derivatives in organic samples is correct. It was demonstrated that in their fifth larval stage, the Trichoptera larvae of the genus *H. angustipennis* are a promising material, feasible as potential accumulative bioindicators of aquatic environment contamination with TNT, that satisfied the prerequisite prompt response to this type of pollution. It seems to be sensible to pursue broader research and implement a monitoring method based on this insect species as an accumulative bioindicator of TNT contamination in aquatic environments.

This research could serve to identify the potential risk of human exposure to TNT released to groundwater. The developed method of analysis of the levels of TNT and its derivatives in the test organisms permits simultaneous analysis of the presence of other explosives. It is planned to perform more experiments with other high explosive materials, such as hexogen (RDX), penthrite (PETN) and tetryl, used in the defence industries and mine blasting, whose residuals are present in areas of explosive storage and former military operations, remaining environmental contamination hazards.

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Opracowanie metody analizy trotylu oraz jego pochodnych w larwach chruścików z gatunku wodosówka potokowa (Hydropsyche angustipennis, Curtis 1834) typowanego jako biowskaźnik akumulacji do wykrywania zanieczyszczenia środowiska wodnego pozostałościami po materiałach wybuchowych

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Streszczenie. Zagrożenia związane z produkcją, przechowywaniem oraz użyciem środków bojowych zawierających kruszące materiały wybuchowe na tle wydarzeń politycznych i społecznych pojawiających się w XXI wieku stanowią nowe wyzwania dla specjalizowanych laboratoriów zajmujących się wykrywaniem skażeń oraz identyfikowaniem źródła jego pochodzenia. Częstym problemem analitycznym w badaniach prowadzonych w ekosystemie jest niskie stężenie badanej substancji – poniżej limitu detekcji urządzenia. Pomocne w rozwiązaniu tego dylematu są biowskaźnik kumulacji, które wydają się doskonałym narzędziem do wykrywania zanieczyszczeń np. wody płynącej. Biomarkery takie umożliwiają stwierdzenie obecności określonych czynników chemicznych jednocześnie są czułym wskaźnikiem reakcji ekosystemu na skażenie. Przedstawiona metoda chromatograficzna pozwala ilościowo i jakościowo oznaczyć 2,6-diamino-4-nitrotoluen, 2,4-diamino-6-nitrotoluen, 1,3,5-trinitrobenzen, trinitrotoluen, 2-amino-4,6-dinitrotoluen, 4-amino-2,6-dinitrotoluen oraz tetryl w matrycy biologicznej. Badano efekty kumulacji w tkance chruścików na roztworach testowych trotylu w czasie od 1 do 24h. Zaobserwowano efekt wysycenia oraz zmierzono stężenie pochodnych trotylu. Zaobserwowane efekty potwierdziły użyteczność wytypowanej larwy jako biowskaźnika akumulacji zanieczyszczeń trotylu w ekosystemie. Zaobserwowane efekty potwierdziły użyteczność wytypowanej larwy jako biowskaźnika akumulacji zanieczyszczeń trotylu w ekosystemie.

Słowa kluczowe: bezpieczeństwo, zanieczyszczenie, HPLC, trotyl, biowskaźnik