Carbon nanomaterial functionalization with pesticide-detoxifying carboxylesterase enzyme

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Abstract

Four carbon materials, spent coffee ground biochar, carbon black, short CNTs and N-doped few-layer graphene were tested for functionalization with a commercial carboxylesterase. Their robustness to variations in time and key physicochemical parameters (temperature and pH) was analysed. In general, carbon nanomaterials showed better performance than biochar, both in terms of binding capacity and resilience in harsh conditions, at statistically significant levels. Among the tested materials, functionalized N-graphene also showed the highest level of inhibition of carboxylesterase by pesticide exposure. Therefore, N-graphene was selected for biotechnological application of pesticide scavenging toxicity in *T. thermophila*, a ciliate bioindicator of water quality. While immobilisation of the enzyme was not effective for carbaryl, a methyl carbamate, in the case of the organophosphorus dichlorvos, a 1- or 30-min contact time with a water solution containing 5 times the LC100 -0.5 mM- allowed 50% and 100% rescue of ciliate survival, respectively. These results indicate that functionalization with carboxylesterase may be of additional benefit compared to bare carbon in water clean-up procedures, especially for highly hydrophilic pesticides such as dichlorvos.

1.Introduction

In the last few decades Nanotechnologies (NTs) has offered ground-breaking innovation in several industrial and technological fields such as coating and painting, automotive, electronics and medicine. Since 1980 a lot of new nanomaterials have been discovered and characterised, among these, carbon-based nanomaterials (NMs), like carbon nanotubes (CNTs), fullerenes and more recently graphene have been the most promising candidates for their unique structural, chemical and physical characteristics (Hirsch et al., 2010; Oliveira et al., 2015; Yan et al., 2019). Particularly, CNTs were discovered in 1991 by Iijima (1991), and are composed by one (single-walled) or more cylinders (MWNTs) formed of graphene sheets with diameters of 1-3 nm or 5-40 nm, respectively. Their structure gives them unique characteristics of rigidity, strength and elasticity compared with other non-nano materials. One major feature is the High Aspect Ratio (HAR) and high thermal and electrical conductivity. Unique optical and electrochemical characteristics of carbon-based NMs make them suitable also for imaging and tracking of (macro)molecules, optical biosensors, drug scaffolding and delivery, or to create catalytically active systems (Oliveira et al., 2015). Carbon-based NMs also have multiple environmental applications, such as fertilisers, plant growth stimulators, soil conditioners, and nano-encapsulated biocides. CNTs have been shown to gain adsorption capacity of microcystins (cyanobacterial toxins), heavy metals (e.g. lead and copper) outperforming standard activated carbons. Likewise, MWCNTs have been used also for immobilising different environmental contaminants like antibiotics, herbicides, pesticides, nitrogen or phosphorus, dyes, pharmaceuticals/drugs, organic pollutants like phenol and derivatives (Zaytsev and Neumann, 2016; Saxena et al., 2020). For these properties one of the most interesting and promising applications for CNTs is their use in wastewater treatment.

Among the various NMs, graphene seems to be the new top material that has attracted the attention of scientists in recent years. Interest in this material has grown thanks to physicochemical properties such as high electrical conductivity or high specific surface area, mechanical strength, among other desirable properties (Carmalin et al., 2016). It was discovered that it is also an remarkable adsorption material, so this ability has been studied for various applications. The structure of graphene itself seems to give it optimal adsorption ability, and the negative surface charge of graphene oxide (GO) or reduced graphene oxide (rGO) helps in the efficient removal of cationic pollutants such as heavy metals. In addition, graphene has been successfully used in the adsorption of organic pollutants in various forms of polycyclic aromatic hydrocarbons, gasoline, and dyes (Nupearachchi et al., 2017; Lu and Astruc, 2020). Graphene is used to remove pharmaceutical traces from water and wastewater; it is able to remove aspirin, acetaminophen and caffeine, GO is used to remove dorzolamide, tetracycline, ciprofloxacin, norfloxacin, diclofenac, sulfamethoxazole, beta blockers. Graphene nanosheets and GO are used to remove naphthalene, 2-naphthol, 1-naphthylamine and tylosin; rGO can adsorb organic compounds such as ketoprofen, carbamazepine and bisphenol (Ji et al., 2010; Yuan et al, 2012; Tang et al, 2013; Ghadim et al, 2013; Liu et al, 2014; Al-Khateeb et al, 2014; Kyzas et al, 2014; George et al, 2015; Nam et al, 2015; Carmalin et al, 2016; Lu and Astruc, 2020). Thanks to the properties of graphene, research is focused on developing and improving new methods and techniques for engineering and biological remediations. Furthermore, graphene has been used successfully as a matrix for enzyme immobilisation in different technological applications (Soozanipour and Taheri-Kafrani, 2018; Zhang et al 2010).

Bioremediation enables the removal of toxic compounds in an environmental compartment, and consists in the chemical decomposition by organisms minimising the formation of intermediates of complicated degradation (Matula et al., 2020). The potential of bioremediation was recognized decades ago by Raymond et al. (1975), who reported the degradation of petroleum-derived hydrocarbons by microbial populations. Nowadays, bioremediation is mainly performed with native or indgenous microorganisms because of restrictive regulations in the USA and Europe that limit or prohibit the use and release of genetically modified organisms into the environment. To overcome this restriction, many researchers have explored the potential use of cell-free enzymes to decontaminate the environment (Thakur et al., 2019). Pollutants that are susceptible for enzymatic bioremediation include organophosphorus compounds (OP) (Oakeshott et al. 2004).

Pesticides remain an integral part of agricultural practice, with tens of thousands of tons used worldwide each year. OP compounds account for over 38% of total pesticides used, and the World Health Organisation confirms that there are three million pesticide poisonings each year. In addition, these compounds are often mobile and can contaminate aquifers and other environmental compartments. All these characteristics make OPs priority targets for bioremediation studies. An ideal candidate for OP enzymatic remediation must be able to rapidly detoxify the contaminant and be environmentally friendly without causing lasting harm to the environment. Several enzymes capable of degrading OPs occur in nature, and much research is being done to improve their efficiency and stability. The efficiency and stability of enzymes have been explored in a variety of ways, from direct modification of the amino acid sequence to immobilisation on solid supports or in membrane vesicles. One example is the use of phosphotriesterase-immobilised on semiconductor quantum dot nanomaterials, creating a highly active enzyme structure with a 2-fold increase in efficiency over the free enzyme (Thakur et al., 2019). From this perspective, the aim of this work was to develop a safe, stable, and efficient application of carbonaceous nano- and non-nano-materials with an OP-inactivating enzyme.

In this study, we compared four carbon (nano)-materials for a biotechnological application to remediate freshwater from OP and methylcarbamate pesticides. We considered three nanomaterials (1D, 2D, and 3D, respectively carbon nanotubes), N-graphene, a commercial carbon black nanoparticle, and biochar derived from spent coffee grounds. After determining the optimal functionalization protocol and the robustness of enzyme immobilisation, we investigated the ability to reduce/abolish pesticide toxicity in the bioindicator ciliate *T. thermophila*.

2. Materials and methods

2.1 Carbon materials.

Biochar from spent coffee grounds used in this study has been previously described in Sanchez-Hernandez (2018). Carbon nanotube NTX1S was manufactured by Nanothinx S.A. (Rio Patras, Greece) into the framework of the H2020 MSCA-RISE project Nanogentools. According to the manufacturer, short NTX1 (sensu MWCNT-6 µm Sweeney et al., 2015), had 97% purity (as carbon content by thermogravimetric analysis), 30.6 mm external diameter and median length 1.1 µm as by scanning electron microscopy image analysis. The Carbon Black nanoparticles used in this work are commercially available under the Vulcan XC-72R™ (Fuel Cell, College Station, TX, USA). The nitrogen-doped graphene (N-graphene) was manufactured by means of exfoliation of graphite rods via pulses of current methods as previously described in Baldea et al., 2020 According to the manufacturer, in this batch the content of graphene-oxide, few layer graphene and monolayer graphene accounted for 6%, 75% and 19%, respectively. Elemental analysis of the sample indicated that the sample contains hetero atoms, nitrogen (2.41 % wt) and sulfur (0.93 % wt).

2.2 Functionalization of carbon materials

Biochar, carbon black, carbon nanotubes, and N-graphene were used for enzymatic functionalization using carboxylesterase as the model enzyme (porcine liver carboxylesterase, Sigma-Aldrich®, Merck, Darmstadt, Germany), following the method described by Sanchez-Hernandez (2018) with minor modifications. Essentially, the carbon materials were incubated for 1 h (continuous agitation) in sodium acetate buffer 0.05 M pH 5.2 at a ratio of 1 mg/mL (W/V) with serial concentrations of the enzyme (20-400 µg/ml) prepared in the same medium. Afterward, the carbon particles were centrifuged at 21460 g for 10 min at 10° C and washed twice in sodium acetate buffer. The supernatants were used to measure the residual enzyme not bound onto the particle surface. The bicinchoninic acid (BCA) method (Thermofisher-Scientifics, Waltham, MA, USA) was used to such a purpose using known concentrations of carboxylesterase as reference standards. In a typical functionalization trial, 1 mg of each carbon material was incubated with 400 µg carboxylesterase in sodium acetate buffer, as described. After the BCA assay, samples were diluted in acetate buffer at a fixed enzyme concentration of 200 µg carbon-bound enzyme mL^{-1} and then stored at 4°C for 1-5 days until needed. When needed, each preparation was diluted 1:10 to 20 µg carbon-bound enzyme mL^{-1} and used for subsequent analyses.

2.3 Enzyme activity assay

Aliquots of each functionalized (20 µg of carbon-bound enzyme) or not-functionalized carbon material were centrifuged for 10 min at 21460 g at 10°C. Pellets and supernatants were tested for carboxylesterase activity in 0.1 M Tris-HCl pH 8.4 in the presence of 2 mM 4-nitrophenyl butyrate (NPB) as substrate. Four independent reactions were continued for 5, 9, 12 and 15 minutes at RT. Samples were centrifuged and the absorbance of the supernatant recorded in a volume of 150 µl using a Infinite® 200 Pro (Tecan Sales AG, Mannedorf, Switzerland) plate reader photometer at 405 nm. For the calculation of the enzyme activity (Vmax) a path length of 0.42 cm was considered along with a molar extinction coefficient for nitrophenol of 18,330 M^{-1} cm^{-1} (Biggs 1954). True enzyme activity was calculated in the linear regime after subtraction of the intrinsic activity of non-functionalized carbon material. Definition of the carboxylesterase enzymatic activity, 1 Unit of enzyme catalyses the hydrolysis of 1 µmol min^{-1} of NPB to 4-nitrophenolate.

2.4 Stability of carboxylesterase activity

Stability of enzymatically functionalized carbons was tested in accordance with Petersen et al., 2011. To test the effect of time, functionalized carbon materials (20 µg of carbon-bound enzyme) prepared in sodium acetate buffer pH 5.2 were left at 25°C in the dark for 24h, 96h and 168h. Subsequently, the residual enzyme activity was evaluated in both pellet and supernatant. For temperature effects, functionalized carbon materials were incubated at 37 °C or 47 °C for 1 h in vertical agitation and residual carboxylesterase activity evaluated in pellet and supernatant. For pH, carbon materials were centrifuged to allow for buffer exchange using either 0.1 M Tris-HCl pH 8.4 or 9.5. After 1 h in vertical agitation at 25°C the residual enzymatic activity was evaluated in the pellet and supernatant.

2.5 Response of carboxylesterase functionalized carbons to pesticides

To test the response of functionalized carbons to selected pesticides, functionalized material (20 µg of carbon-bound enzyme) were incubated in sodium acetate buffer in the presence of 0.5 µM 2,2-dichloroethenyl dimethyl phosphate (dichlorvos) (Sigma-Aldrich®, Merck, Darmstadt, Germany) or 30 µM naphthalen-1-yl N-methylcarbamate (carbaryl) (Sigma-Aldrich®, Merck, Darmstadt, Germany) prepared from concentrated stock solution (10 mM dichlorvos in ethanol; 6 mM carbaryl in DMSO). Samples were set in vertical agitation for 1 h at 25°C. After centrifugation at 21460 g, 10 min at 10°C, carboxylesterase activity was determined in the pellet and supernatant as previously described.

2.6 Protective effect of carboxylesterase bound graphene against pesticide toxicity in the ciliate T. thermophila

T. thermophila CU427 was grown axenically in PPY medium to late log phase -10^6cells / ml density- at 30° C with agitation as previously described (Smith and Doerder 1992).

To determine the range of pesticide toxicity, approximately 10^{-4} ciliates were exposed to increasing concentrations of carbaryl or dichlorvos (1, 5, 10, 30, 50, 100, 500 µM) in physiological saline, 5 mM Tris-HCl pH 7.5, for 48 h. For survival assessment, organisms were counted after serial dilution in concave wells at 50X magnification. Cells were considered alive if swimming. The least full effective concentration for immobilisation (as an indicator of mortality) was 30 µM and 100 µM, respectively. This concentration is known as LC100.

Carboxylesterase-bound graphene was prepared in larger functionalization batches of 10 or 25 mg at a standard ratio of 400 μg carboxylesterase / mg carbon material, essentially as previously described. Carbaryl or dichlorvos were dissolved in 5 mM Tris-HCl, pH 7.5 at 100 or 500 μM, respectively (higher carbaryl preparations showed precipitation). Ten (10) or 25 mL of pesticide solution were incubated respectively with 10 or 25 mg of carboxylesterase functionalized or non-functionalized N-graphene for 1 or 30 min under vertical agitation. The contacted pesticide solutions were recovered by centrifugation at 4500 g for 20 min at 10°C in conical tubes and used for toxicity assessment. Residual toxicity in *T. thermophila* was determined as previously described.

2.7 Statistics

Data from at least four independent experiments were analysed by means of ANOVA after square root transformation and checking for homogeneity (Levene's test) and normality (Lilliefors K-S and Shapiro-Wilk tests). When ANOVA criteria could not be fulfilled, data were analysed using non parametric statistics (Kruskal-Wallis and Kolmogorov-Smirnov two sample tests) at $p \leq 0.05$. Other statistical details may be found in text. The Systat 12 (Systat Software, Inc, San Jose, CA, USA) statistical software package was used for computation. Stabilisation of carboxylesterase activity onto carbonaceous material was evaluated by measuring periodically the enzyme activity in the carbonaceous particles for a week. The enzyme activity was fitted to the first-decay kinetic mode:

$$EA = EA0 \times e^{(-kt)}$$

Where EA is the initial enzyme activity a time *t* (hours) after incubation the materials with a carboxylesterase solution, EA0 is the initial enzyme activity and k is the lost rate of enzyme activity. The model allowed us to calculate the half-time (t1/2) of activity lost.

3. Results

3.1 Functionalization of carbon materials with the carboxylesterase enzyme

Using a range between 20-400 µg/mL carboxylesterase enzyme, we tested the binding capacity of the different carbon materials for their surface functionalization capability. Statistically significant differences were observed for both the type of material and concentration (Kruskal-Wallis Test, p-value < 0.01). In general, results showed that the enzyme saturated the biochar surface at a much lower level than nanomaterials (Figure 1). Pairwise comparisons confirmed these effects, particularly for CB and NTX1, which had higher overall binding capacity (KS Kolmogorov-Smirnov Two Sample Test, p-value < 0.05).

[Figure 1] approximatively here

The average basic activity of the bound-carboxylesterase enzyme was 770 U / mg protein and showed no significant differences between the different carbon materials (see Figures 2-4). After examining the basic activity of the bound carboxylesterase enzyme, we evaluated its robustness as a function of time, pH, and temperature.

3.2 Chemical-physical robustness of functionalized materials

In the first step of the assay, the residual activity of carboxylesterase was determined periodically over a period of 7 days post-functionalization under standard conditions (25°C and pH 5.2). There was a significant effect of time on enzymatic activity (Kruskal-Wallis test statistic, p-value < 0.0001) with some marginally significant differences between materials driven by N-graphene showing the most performing functionalization half-life (Figure 2). In general, most of the activity was lost within the first 96 hours, after which it tends to stabilis, varying between 150 and 200 U.

[Figure 2] approximatively here

We tested the enzymatic activity on functionalized carbons at two temperatures, i.e., 37°C and 47°C (Figure 3). The temperature had a significant inhibitory effect on the enzyme activity (Kruskal-Wallis test statistic, $p< 0.0001$). The enzyme activity drops (>60% of the initial activity) between the standard value (25°C) and the two contrasting temperature ranges. These changes were statistically significant as well as the effect between 37°C and 47°C (two side probability from Kolmogorov-Smirnov Two Sample test, $p <0.0001$ or 0.002, respectively) Conversely, there were no statistically significant differences between the material types.

[Figure 3] approximatively here

Likewise, pH was another variable with a significant impact on enzyme activity(Kruskal-Wallis test, $p < 0.0001$). Although an enhanced effect was observed with increasing pH, the differences were not significant. Biochar lost a significant amount of enzyme activity at extremely alkaline pH than carbon nanotubes, which displayed the greatest robustness against strong variation of pH (Figure 4).

[Figure 4] approximatively here

In general, it is important to highlight that no enzymatic activity was detected in the supernatant in the trials examining the effects of time, temperature or pH (data not shown). This finding suggests that the particle-bound enzyme was inhibited in any way instead of a lack of activity by desorption from the particle surface.

3.3. Pesticide inhibition of carboxylesterase bound enzyme activity.

Functionalized carbons were exposed to two different pesticides, carbaryl and dichlorvos, at concentrations of 30 µM and 0.5 µM, respectively, which correspond to the average EC50 value for the acute test with water flea obtained from the EPA Toxicity Database (https://cfpub.epa.gov/ecotox/).

The effect of carbaryl on carboxylesterase activity was highly statistically significant (Two way ANOVA, $p < 0.0001$), but not that of carbon type and their interaction. After exposure to carbaryl, all functionalized carbons showed a significant decrease in enzymatic activity compared to the control, with the exception of biochar. The greatest inhibition was observed for the enzyme bound to N-graphene, which had a residual activity of 30%. For NTX1, CB and B, the residual activity was 44%, 53% and 60%, respectively (Figure 5). N-graphene activity was also statistically different from that of Biochar according to Two Way ANOVA ($p < 0.05$). The effects of dichlorvos were also statistically significant ($p = 0.001$), but they were clearly driven by N-graphene (Figure 5). The enzyme bound to 2D nanomaterial, in fact, showed the greatest inhibition (44% residual activity) and was the only one that was statistically significant. For the other materials, residual activity was determined to be 61%, 72%, and 65% for B, CB, and NTX1, respectively.

[Figure 5] approximatively here

3.4 Protective effect of carboxylesterase bound graphene

We tested carboxylesterase-functionalized N-graphene for its ability to protect the bioindicator *Tetrahymena thermophila* from pesticide toxicity (Figure 6). Our data showed that functionalization (yes or no), type of pesticide (carbaryl or dichlorvos), and contact time of N-graphene with pesticide (1 or 30 min) were highly significant factors for survival (two-way ANOVA, $p < 0.0001$; $p = 0.004$ for functionalization). Moreover, the enzymatic functionalization of N-graphene was beneficial only in the case of exposure to dichlorvos, while in the case of carbaryl the protective effect appeared independent of enzyme functionalization (Figure 6). This interaction was also evident in the two-way ANOVA analysis ($p < 0.0001$).

[Figure 6] approximatively here

4. Discussion

In this study, we compared the performance of four different carbon materials with respect to their use in the biotechnological application of water clean up from pesticides. The materials selected for testing were high-purity (97%) short (average length 1.1 micron) carbon nanotubes (NTX1), nitrogen-doped graphene (2.41 % N), a commercial carbon black (CB) and biochar (B). With the exception of biochar, these substances represent 1, 2 and 3 D nanomaterials. Charcoal has been used since ancient times to bind harmful chemicals in water purification technologies (Bandosz, 2006) and more recently, carbon nanomaterials have demonstrated higher performance, at least at low technology readiness level lab-scale applications. In fact, carbon nanomaterials such as CNTs (either single- or multi-walled) showed exceptional ability to adsorb antibiotics such as tetracycline with 1-2 orders of magnitude higher than bulky activated carbons. Similarly, CNTs showed high efficiency in removing heavy metals, pharmaceutical dyes, organic contaminants, and pesticides from wastewater (Ji et al., 2009; Youssef et al., 2018; Saxena et al., 2020). Graphene also showed a great ability to adsorb a large number of pharmaceuticals, as well as inorganic pollutants (i.e., metal ions and other ions), organic pollutants such as dyes, aromatic compounds, and gasoline (Sophia et al., 2016; Nupearachchi et al., 2017; Lu and Astruc, 2020). Biochar is charcoal obtained by pyrolysis from solid organic residues as diverse as agri-waste, biosolids, pine needles, coffee grounds, fruit pits, and so on (Lehmann and Joseph, 2009). Biochar has demonstrated to be an efficient heavy metal adsorbent from water (Park et al., 2016) as well as polycyclic aromatic hydrocarbons from pore water (Oleszczuk et al., 2012). In all the aforementioned studies, carbon materials were used for their remarkable feature of non-specific adsorption. The novelty of our study was to examine this remediation capacity of biochar and other nanocarbon materials using upgrading materials. Such an upgrading consisted in the enzymatic activation with a pesticide-detoxifying esterase for clean up of two pesticides with markedly different physicochemical properties. For this low TRL application, a low-cost enzyme such as porcine carboxylesterase was selected, which is capable of forming stable ligands with organophosphorus (dichlorvos) and methyl carbamate (carbaryl) pesticides (Sanchez-Hernandez, 2018). The first step of the process was to compare the carboxylesterase enzyme immobilization capacity. As expected, all nanocarbons showed higher performance than biochar, which was more easily saturated with the enzyme. In this regard, CNT and carbon black also outperformed N-graphene (Figure 1). The best performance of carbon nanomaterials could be explained by their unique property of high surface area to volume ratio (Asuri et al., 2006; Zhang et al. 2012). In evaluating enzymatic activity, not only the ability to link enzymes became important, but also whether the linkage created a condition for their activation. Starting from the same functionalization level (20 μg), the basal enzymatic activity showed no significant differences between the different materials including biochar, suggesting an equal level of interaction between the carbon materials and the enzyme.

The effect of temperature rise was marked for all functionalized materials both at 37°C and 47 °C (Figure 3), with residual activity ranging from about 40 % to 20 %. This behaviour is in line with previous reports (Srivastava et al., 2014). pH effects were tested at values higher than the optimal one for functionalization, 8.4 and 9.4, the latter being the upper law-limit for wastewaters according to the Italian decree-law 152/2006. pH increase above 5.2 significantly impacted carboxylesterase activity, with carbon nanotubes showing the best robustness and biochar the worst one (Figure 4). Literature data are consistent with our observations (Antón-Millán et al., 2018; Sanchez- Hernandez, 2018; Srivastava et al., 2014). Non-covalent enzyme immobilization, in fact, is usually favoured at acidic pH for the formation of electrostatic interactions among positivized protein residues and negative charges onto carbon materials. As shown in Lu and Astruc (2020), the adsorption of organic compounds on carbon-based nanomaterials is based on the π-π-electron interaction between the surface of the material and the compound. In this π-π-electron interaction, pH played an important role because a change in pH led to a change in surface charge and thus a different interaction between the two compounds.

The final step of the evaluation was to test the system for the effects of pesticides. Pesticides are still one of the main problems of environmental sustainability. They are, in fact, an integral part of agriculture, and tens of thousands tons are applied worldwide each year. OPs account for over 38% of the total pesticides used, also causing three million poisonings each year, while carbamates and pyrethroids practically make up the rest of the total insecticides used for parasite control in agriculture and residential areas and public facilities worldwide (Thakur et al., 2019; Matula et al., 2020). Thus, improving the bioremediation of pesticides is a topic of great interest, especially when new materials with particular surface-to-volume ratio performances could be used. Pesticide bioremediation can be performed by means of selected enzymes, some of which have been identified over the year for OPs such as diisopropyl fluorophosphatase, paraoxonase, phosphotriesterase, organophosphate hydrolase, carboxylesterase etc. (Thakur et al., 2019). In a test for the degradation of OPs, it was found that the functionality of phosphotriesterase is increased when it is immobilised on a solid surface. This system, in fact, displayed an improvement in thermal stability, pH effect and Vmax (Breger et al, 2015; Karami et al, 2016; Raynes et al. 2011; Thakur et al, 2019). In our case, we chose to functionalize different carbon nanomaterials with a low cost, commercially available carboxylesterase which is also active towards carbamates (Sterri and Fonnum 2009) as a relatively simple test bed to characterise a hybrid material system and demonstrate its practical efficacy in a pesticide scavenging application. Enzyme functionalized N-doped graphene was finally selected because this system showed overall good performance in terms of enzyme binding, resilience of enzymatic activity and robustness to changes in physicochemical conditions. Moreover, it also showed the highest inhibition after both pesticide exposures, suggesting the formation of larger amounts of stable enzyme-pesticide ligands and thus a higher scavenging potential. Last but not least, it is worth noting N-graphene is not toxic (Baldea et al., 2020), while there is a growing literature on the hazard of other carbon nanomaterials, in particular CNTs (Sawicka et al., 2021). The exposition of *T. thermophila* to pesticides allowed us to evaluate the scavenging effect of carboxylesterase functionalized N-graphene towards ciliate survival. We found that contact between graphene and pesticides generally leads to a reduction in toxicity, with the greatest reduction obtained with increasing duration of contact. We also could find that functionalized and non-functionalized graphene scavenge pesticide toxicity. However, there was a different response between the two pesticides. While carbaryl toxicity is scavenged either by functionalized or non-functionalized graphene at the same extent suggesting no role for the enzyme, the biotest response revealed an interesting role of functionalization for dichlorvos.

The effect of carboxylesterase in fact, was decisive when survival of *T. thermophila* was challenged by 500 μM dichlorvos, a concentration 5 times higher than that giving a full mortality effect (LC_{100}). Pretreatment for only 1 min improved survival from 10 to 50% and from 50 to 100% after 30 min compared to N-graphene alone. In contrast, in the case of carbaryl, functionalization provided no advantage over the already excellent scavenging ability of bare N-graphene, which was able to prevent 70% and 100% of carbamate mortality in just 1 min or 30 min of contact, at a concentration of 100 μM, three times more than LC_{100} (higher concentrations could not be tested as they were not stable in the aqueous exposure medium). Although various forms of graphene, including pyridine (N)-doped graphene, showed unfavourable endothermic physisorption with carbaryl (Mandeep et al., 2021), the graphite rod exfoliated N-graphene presented in this study performed well. It is worth noting that this carbon nanomaterial consisted of monolayer graphene (19%), few layer graphene (75%) and graphene oxide (6%) (Baldea et al., 2020) and besides oxygen, other heteroatoms such as nitrogen (2.41%) and sulphur (0.93%) were detected. Such a high level of defects and complexity in the graphene structure would allow for strong exothermic interactions in nature either by physisorption or chemisorption. Indeed, electrostatic interactions, hydrophobic interactions, hydrogen bonds, and/or chemical bonds may occur between adsorbate and adsorbent. Further studies such as the Langmuir-Freundlich isotherm model and density functional theory may help to clarify their nature. The structure of the two molecules allows some speculations. Indeed, these two pesticides have different degrees of lipophilicity, as indicated by their different solubility in water (8 g vs. 0.11 g L^{-1}) and their average computed logP value, 1.3 against 2.94 (calculated by means of the ALOGPS2.1 software, Tetko and Tanchuk, 2002). The OP compound tends to form H-bonds between its electronegative O-atom and the positively charged hydrogen atoms in defects of N-graphene as shown for other carbon materials such as coconut fibre biochar (An Binh and Kajitvichyanukul, 2019). However, there would also be an obvious competition with H-atoms of water molecules. Formation of stable bonds with carboxylesterase can explain the removal of dichlorvos from solution and subsequent survival of the ciliate. Carbaryl has a biplanar conformation, in which the first level contains the naphthalene ring with a pi-electron cloud. With the exception of the carbon atoms bonded to the more electronegative oxygen atoms of the side chain, all carbon atoms of carbaryl have a small negative charge. In the side chain the oxygen atoms and the nitrogen atom are negatively charged due to their high electronegativity, while the hydrogen atoms are positively charged due to their electropositive nature (Mandeep et al., 2021). It also has a large lipophilic naphthoxy moiety and a weakly hydrophilic carbonyl group (Sandoz et al., 2000). This structure makes it predominantly a lipophilic molecule that tends to escape from water e.g. by means of hydrogen bonding with N-graphene. However more energetic chemical binding cannot be excluded due to the good electrophilic nature of carbaryl (Mandeep et al., 2021) and the richness of nucleophilic heteroatoms in N-graphene (Baldea et al 2020).

5. Conclusions.

Results of the current study proved that the use of carbon materials such as graphite rod exfoliated N-doped graphene can be an optimal solution for enzymatic remediation of pesticide-contaminated water. Enzymatically functionalized materials showed a high capacity to remove organophosphate pesticide (dichlorvos) probably via phosphorylation of the active site of the carboxylesterase bound onto the surface of carbonaceous materials. It is well known that such pesticide-enzyme interaction is irreversible, which means that the remediation system may be highly effective but saturated when all enzyme molecules are phosphorylated. Future research should focus on scaling-up the readiness level of this application assessing more pesticides, mixtures, real water effluents, and exploring the advantage of extremozymes with covalent functionalization for a longer durability. In addition, more studies on the hazard level of N-graphene are due, accomplishing a "safe by design" approach for new nanomaterials and products containing nanomaterials, thus minimising environmental and health risks.

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References

1. Al-Khateeb, L.A., Almotiry, S., Salam, M.A., 2014. Adsorption of pharmaceutical pollutants onto graphene nanoplatelets. Chem Eng J. 248, 191–199.
2. An Binh, Q., Kajitvichyanukul, P., 2019. Adsorption mechanism of dichlorvos onto coconut fibre biochar: the significant dependence of H-bonding and the pore-filling mechanism. Water Sci Technol. 75, 886-876.
3. Antón-Millán, N., García-Tojal, J., Marty-Roda, M., Garroni, S., Cuesta-López, S., Tamayo-Ramos, J.A., 2018. Influence of Three Commercial Graphene Derivatives on the Catalytic Properties of a *Lactobacillus plantarum* α-l-Rhamnosidase When Used as Immobilization Matrices. ACS Appl Mater Inter. 10, 18170–18182.
4. Baldea, I., Olteanu, D., Filip, G. A., Pogacean, F., Coros, M., Sociu, M., Tripon, S. C., Cenarium, M., Megarusan, L., Staden, R. S., Pruneanu,S. , 2020. Cytotoxicity mechanisms of nitrogen-doped graphene obtained by electrochemical exfoliation of graphite rods, on human endothelial and colon cancer cells. Carbon. 158, 267-281.

5. Bandosz, T.J., 2006. Activated carbon surfaces in environmental remediation. Elsevier, Oxford.
6. Biggs, A.I., 1954. A spectrophotometric determination of the dissociation constants of p-nitrophenol and papaverine. T Faraday Soc. 50, 800-802.
7. Breger, J.C., Ancona, M.G., Walper, S.A., Susumu, E.O.K. , Stewart, M.H., Deschamps, J.R., Medintz, I.L., 2015. Understanding How Nanoparticle Attachment Enhances Phosphotriesterase Kinetic Efficiency. ACS Nano. 9, 8491-8503.
8. Garcia, J.A., Di Paola R., 2015. Chemohormonal Therapy in Metastatic Hormone-Sensitive Prostate Cancer. New Eng J Med. 373, 737-746.
9. George, Z.K., Koltsakidou, A., Stavroula, G.N., Dimitrios, N.B., Dimitra, A.L., 2015. Removal of beta-blockers from aqueous media by adsorption onto graphene oxide. Sci Total Environ. 537, 411–420.
10. Ghadim, E., Manouchehri, F., Soleimani, G., Hosseini, H., Kimiagar, S., Nafisi, S., 2013. Adsorption properties of tetracycline onto graphene oxide: Equilibrium, kinetic and thermodynamic studies. PLoS ONE. 8, 1–9.
11. Hirsch A., 2010. The era of carbon allotropes. Nature Materials. 9 (11), 868–71.
12. Iijima, S., 1991. Helical microtubules of graphitic carbon. Nature. 54 (6348), 56-58.
13. Ji, L., Chen, W., Duan, L., Zhu, D., 2009. Mechanisms for strong adsorption of tetracycline to carbon nanotubes: A comparative study using activated carbon and graphite as adsorbents. Environ Sci Technol. 43, 2322–2327.
14. Ji, L., Liu, F., Xu, Z., Zheng, S., Zhu, D., 2010. Adsorption of pharmaceutical antibiotics on template-synthesized ordered micro-and mesoporous carbons. Environ Sci Technol. 44, 3116–3122.
15. Karami, R., Mohsenifar, A., Mesbah Namini, S. M., Kamelipour, N., Rahmani- Cherati, T., Roodbar Shojaei, T., et al., 2016. A novel nanobiosensor for the detection of

paraoxon using chitosan-embedded organophosphorus hydrolase immobilized on Au nanoparticles. Prep Biochem Biotech. 46, 559–566.

16. Kashefi, S., Borghei, S. M. , Mahmoodi, N.M., 2019. Superparamagnetic enzyme-graphene oxide magnetic nanocomposite as an environmentally friendly biocatalyst: Synthesis and biodegradation of dye using response surface methodology. Microchem J. 145, 547–558.
17. Kishore, D., Talat, M., Srivastava, O.N., Kayastha, A.M., 2012. Immobilization of b-Galactosidase onto Functionalized Graphene Nano-sheets Using Response Surface Methodology and Its Analytical Applications. PLoS ONE. 7, e40708.
18. Kyzas, G.Z., Bikiaris, D.N., Seredych, M., Bandosz, T.J., Deliyanni, E.A., 2014. Removal of dorzolamide from biomedical wastewaters with adsorption onto graphite oxide/poly(acrylic acid) grafted chitosan nanocompos- ite. Biores Technol. 152, 399–406.
19. Lehmann, J. and Joseph, S., 2009. Biochar systems. In: Lehmann, J. and Joseph, S. (Eds), Biochar for environmental management. Routledge, London. 147-168.
20. Liu, F.F., Zhao, J., Shuguang, W., Peng, D., Baoshan, X., 2014. Effects of solution chemistry on adsorption of selected pharmaceuticals and personal care products (PPCPs) by graphenes and carbon nanotubes. Environ Sci Technol. 48, 13197–13206.
21. Lu, F. and Astruc, D., 2020. Nanocatalysts and other nanomaterials for water remediation from organic pollutants. Coordin Chem Rev. 408, 213180.
22. Mandeep, G.A., Kakkar, J, Kakkar, R., 2021. DFT study of carbaryl pesticide adsorption on vacancy and nitrogen-doped graphene decorated with platinum clusters. Struct Chem. 32, 1541–1551.
23. Matula, M., Kucera, T., Soukup, O., Pejchal, J., 2020. Enzymatic Degradation of Organophosphorus Pesticides and Nerve Agents by EC: 3.1.8.2. Catalysts. 10, 1365

24. Mehnati-Najafabadi, V., Taheri-Kafrani, A., Bordbar, A., 2018. Xylanase immobilization on modified superparamagnetic graphene oxide nanocomposite: Effect of PEGylation on activity and stability. Int J Biol Macromol., 107, 418-425.
25. Movahedi, M., Shariat, S.Z.A.S., Nazem, H., 2019. Immobilization of Lactoperoxidase on Graphene Oxide Nanosheets and Copper Oxide Nanoparticles and Evaluation of Their Stability. Catal Lett. 149, 562–573.
26. Nam, S., Jung, C., Hang, L., Miao, Y., Joseph, R.V.F., Linkel, K.B., Namguk, H., Kyung-Duk, Z., Yeomin, Y., 2015. Adsorption characteristics of diclofenac and sulfamethoxazole to graphene oxide in aqueous solution. Chemosphere. 136, 20–26.
27. Nupearachch, C.N., Mahatantila, K., Vithanage, M., 2017. Application of graphene for decontamination of water; Implications for sorptive removal. Groundwater Sust Develop. 5, 206–215.
28. Oleszczuk, P., Hale, S.E., Lehmann, J. and Cornelissen, G., 2012. Activated carbon and biochar amendments decrease pore-water concentrations of polycyclic aromatic hydrocarbons (PAHs) in sewage sludge. Biores Technol. 111, 84-91.
29. Oliveira, S.F., Bisker,G., Naveed, Bakh, A. , Gibbs, S.L., Landry, M.P., Strano, M.S., 2015. Protein functionalized carbon nanomaterials for biomedical applications. Carbon. 95, 767-779.
30. Park, J.H., Ok, Y.S., Kim, S.H., Cho, J.S., Heo, J.S., Delaune, R.D., Seo, D.C., 2016. Competitive adsorption of heavy metals onto sesame straw biochar in aqueous solutions. Chemosphere. 142, 77–83.
31. Petersen, E. J., Zhang, L., Mattison, N. T., O’Carroll, D. M., Whelton, A. J., Uddin, N., Nguyen, T., Huang, Q., Henry, T. B., Holbrook, R. D., Chen K. L., 2011. Potential Release Pathways, Environmental Fate, And Ecological Risks of Carbon Nanotubes. Environ Sci Technol. 45 (23), 9837-9856.

32. Raymond, J.L., Jamison, V.W., Hudson, J.O., 1975. Final report on beneficial stimulation of bacterial activity in ground water petroleum products. AlChE Symposium Series. 73, 390.

33. Raynes, J.K., Pearce, F. G., Meade, S. J., Gerrard, J. A., 2011. Immobilization of organophosphate hydrolase on an amyloid fibril nanoscaffold: towards bioremediation and chemical detoxification. Biotechnol Prog. 27, 360–367.

34. Sanchez-Hernandez, J.C., 2018. Biochar activation with exoenzymes induced by earthworms: A novel functional strategy for soil quality promotion. J Hazard Mat. 350, 136-143.

35. Sandoz, C., Lesca, P., Narbonne, J., Carpy, A., 2000. Molecular Characteristics of Carbaryl, a *CYP1A1* Gene Inducer. Arch Biochem Biophys. 373, 275–280.

36. Singh, N., Srivastava, G., Talat, M., Raghubanshi, H., Srivastava, O. N., Kayastha, A. M., 2014. Cicer a-galactosidase immobilization onto functionalized graphene nanosheets using response surface method and its applications. Food Chem. 142, 430–438.

37. Smith, D.L., Doerder, F.P., 1992. Multiple effects of mutation on expression of alternative cell surface protein genes in tetrahymena thermophila. Genetics. 130 (1), 97-104.

38. Soozanipour, A., Taheri-Kafrani, A., 2018. Enzyme immobilization on functionalized graphene oxide nanosheets: efficient and robust biocatalysts. Methods in Enzymology. Academic Press. 608, 371-403.

39. Sophia, C.A., Lima E.C., Allaudeen N., Rajan S., 2016. Application of graphene based materials for adsorption of pharmaceutical traces from water and wastewater- a review. Desal Water Treat. 57, 27573–27586.

40. Srivastava, G., Singh, K., Talat, M., Srivastava, O.N. , Kayastha, A.M., 2014. Functionalized Graphene Sheets As Immobilization Matrix for Fenugreek b-Amylase: Enzyme Kinetics and Stability Studies. PLoS ONE. 9, e113408.
41. Sterri, S.H. and Fonnum, F., 2009. Role of Carboxylesterases in Therapeutic Intervention of Nerve Gas Poisoning. In: Gupta, R.C. (Ed.), Handbook of Toxicology of Chemical Warfare Agents. Academic Press/Elsevier, New York, NY, pp. 1033-1040.
42. Sweeney, C. J., Chen, Y., Carducci, M., Liu, G., Jarrad, D. F., Eisenberg, M., Wong, Y., Hahn, N., Kohli, M., Cooney, M. M., Dreicer, R., Vogelzang, N. J., Picus, J., Shevrin, D., Hussain, M., Jarcia, J. A., Di Paola, R.S., 2015. Chemohormonal therapy in metastatic hormone-sensitive prostate cancer. New Eng J Med. 373, 737-746.
43. Tang, Y., Guo, H., Xiao, L., Yu, S., Gao, N., Wang, Y., 2013. Synthesis of reduced graphene oxide/magnetite com- posites and investigation of their adsorption performance of fluoroquinolone antibiotics. Colloid Surface A. 424, 74–80.
44. Tetko, I.V., Tanchuk, V.Y., 2002. Application of associative neural networks for prediction of lipophilicity in ALOGPS 2.1 program, J Chem Inf Comput Sci. 42, 1136-45
45. Thakur, M., Medintz, I.L., Walper, S.A., 2019. Enzymatic Bioremediation of Organophosphate Compounds—Progress and Remaining Challenges. Enzymes for Organophosphate Bioremediation. 7, 289.
46. Yan, L., Zhao, F., Wang, J., Zu, Y., Gu, Z., Zhao, Y. ,2019. A Safe-by-Design Strategy towards Safer Nanomaterials in Nanomedicines. Adv Mat. 31, 1805391.
47. Youssef, A.M., El-Naggar, M.E., Malhat , F.M., El Sharkawi, H.M., 2019. Efficient removal of pesticides and heavy metals from wastewater and the antimicrobial activity of f-MWCNTs/PVA nanocomposite film. J Clean Prod. 206, 315-325

48. Yuan, G., Yan, L., Liang, Z., Hui, H., Junjie, H., Syed, M.S., Xingguang, S., 2012. Adsorption and removal of tetra- cycline antibiotics from aqueous solution by graphene oxide. J Colloid Interf Sci. 368, 540–546.

49. Zaytseva, O., Neumann, G., 2016. Carbon nanomaterials: production, impact on plant development, agricultural and environmental applications. Chem Biol Technol Agri. 3, 17.

50. Zhang, J., Zhang, F., Yang, H., Huang, X., Liu, H., Zhang, J., Guo, S., 2010. Graphene oxide as a matrix for enzyme immobilization. Langmuir. 26 (9), 6083-6085.

51. Zhang, M., Gao, B., Yao, Y., Xue, Y., Inyang, M., 2012. Synthesis, characterization, and environmental implications of graphene-coated biochar. Sci Tot Env. 435–436, 567–572.

Figure captions

Figure 1. Binding capacity of functionalized carbon nanomaterial.

Shown are bound-enzyme fractions (average +/- standard errors). Increasing amounts of carboxylesterase (20, 40, 100, 400 μg) were used to functionalize 1 mg of each of the four different carbon materials. The quantity of carbon-bound enzyme was determined indirectly by the unbound residual amount in the functionalization buffer. Groups / bars that do not share a common letter are significantly different from each other. Capital letters refer to differences among groups of carbon materials. Lowercase letters refer to differences within the same group (two side probabilities from Kolmogorov-Smirnov Two Sample Test, $p < 0.05$). Legend: B (biochar); CB (carbon black); G (N-graphene); NTX1 (carbon nanotubes).

Figure 2. Stability of enzyme activity over time.

Carbon materials were incubated in the presence of carboxylesterase (20 μg/mL), and the hydrolytic activity was measured at different times using the substrate 4-nitro-phenylbutyrate. Symbols represent the mean value (+/- SEM) of four independent trials. Legend: NTX1 (CNT), CB (Carbon black), B (Biochar), G (N-graphene). Other statistical details can be found in text.

Figure 3. Effects of temperature on carboxylesterase activity.

Explanation and legend as for Figure 2 except temperature was considered. No statistically significant differences were found among groups (carbon types). Other statistical details can be found in text.

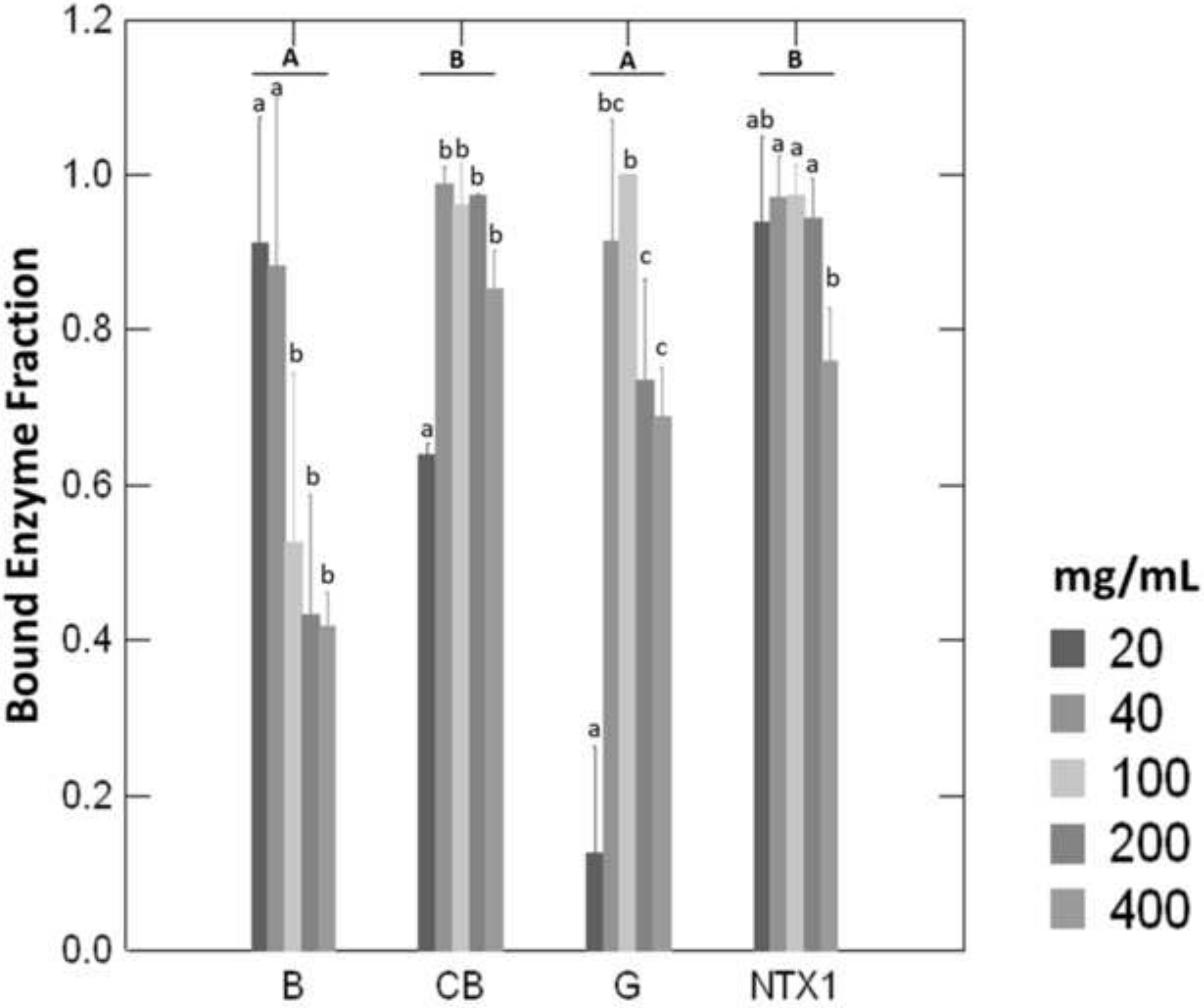
Figure 4. Effects of pH on carboxylesterase activity.

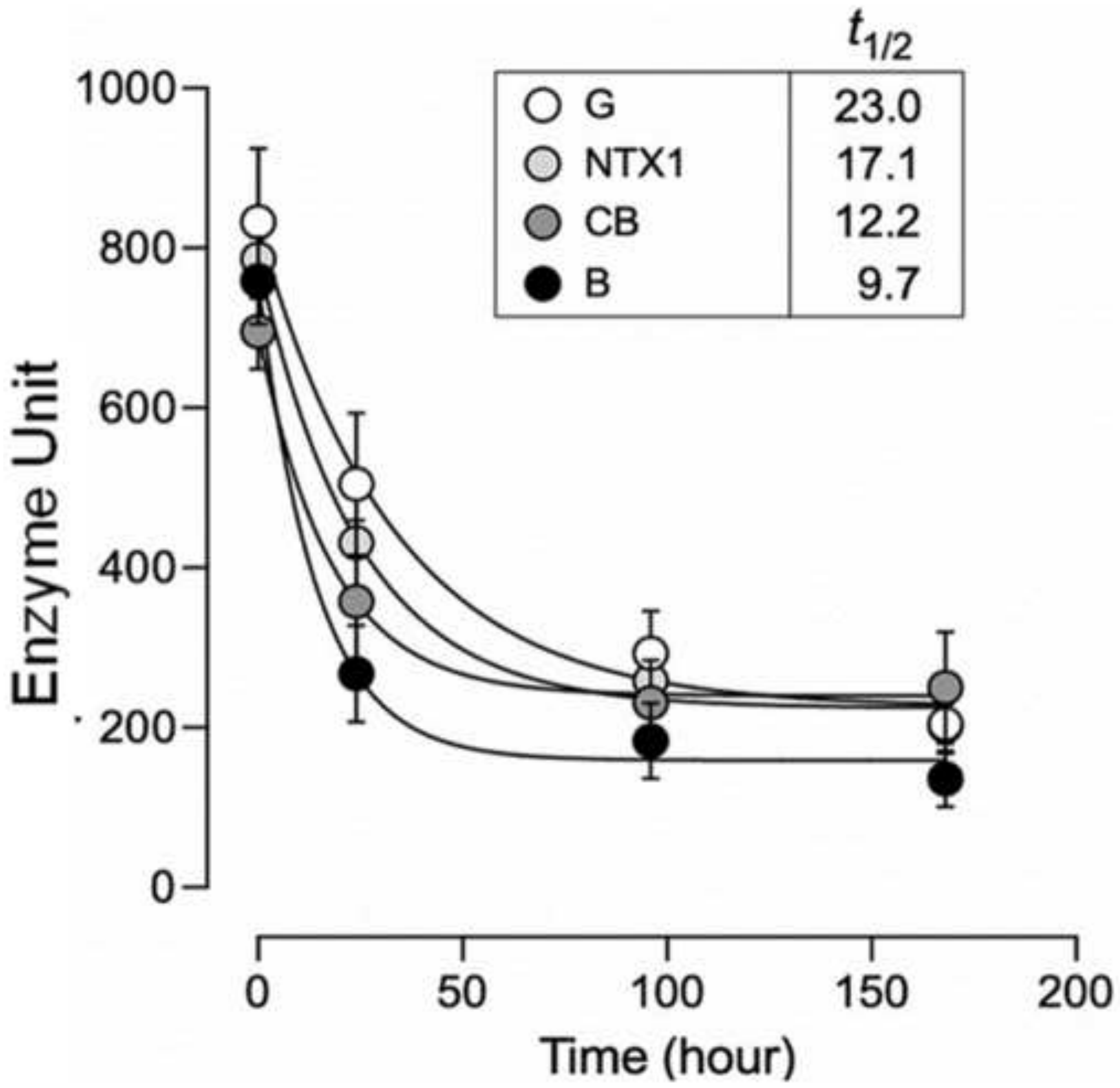
Explanation, legend and statistics as for Figure 2 except pH was considered. Two side probabilities (p-values) from Kolmogorov-Smirnov Two Sample Test for NTX1 were 0.004, 0.079, and 0.050 respectively for B, CB and G; CB vs B, 0.065. Other statistical details can be found in text.

Figure 5. Effects of pesticide on bound carboxylesterase activity.

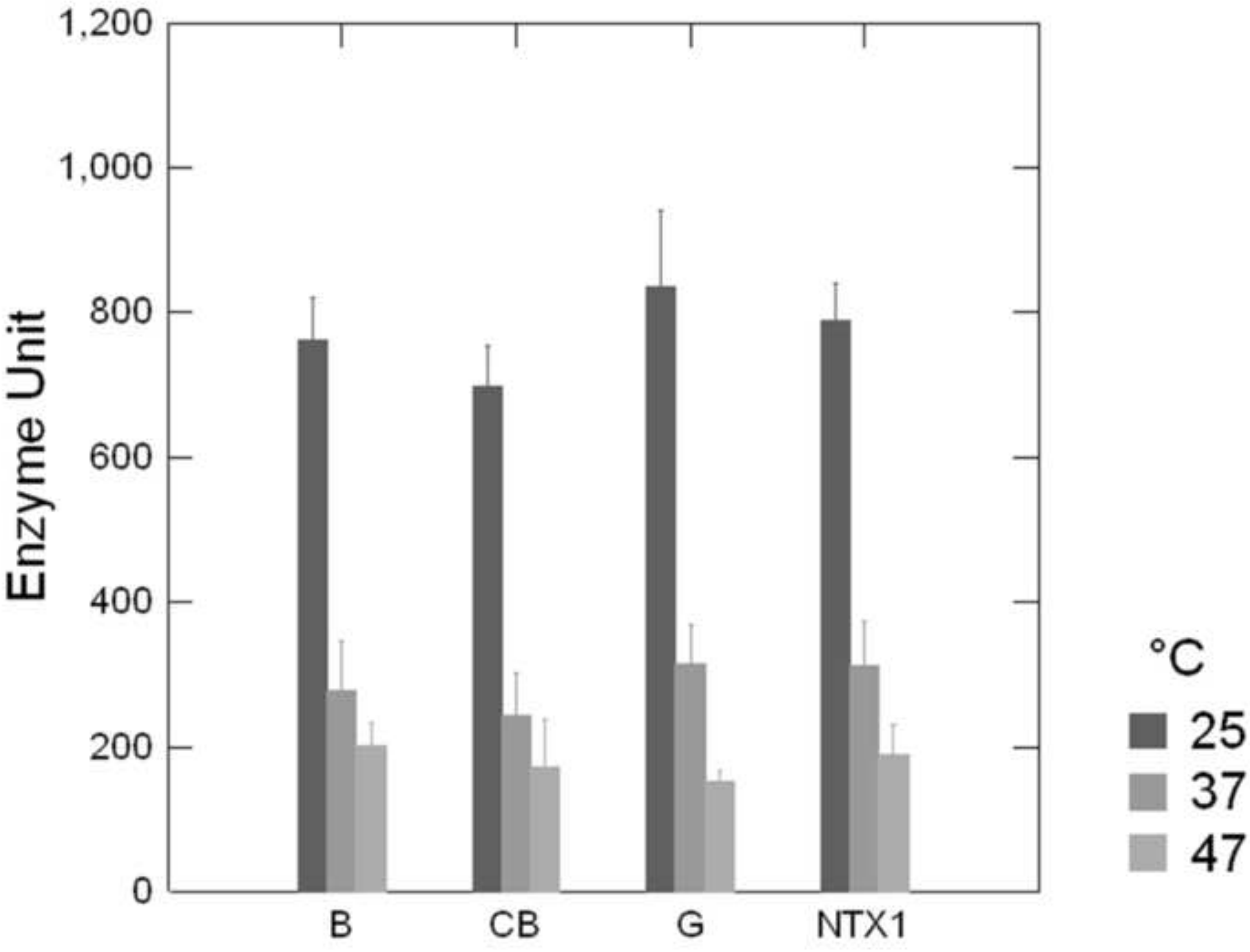
Residual activity of bound carboxylesterase for the effects of pesticide treatment (average +/- standard errors from four independent experiments). Statistical differences from control, *p < 0.05; ** p < 0.01, (Two Way ANOVA, Fisher post hoc test for pairwise comparison). Legend: NTX1 (CNT), CB (Carbon black), B (Biochar), G (N-graphene).

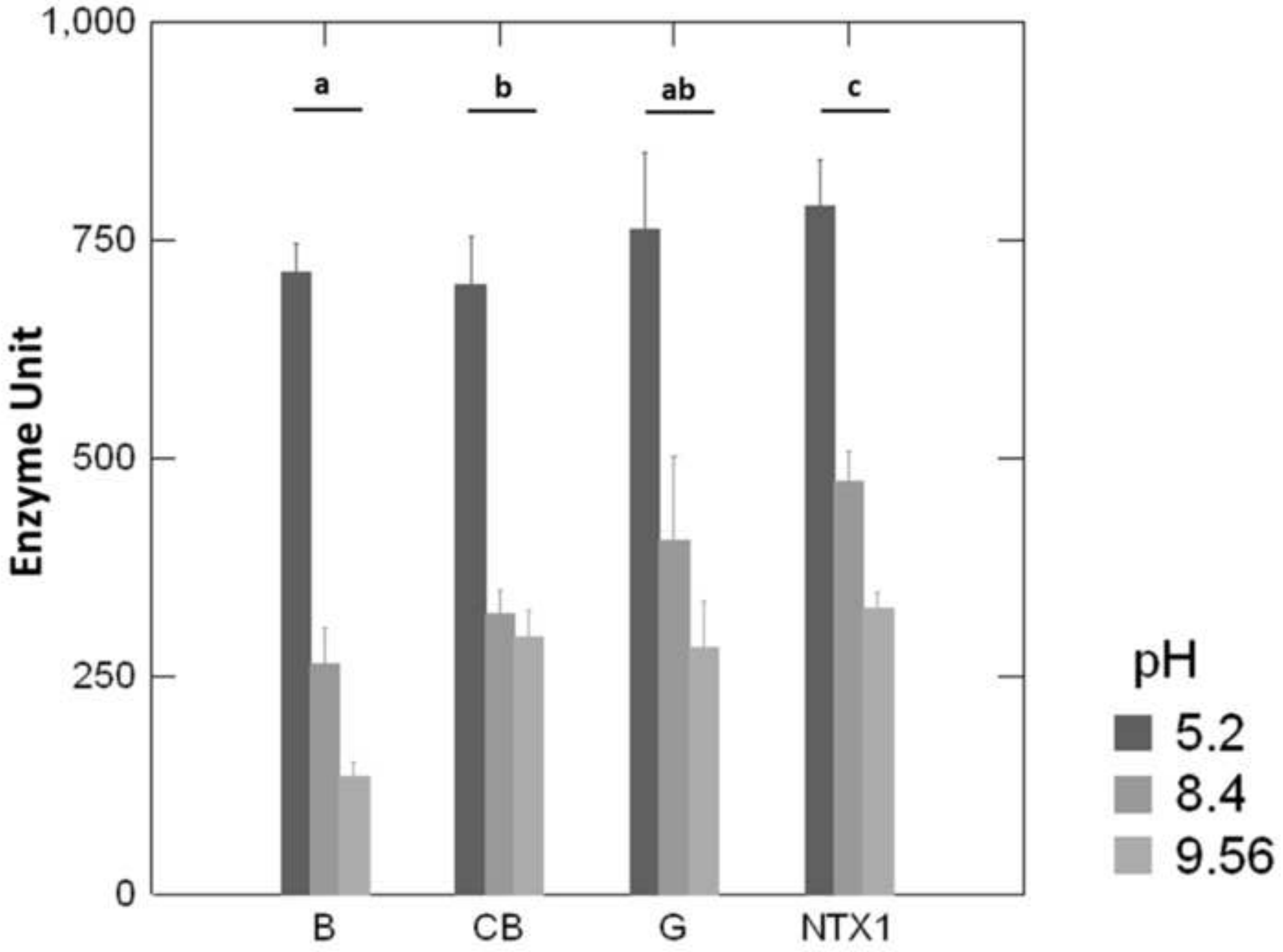
Figure 6. Protective effect of carboxylesterase functionalized N-graphene in T. thermophila. Shown are fractions +/- standard errors (four independent experiments) of living cells after exposure of ciliates to a full lethal concentration of carbaryl or dichlorvos, respectively 100 or 500 microM, that has been previously conditioned with carboxylesterase functionalized N-graphene for 1 or 30 minutes (see methods for experimental details). Bars that do not share a common letter are significantly different from each other (two-way ANOVA, p < 0.05, Tukey's post hoc test for pairwise comparison) Legend: (NF, 1), 1 min contact time, not functionalized N-graphene; (NF, 30), 30 min contact time, not functionalized N-graphene; (F, 1), 1 min contact time, functionalized N-graphene; (F, 30), 30 min contact time, functionalized N-graphene. CA, carbaryl; DI, dichlorvos.

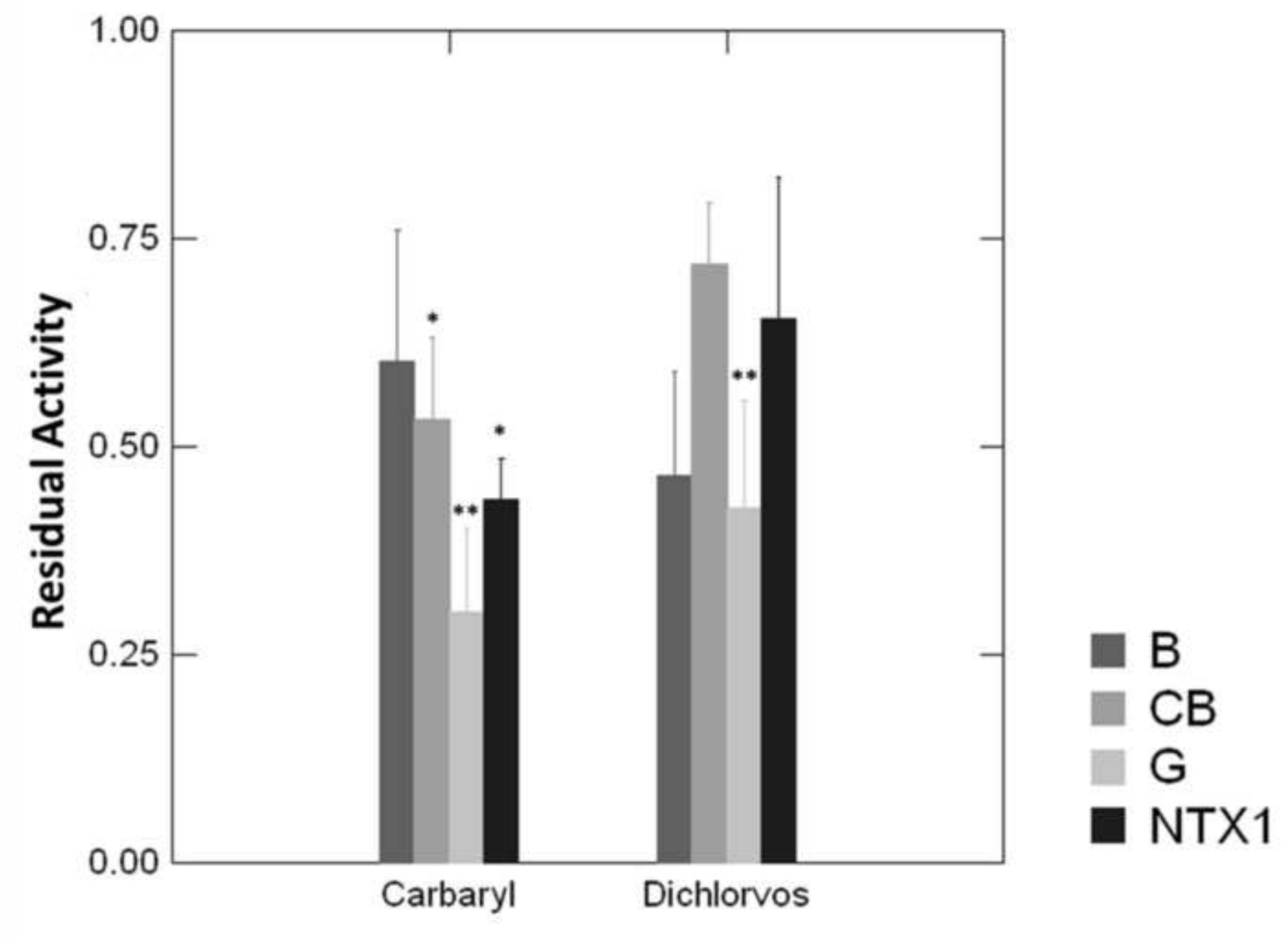

Bound Enzyme Fraction
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
B
A
B
a
a
b
b
b
a
b b
b
b
a
bc
b
c
c
ab
a
a
a
b
B
CB
G
NTX1
mg/mL
20
40
100
200
400

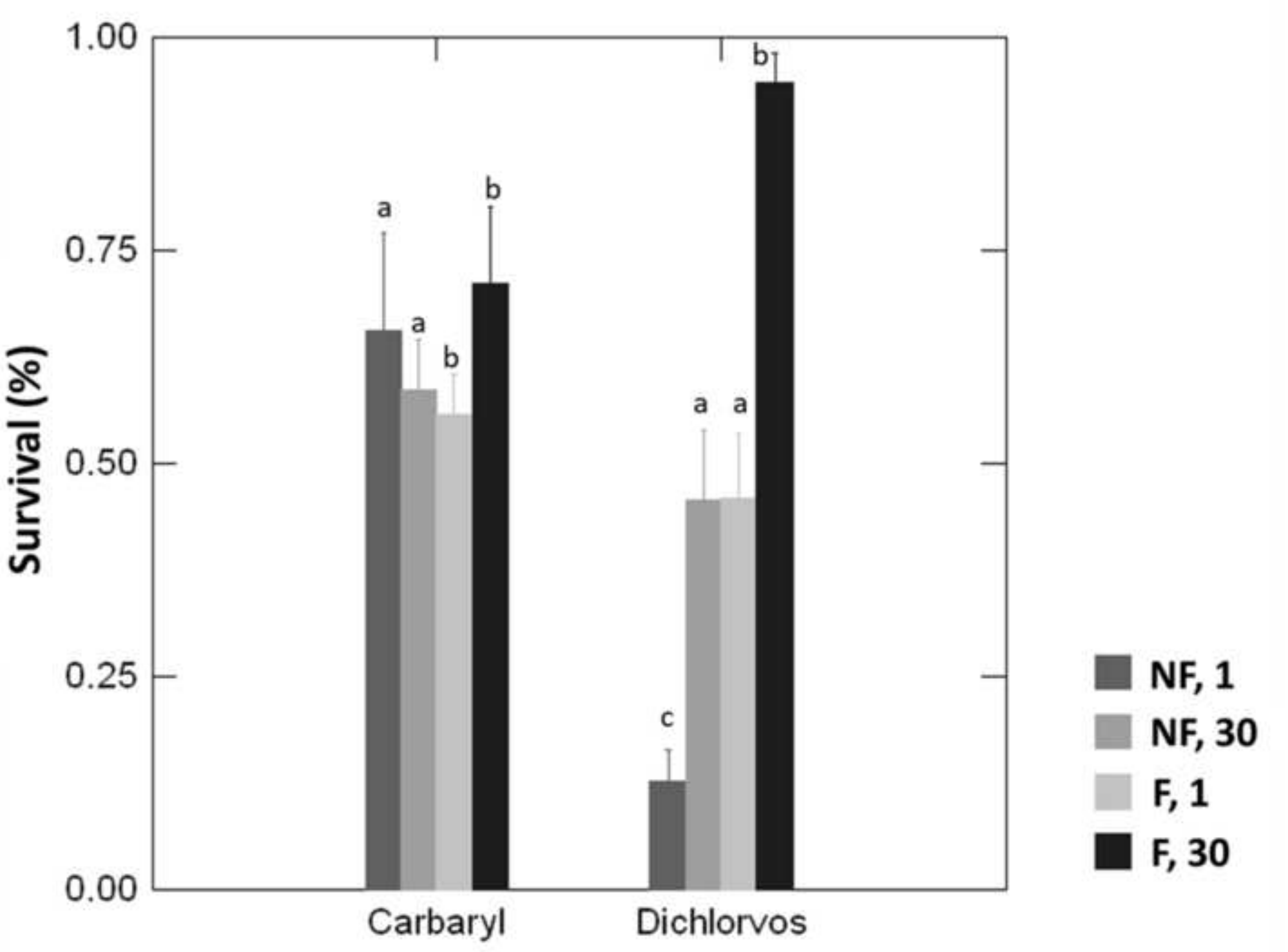


$t_{1/2}$
○ G 23.0
○ NTX1 17.1
● CB 12.2
● B 9.7
Enzyme Unit
1000
800
600
400
200
0
0
50
100
150
200
Time (hour)


Enzyme Unit
1,200
1,000
800
600
400
200
0
B
CB
G
NTX1
°C
25
37
47


1,000
750
500
250
0
Enzyme Unit
a
b
ab
c
B
CB
G
NTX1
pH
5.2
8.4
9.56


1.00
0.75
0.50
0.25
0.00
Residual Activity
*
**
*
**
Carbaryl
Dichlorvos
B
CB
G
NTX1


1.00
0.75
0.50
0.25
0.00
Survival (%)
a
a
b
b
c
a
a
b
Carbaryl
Dichlorvos
NF, 1
NF, 30
F, 1
F, 30

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All authors have read and agreed to the published version of the manuscript.