Perchlorate detection via an invertebrate biosensor

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Improvised explosive devices (IEDs) are constructed from easily obtainable ingredients that are often unregulated and difficult to trace. Salts of the oxyhalide perchlorate are frequently used as oxidisers in IEDs and in commercially available munitions, thus a reliable detection is needed to aid forensic investigations and the tracing of environmental ground or surface water contamination. We introduce the nematode Caenorhabditis elegans as a biosensor for the presence of perchlorate, a promising alternative to the costly, technically challenging and time-consuming current perchlorate detection methods. Perchlorate uptake dynamics in C. elegans were first validated using ion exchange chromatography followed by assessing the effects of perchlorate on key life-point indices to verify the suitability of the nematodes as a forensic biosensor. Whole genome microarrays and qPCR analyses established that a set of immune and stress response genes were enriched during perchlorate exposure. A nematode strain (agls219) containing an integrated copy of the significantly overexpressed t24b8.5 gene promoter followed by a GFP reporter gene was shown to fluoresce in a perchlorate dose dependent manner with a limit of detection (LOD) of 0.5 mg mL⁻¹. Whilst chemicals commonly used in the construction of IEDs did not induce fluorescence, exposure to other oxyhalides did, highlighting the presence of possible shared stress response pathways. Burnt wire sparklers containing potassium perchlorate elicited fluorescence while other non-perchlorate containing post-blast explosion matrices did not. This demonstrates how C. elegans can be used to screen for perchlorate at environmental hotspots, an optimization, possibly with other target transgenes, is required to enable the detection of perchlorate at concentrations below 0.5 mg mL⁻¹.

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transduces the response generated by the biological component to perchlorate into optical or electrical signals.\textsuperscript{27} Numerous biosensors have been developed to detect high order explosives, including canines, rats, bees, wasps, moths and even genetically modified microorganisms,\textsuperscript{28–32} however a biosensor for perchlorate is, to date, elusive. To enable the detection of perchlorate, the candidate biosensor should be amenable for genetic manipulation and the yielded optical signals easily accessible. We therefore propose the use of the model organism \textit{Caenorhabditis elegans}, as it possesses a very simple anatomical structure, is transparent, easy to cultivate in a laboratory setting, has a compact genome and a very short life cycle, and most importantly is suitable for genetic analysis and manipulation.\textsuperscript{33}

**Experimental section**

**Reagents and materials**

All reagents used were of analytical or reagent grade. All chemicals (unless stated) were prepared from their sodium salts, including perchlorate, iodide, periodate, chlorate, chloride, bromate, sulphate, ammonium, nitrate, nitrite, phosphate and sucrose (Sigma-Aldrich, Gillingham, Dorset, UK). Perchlorate, nitrite, chlorate, periodate, iodide, bromate and phosphate were prepared fortnightly (100 mg mL\(^{-1}\)) in ultrapure water and were stored in the dark at 4 °C from which working solutions were prepared daily. Eluents for IC were prepared using a 50% w/v solution of sodium hydroxide in water. Commercial products containing perchlorate as an oxidizer used here for biosensor application were the black powder substitute Pyrodex® (Wilson & Wilson, Fieldsports Ltd., Ramsgate, Kent, UK) and burnt wire sparklers (i.e., fireworks). RDX post-explosion residues (exploded car parts were provided by Event Horizon Precision Energetics, Ashcott, Somerset) were also utilised as standards were prepared by dissolving the residues in ultrapure water overnight. The chemicals were prepared by dissolving the appropriate amount of their powders/salts in filtered double autoclaved ultrapure deionized water (18.2 MΩ cm) delivered from a Millipore Synergy water ultra-purification system (Millipore, Bedford, MA, USA). All chemicals were kept in glass bottles at 4 °C and filtered before each use with a 0.22 μM filter. The wild type \textit{C. elegans} strain N2 (Bristol, Great Britain) was utilized as well as a transgenic strain \textit{agIs219}

![Fig. 1](image)

(A) Overlaid IC chromatogram of sodium perchlorate standard at (a) 1000 ng mL\(^{-1}\), (b) 750 ng mL\(^{-1}\), (c) 500 ng mL\(^{-1}\) and (d) 100 ng mL\(^{-1}\). (B) Matrix-matched standards of unexposed nematodes with extracts spiked with (a) 2.5 mg mL\(^{-1}\), (b) 1 mg mL\(^{-1}\), (c) 0.5 mg mL\(^{-1}\) or (d) 1 μg mL\(^{-1}\) perchlorate for 24 hours from L1 stage. (C) Overlaid IC chromatograms of (a) a 1 μg mL\(^{-1}\) sodium perchlorate fortified lysate retrieved from nematodes exposed to 0.5 mg mL\(^{-1}\) perchlorate, (b) an extract of nematodes exposed to 0.5 mg mL\(^{-1}\) perchlorate only and (c) last washout solution of the exposed nematodes. (D) A comparison between external perchlorate concentration (applied dose) and internal perchlorate concentration (ingested).
worms were added to plates containing 1 ng mL\(^{-1}\) perchlorate, added to both the agar and \(E.\ coli\) OP50, for 24 hours at 20 °C. The worms were washed 7 times with ultrapure deionized water to eliminate any exogenous sources of perchlorate including the agar and the \(E.\ coli\) OP50. The last washout solution was saved to verify using IC that the perchlorate concentration was uniform. The Worm pellets were then submerged in 3 mL of ultrapure deionized water supplemented with glass beads and lysed by ultrasonication for 10 minutes. The lysate was passed through a 0.45 micron filter prior to sample injection. The worm lysate was analysed directly by IC in triplicate followed by a matrix-match standard containing 1 µg mL\(^{-1}\) perchlorate.

Sample normalisation by Bradford assay

The nematodes were normalised to protein content via the Bradford Coomassie brilliant blue assay prior to IC analysis. The worms (\(\sim 40\,000\) L1 nematodes) were treated with perchlorate (1 µg mL\(^{-1}\), 0.5 mg mL\(^{-1}\), 1 mg mL\(^{-1}\) or 2.5 mg mL\(^{-1}\)) for 24 hours at 20 °C. The samples were normalised to protein content using the Bradford assay at 595 nm. The lysates were then measured for perchlorate content using IC.

Perchlorate effect on key life-point indices

The effects of perchlorate on the growth, eating behaviour, reproduction and lifespan were investigated. Synchronized L1 worms were added to plates containing 1 ng mL\(^{-1}\), 0.5 µg mL\(^{-1}\), 1 µg mL\(^{-1}\), 0.5 mg mL\(^{-1}\), 1 mg mL\(^{-1}\) or 3 mg mL\(^{-1}\) sodium perchlorate in both the agar and the \(E.\ coli\) OP50 bacteria. Randomly selected worms (\(n = 10\) worms per condition) were photographed once a day for 5 days using an upright microscope/camera system (SMZ1500, Nikon). The growth was assessed by measuring the flat volumetric surface area of the photographed nematodes using Image-pro Express 5.1 Software (Media Cybernetics). The total brood size of sodium perchlorate challenged worms was determined until the egg laying period was completed by plating synchronized L1 stage worms on NGM plates containing perchlorate concentrations equal to those utilised in the growth assay. The worms were then allowed to grow until they reached L4 stage and individual worms (12 worms per condition) were transferred to single NGM agar containing wells seeded with \(E.\ coli\) OP50 supplemented with the relevant perchlorate concentration. Worms were allowed to reach adulthood and lay eggs for one day, thereafter each worm was transferred to a new well in a new 12 well plate and allowed to lay eggs for the second day of adulthood. The number of hatched progeny of each worm was counted one day after egg laying. The process of transferring the worms and counting the progeny was performed for 6 days during which the total number of eggs per worm in each condition was counted. The life span of approximately 400 synchronized L1 stage worms was investigated upon addition of 1 ng mL\(^{-1}\), 1 µg mL\(^{-1}\) or 1 mg mL\(^{-1}\) sodium perchlorate to both the agar and the \(E.\ coli\) OP50. The worms were transferred on a daily basis to fresh NGM plates containing the same concentration of sodium perchlorate until egg laying ceased. Thereafter, the worms were transferred to new plates every two days. The number of dead worms was scored on a daily basis for approximately three weeks (18 days) until no viable worms were observed. To assess the effects of perchlorate on the eating behaviour of the nematodes, synchronized L1 stage worms were placed onto unexposed NGM plates or plates containing sodium perchlorate (1 ng mL\(^{-1}\), 10 ng mL\(^{-1}\), 100 ng mL\(^{-1}\), 1 µg mL\(^{-1}\), 175 µg mL\(^{-1}\), 0.5 mg mL\(^{-1}\) or 1 mg mL\(^{-1}\) added to both the agar and the \(E.\ coli\) OP50). The worms were allowed to grow at 20 °C until they reached late L4 stage when their internal organs can be easily visualised under a microscope. The number of pharyngeal pumps was determined by manual counting over a 30 seconds time frame from at least 10 worms per condition. To assess if perchlorate disrupts the defecation cycles of \(C.\ elegans\), the time required for ten worms to defecate in two successive defecation cycles was recorded.

DNA microarrays and qPCR analysis

A whole genome microarray was utilised from cDNA samples derived from nematodes raised from L1 to L4 stage in the presence or absence of 1 mg mL\(^{-1}\) perchlorate. In detail, the total RNA of the synchronized nematodes was extracted and subsequently converted to cDNA which was then hybridized to the Affymetrix GeneChip\textsuperscript{TM} \(C.\ elegans\) Gene 1.0 ST array according to the manufacturer’s protocol. The microarray data was normalised using the RMA algorithm, and the Principal Component Analysis (PCA) and gene expression data analysis were performed using Transcriptome Analysis Console (TAC) Software 4.0 and Quilcore Omics Explorer version 3.4, respectively. The differentially expressed genes that met the cut-off criteria (>1.4 or <0.7) were analysed using the Search Tool for...
the Retrieval of Interacting Genes/Proteins (STRING), software version 10.5. The microarray data were validated using qPCR analysis of t24b8.5, zip-10, lys-7, sms-9, sek-1 and vhp-1 in multiple cDNA samples of worms treated with 1 mg mL\(^{-1}\) perchlorate for 48 hours from L1 to L4 stage.

**Exposure of transgenic worms and assessment of fluorescence**

A synchronized L1 population of the agls219 transgenic worms was raised for 24 hours at 20 °C with or without perchlorate (unexposed or 1 \(\mu\)g mL\(^{-1}\), 0.5 mg mL\(^{-1}\), 1 mg mL\(^{-1}\), 2.5 mg mL\(^{-1}\) or 5 mg mL\(^{-1}\) added to both the E. coli OP50 and the agar). Worms \((n = 15\) per condition\) were immobilized on glass slides using sodium azide and their fluorescence emission was investigated using a Nikon (Eclipse TE2000) inverted fluorescence microscope with blue laser scanning fluorescence \((\lambda_{ex} = 450–490\) nm\).

**Assessing the specificity of the agls219 worms to perchlorate**

The agls219 nematodes \((n = 10–15\) per condition\) were exposed to 2.5 mg mL\(^{-1}\) ammonium chloride \((\text{NH}_4\text{Cl})\), sodium sulphate \((\text{Na}_2\text{SO}_4)\), trisodium phosphate \((\text{Na}_3\text{PO}_4)\), sucrose \((\text{C}_{12}\text{H}_{22}\text{O}_{11})\), sodium nitrite \((\text{NaNO}_2)\), or sodium nitrate \((\text{NaNO}_3)\) from L1 stage for 24 hours. The worms were also exposed to sodium bromate \((\text{NaBrO}_3)\), sodium iodide \((\text{NaI})\), sodium chloride \((\text{NaClO}_3)\) or sodium periodate \((\text{NaIO}_4)\) at 1 mg mL\(^{-1}\) for 24 hours at 20 °C. A 1 mg mL\(^{-1}\) concentration was used instead of 2.5 mg mL\(^{-1}\) because of the relevant toxicity of these chemicals. Imaging was conducted using the Nikon (Eclipse TE2000) inverted fluorescence microscope with blue laser scanning fluorescence \((\lambda_{ex} = 450–490\) nm\) after immobilisation on a glass slide using sodium azide. The nematodes were also exposed to Pyrodex®, a black powder substitute containing potassium perchlorate as an oxidizer, burnt wire sparklers initially containing potassium perchlorate in their unburnt form and RDX post-explosion residues.

**Results and discussion**

**Uptake dynamics of perchlorate**

Biosensors, if optimised correctly, offer a fast, inexpensive and highly sensitive alternative to traditional detection methods. Here we propose the use of genetically engineered Caenorhabditis elegans strains to serve as an alternative detector for perchlorate. The ability of the worms to serve as a biosensor for perchlorate depends on the uptake of perchlorate by nematodes and any subsequent effects of perchlorate on the animal. It was important to also assess the effects of the drug on the bacterial food source. After obtaining which perchlorate concentrations are tolerated by the bacteria, the actual effects of perchlorate on the nematodes’ growth were investigated. The growth of E. coli OP50 was not affected when perchlorate was added at doses up to 10 mg mL\(^{-1}\), whilst a dose of 25 mg mL\(^{-1}\) was growth inhibitory (data not shown). Although microbes such as proteobacterium Azospira suillum strain PS (formally Dechlorosoma suillum strain PS) can use perchlorate as an electron donor for microbial metabolism, E. coli OP50 is neither a halophile nor a perchlorate reducing bacteria.\(^{33–40}\)

To assess the suitability of utilizing C. elegans as a perchlorate biosensor required the uptake dynamics of perchlorate in the nematodes to first be assessed via 1C analysis of unexposed worm lysates spiked with perchlorate and compared to perchlorate standards (Fig. 1A and B). The lysates of worms exposed to mg mL\(^{-1}\) of perchlorate contained \(\mu\)g mL\(^{-1}\) quantities of perchlorate revealed a dose dependent increase in perchlorate but indicated that the worms are most likely not able to bio-concentrate perchlorate from the outside environment (Fig. 1C and D) and therefore present a similar model of perchlorate activity as in humans.\(^{4}^{6}–41\) Several studies have reported that perchlorate is excreted with a half-life of 6–8 hours and is not metabolized because the chemical reduction induced by the strong chlorine–oxygen bonds is rare.\(^{42}\) In addition, although perchlorate exhibits a high redox potential, it was shown to be excreted virtually unchanged in rats.\(^{42}\)

**Perchlorate effects on key life-cycle endpoints**

Evaluating the physiological effects that perchlorate exerts on the worms was of paramount importance, as an understanding of its toxicity is required to define the worms limitations and in turn assess their suitability as perchlorate biosensors. In detail, exposure to increasing concentrations of perchlorate resulted in a gradual decrease in body size (Fig. 2A). The decreased body size is a common concept in the toxicology of C. elegans where various toxins have been shown to affect the growth of the nematodes by decreasing the growth rate or reducing the nematodes size as observed, for example, with Aflatoxin B1 (AFB1).\(^{43}\) In addition, the nematodes were reported to shrink in hypertonic solutions which might have been caused by elevated perchlorate salt concentrations.\(^{44}\) However, a perchlorate dose of 5 mg mL\(^{-1}\) is very toxic to the worms as indicated by their developmental arrest at the L1 stage.

A direct indication of the effects of potential toxic compounds on nematodes can be indicated by a change in reproduction.\(^{45}\) The total brood size of sodium perchlorate treated worms decreased upon perchlorate exposure and it was reduced by almost 50% at 3 mg mL\(^{-1}\) with a 24 hour delay in the onset of reproduction (Fig. 2B). This dose caused a significantly reduced growth rate of the treated worms which explains the delay in the onset of egg laying. However, many other compounds confer reproductive toxicity in C. elegans, etoposide for example, a clinical anticancer drug, was found to severely decrease the reproduction rate of the worms.\(^{46}\) Upon testing the effects of perchlorate on the life span of C. elegans, it was evident that there were no significant changes upon exposure to 1 \(\mu\)g mL\(^{-1}\) sodium perchlorate in comparison to unchallenged control worms. Exposure to the same dose did not alter the median survival time, namely 14 days was equivalent to the median survival of unexposed worms (Fig. 2E). In contrast, worms treated with 1 mg mL\(^{-1}\) sodium perchlorate were marked by a statistically significant decrease in median survival (by one day), indicating the potency of this dose on the life span of the nematodes. A one-day decrease, despite being statistically
significant, will not limit C. elegans utilisation in subsequent experiments to test for the ability of the worms to detect perchlorate. As both pharyngeal pumping and defection rates were not affected by perchlorate exposure, the reduced growth, brood size and survival after perchlorate exposure were the result of the chemical itself and not a consequence of an altered eating behaviour (Fig. 2C and D). For example, it was reported that Reductil®, a drug used to treat obesity, can affect key life point indices by altering the eating behaviour of the worms as nematodes exhibited decreased growth rates as a consequence of reduced defection and pharyngeal pumping frequencies. The analyses presented here therefore demonstrate that perchlorate is well tolerated at high levels which indicate that the worms can be used as a biosensor of perchlorate exposure over a wide range of doses. However, growth and reproduction are affected at doses reaching 3 mg mL\(^{-1}\) and lethality is observed at concentrations higher than 5 mg mL\(^{-1}\), which are levels that are rarely encountered in nature and can only be present at extreme spill or explosion sites.3

**Microarray, qPCR analysis**

In search for a perchlorate responsive gene, a whole genome microarray was conducted comparing unexposed worms with counterparts challenged with 1 mg mL\(^{-1}\) perchlorate. This revealed the enrichment of pathways implicated in growth, morphogenesis, stress and defense response as reflected in the observed phenotypic changes induced by perchlorate (Fig. 3A and B). Interestingly, perchlorate was previously reported to influence the mitochondrial metabolic activity and to even enhance the formation of Reactive Oxygen Species (ROS) in the mitochondria and increase the mitochondrial permeability transition pore (mPTP) openings in the goldfish Carassius auratus causing hepatocytes destruction.48,49 The generated ROS were hypothesized to enhance DNA damage based on perchlorate induced DNA fragmentation in the isolated mitochondria.50,51 Indeed, several antioxidant genes and oxidoreductases were enriched following perchlorate exposure in the nematodes. In fact, 2.4% of the enriched genes were oxidoreductases indicating that perchlorate may well be implicated in the formation of ROS which might have induced oxidative stress in the worms. Incidentally, one of the mechanisms for pathogen elimination is through the generation of ROS. For instance, in *Drosophila*, a NADPH dual oxidase system (DUOX) is activated in response to infection by the bacterial derived uracil which induces the formation of ROS in the intestines as a defence mechanism.52 This is also true for *C. elegans*,53 whereby a reduced expression of the DUOX, bli-3 for example, was
reported to lead to increased susceptibility to pathogens and to reduce hydrogen peroxide production.\textsuperscript{34} Moreover, immunity is linked to stress response primarily through the activation of p38 MAPK pathway and JNK pathway in mammals, and in fact, \textit{C. elegans} innate immunity has been reported to be linked to abiotic stress, including fluctuations in temperatures, osmolality or exposure to immunity and response to xenobiotic stress.\textsuperscript{35} The expression of \textit{t24b8.5} exhibited a consistent increase in expression in 5 independently generated samples of worms treated with 1 mg mL\textsuperscript{-1} perchlorate (Fig. 3C). For this reason we opted to use a transgenic worm strain to investigate the utility and sensitivity as a putative perchlorate biosensor.

**Biosensor sensitivity**

The transgenic strain \textit{agls219} (\textit{P}t24b8.5::GFP::\textit{unc}-54-3'UTR and \textit{Pttx-3}:GFP::\textit{unc}-54-3'UTR) expresses GFP driven by the \textit{t24b8.5} promoter but also \textit{Pttx-3}:GFP as a positive selection marker (\textit{txt-3} expression is limited to AIY interneurons and surrounding muscles in the head of the worm). \textit{Agls219} has been used to study innate immunity response of \textit{C. elegans} during infection as it is strongly inducible upon the activation of PMK-1, the major regulator of innate immunity in the nematodes. Moreover, \textit{T24B8.5} is a ShK-like toxin peptide and its expression is regulated by the PMK-1/p38 MAPK pathway and the transcription factor ATF-7.\textsuperscript{34} The \textit{agls219} worms responded to a perchlorate challenge via a gradual increase in fluorescence intensity at 0.5 mg mL\textsuperscript{-1}, 1 mg mL\textsuperscript{-1} and 2.5 mg mL\textsuperscript{-1}. Although the toxicity of perchlorate affected the fluorescence at the 5 mg mL\textsuperscript{-1}, the signal remained statistically significantly higher than the unexposed controls (Fig. 4A and B). Whether perchlorate induces its effects through the activation of PMK-1 via ROS or through other effectors remains to be elucidated. Indeed many toxic compounds can induce stress through PMK-1 by either the
TIR-1, NSY-1 and SEK-1 module, through PMK-1 directly, through VHP-1 or by means of the KGB-1 pathway. Other stress pathways that may be involved in the stress response to perchlorate may include the activation of DAF-16 or JNK-1 pathways. The absolute LOD of the biosensor was determined to be 0.1 ng (by mass), which is the more frequent means to report LOD in explosives analysis of bulk materials. This lack of sensitivity somewhat limits its utility for trace analysis of explosives and certainly cannot claim to match the sensitivity of IC and IC-MS (LOD: 0.7 pg to 1.2 ng).

Nevertheless this proof of concept highlights the potential of biosensors as an alternative/complementary approach to classical forensic analysis.

**Biosensor specificity**

IEDs contain other constituents such as sugar, phosphate, sulphur or nitrate, therefore an experiment was required to test for the reactivity of the worms to a set of chemicals commonly used in homemade explosives. An exposure to 2.5 mg mL\(^{-1}\) of each compound did not induce fluorescence, thereby verifying the selectivity of the transgenic nematodes to perchlorate. Perchlorate therefore seems to generate a stress response that is most likely quite specific to at least its class of chemicals. An exposure to other oxyhalides that are closely related to perchlorate and share common chemical characteristics was also conducted. Namely, the nematodes were exposed to...
bromate, chlorate and periodate in addition to sodium iodide since perchlorate competes with the latter for binding to the thyroid receptors in vertebrates and therefore may act in a similar manner. The exposures were performed at 1 mg mL⁻¹, as these compounds are highly toxic to the worms, but with the exception of periodate (perchlorate’s most similar compound) none were able to trigger a significant increase in fluorescence (Fig. 4C). Whilst the limited range in dose-dependent response and cross-reactivity with periodate would preclude its consideration in higher category tests by TWGFX, for example, its selectivity in the presence of a number of other closely related compounds could still enable its classification as a new Category 3 or 4 test for intact bulk explosive material for forensic purposes.⁵

While exposure to perchlorate in its pure form elicited a significant response, such scenario is far from reality as a complicated matrix is more likely to be encountered. The nematodes were therefore challenged with various concentrations of Pyrodex®, all of which failed to elicit fluorescence. Interestingly, low doses of Pyrodex® spiked with perchlorate caused a significant increase in fluorescence in comparison to high doses of Pyrodex® also spiked with perchlorate indicating that the latter might not be bioavailable for immediate uptake by the nematodes in complex matrices (Fig. 4D). However, exposure to burnt wire sparklers invoked fluorescence which, although was statistically not very robust, increased in comparison to worms challenged with RDX containing post explosion residues, a high order explosive that does not contain perchlorate (Fig. 4E).

Conclusion

The ability of the worms to detect perchlorate and other similar compounds is a major advantage within a forensic and environmental context. We believe this to be the first whole animal biosensor that is able to detect this class of chemicals at low cost with the ability to be deployed on a large(er) scales. The transgenic worms have the potential to be developed as a presumptive/indicative test before a confirmatory test is performed for bulk forensic explosives analysis (e.g. using a conventional ion chromatography technique) to reveal the culprit anion. However, the current LOD of the current biosensor is 0.5 mg mL⁻¹, which is a concentration encountered in environmental hotspots.⁵ To increase the specificity of the nematodes additional, perhaps other more sensitive transgenes should be explored or could be optimized through targeted gene deletions of up or downstream enhancers. In addition, further mechanistic studies would aid in defining the underlying cause for the observed decrease in perchlorate bioavailability in complex mixtures. Despite these caveats, this study introduces the nematode’s potential as an orthogonal biosensor for the forensic detection an environmental contamination of perchlorate.

Author contributions

Conceptualization – SAA, LB and SS; formal analysis – SAA; investigation – SAA; methodology – SAA, LB and SS; project administration – SS; resources – SS and LB; validation – SS and LB; visualization – SAA; writing (original draft) – SAA; writing (review and editing) – SS; writing (final refinement) – SAA, LB and SS.

Conflicts of interest

The authors declare no competing financial interest.

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