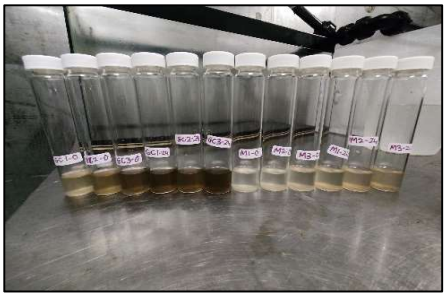
ATTRACTANT REPORT

Provision of services to research and develop an alternative viable and sustainable commercial bait for the Irish whelk, *Buccinum undatum*, pot fishery.



Report as part of BIM Whelk Bait Project currently ongoing at the Marine and Freshwater Research Centre in ATU Galway.

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BIM Ireland's Seafood Development Agency

EXECUTIVE SUMMARY

A series of whelk bait trials are underway as part of a collaboration between BIM and the Marine and Freshwater Research Centre in ATU Galway. One objective of these trials is to identify potential attractants which may play a role in eliciting foraging behaviour in the common whelk (*Buccinum undatum)*, which could warrant more detailed investigation in a future project. These chemically induced cues emanate from effective bait materials which are used in the pot fishery for whelk in Ireland. The bait materials included in this preliminary investigation of potential attractants are:

- Raw green crab (*Carcinus maenas*),
- Mussel (*Mytilus edulis*),
- Brown crab (*Cancer pagurus*) and
- Spiny sea star material (*Marthasterias glacialis*).

Seawater surrounding each sample of bait material was analysed using mass spectrometry analytical techniques. Samples were obtained using three separate methods: solid phase microextraction (SPME) using a polydimethylsiloxane (PDMS) fibre, direct injection of seawater containing bait material odour plume into the analytical machine and solid phase extraction (SPE) of the seawater – the latter aiming to concentrate analytes being described by the analytical system. The SPE method was used to concentrate analytes before analysis. Direct injection of the seawater combined with the SPE method was more effective when compared to the SPME fibre sampling method.

A range of compounds were found within the resolution limit of the instrument across the four bait materials. These include:

- Long chain fatty acids, both saturated and unsaturated,
- Pheromones and aromatic compounds,
- Steroids,
- Cholesterols/cholestenoids and
- Amino acid derivatives.

Some additional substances were identified as contaminants from the equipment, or the sampling methods employed, and were excluded from further consideration. A qualitative screening method was used to detect as wide a range of potential attractants as possible. Therefore, concentrations of these compounds in the baits cannot be determined. However, a wider range of compounds was detected in the crab material. In addition, visual inspection confirmed that more organic material dissipated from crab bait material types when compared to mussel or sea star material. These properties may contribute to the greater attractiveness of the crab material, as demonstrated in the live holding trials.

The wide variety of organic compounds found in the samples do indicate that the chemical attraction of whelk to their prey may be a multi layered and complex process which may use one or more of the chemicals found in the samples, moreover the relative concentrations of each compound in the odour plume may convey complex information to the whelk which needs to be understood before foraging behaviour can be incited in the marine gastropod.

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1. INTRODUCTION

The BIM funded Whelk Bait Project (*BIMWBP*, BIM RFT 195131) is a collaboration between BIM and the Marine and Freshwater Research Centre (MFRC) at ATU Galway, which aims to develop a sustainable alternative bait for the Irish whelk, *Buccinum undatum*, pot fishery, to promote the survival of the economically important fishery while also conserving stocks of natural bait species currently under pressure such as brown crab (*Cancer pagurus*). A series of live holding experiments are underway as part of this project to test the effectiveness of various bait materials under laboratory conditions and to identify potential attractants which may play a role in eliciting foraging behaviour in the whelk. This report describes the results from a qualitative screening of the chemical constituents released to seawater from four bait materials using Gas Chromatography Mass Spectrometry (GCMS). The bait materials compared in this analysis were:

- Raw green crab (*Carcinus maenas),*
- Mussel (*Mytulis edulis*),
- Brown crab (*Cancer pagurus*) and
- Spiny sea star (*Marthasterias glacialis*).

B. undatum forages by chemotaxis, so the development of a formulated bait must utilise this key foraging trigger. In laboratory and field trials, brown crab outperformed alternative baits in eliciting a foraging response, prompting the recommendation that future efforts to develop alterative baits for the whelk fishery further explore the attractants in brown crab (Sikavuopio et al. 2017). However, the properties of brown crab that make it attractive to whelk are not known; limited published information exists on the key induction cues and triggers that elicit whelk foraging behaviour in the wild or in land-based holding trials. The work described in this report represents an important first step in identifying the bait constituents that may play a role in the foraging response of *B. undatum.* The objective was not to isolate and describe the key attractants, but to identify a range of constituents that may warrant further investigation as potential attractants in a more detailed follow-on study.

2. METHODS

Initial chemical analysis was completed using Solid Phase Microextraction (SPME) which offers a matrix that many chemicals in the marine environment will adsorb to. The results derived from sampling during raceway behaviour trials did not show any compounds of interest apart from siloxanes which are common contaminants found using SPME fibres. Further chemical analysis was performed to ensure greater coverage of the sampling methods and use of the analytical technology available. This adaption included soaking bait materials in a smaller volume of seawater before sampling and analysing samples collected using Solid Phase Extraction (SPE) and direct injection (DI) of the holding water into the analytical instrument.

The protocol used for the chemical analysis of seawater was simplified with the number of samples taken, and the manner in which they were collected changed, following the rationale outlined below:

1. **Sensitivity** – The bait material samples were left to soak in a smaller volume of seawater as this has the effect of concentrating the chemical compounds/cues at play. Sampling could then occur over a longer period for some of the bait material types to ensure adequate time for any compounds to diffuse from the bait material and become dispersed in the surrounding seawater during smaller scale soak trials.
2. **Sampling method** – The original sampling method (SPME fibre) is usually an excellent method for the analysis of compounds in water however to ensure that all compounds possibly in the water are sampled and analysed, two additional sampling methods were also performed. A micro syringe was used to collect holding water containing the bait material odour plume which was injected directly (DI) into the analytical instrument. An SPE cartridge was also used to concentrate the compounds further. Both additional methods, DI and SPE, were processed using the same analytical instrument.
3. **Duration** – Sampling of the holding water soaking the various bait material types was carried out over a 48-hour period in seawater initially to see if there was a change in compounds found in the holding water over time. Samples were collected at 0-hour, 24-hours, and 48-hours for half of the bait materials tested. Some materials tested were sampled after soaking for 2-hours.

Sampling methods and processing

Soak trials, sampling and chemical analysis occurred between 10th – 17th January 2023. Approximately 150g of each bait material was placed in a container and immersed in 750ml of filtered seawater and left soak. Soakage in a small volume of water ensured that any chemical compounds emitted from the bait were sufficiently concentrated to be detected by the machine when analysing in qualitative screening mode. Bait soaking containers were agitated prior to water collection. A polydimethylsiloxane (PDMS) fibre was placed for 30 minutes in the soaking containers which contained brown crab and spiny sea star to collect compounds from the water. At intervals, a sample of water was taken from each soak container using a syringe (Agilent 10µl GC syringe) for direct injection into the GCMS. A second 10ml water sample was collected using a glass pipette, stored in a universal glass container and refrigerated for subsequent solid phase extraction (SPE) to concentrate the constituents prior to direct injection into the GCMS. The containers holding green crab and

mussel were sampled at 0-hour (T0), 24-hours (T24) and 48-hours (T48) while the containers holding brown crab and spiny sea star material were sampled at 2-hours (T2). The latter were left soak for a shorter time period as no apparent difference in identified compounds was found on previously sampled soaking periods, suggesting time did not increase the range of material dissipated into the seawater surrounding the bait sample. A time of 2-hours was used to allow some dissipation prior to agitation and water sampling, a longer soaking time was found to be unnecessary at this level of analysis, yielding no additional compounds of interest with time. SPME fibre sampling was also excluded for the latter as was found to be an ineffective sampling method. The methods described above can be seen in Figure 1 below.

Compounds bound to the PDMS fibre were thermally desorbed into the instrument inlet for analysis. The 10µl water sample was then injected into the analytical machine and analysed in the same way. The 10ml water sample was filtered through a SPE cartridge using a vacuum pump, shown in Figure 1 below. The SPE cartridge was cleaned after filtration with 2ml of methanol. The remaining analyte was collected and provided a more concentrated SPE sample for analysis.

Analytical instrumentation and sample description

The system used for analysis comprised of an Agilent 7820 GC System for gas chromatography combined with an Agilent 5977E MSD System for mass spectrometry analysis run in EI mode with a DB5 30m x 0.25cm x 0.25cm column. The column oven temperature was set at 80^{o}C and ramped up to 280^{o}C at 5 ^{o}C/min followed by a ramp up to 300^{o}C and a hold of 15 minutes totalling a run time of 30 minutes. Helium gas was used with a flow rate of 1 mL/min. The MSD System was set to scan mode from 0 to 500 amu.

A series of chromatograms were produced for each water sample and the NIST MS (National Institute of Standards and Technology for Mass Spectrometry) reference software was used to identify the chemical compounds represented in each chromatogram. Chromatogram peaks with a height less than 3 times the background were excluded. The accuracy of the compound identification was measured using the match factor, reverse match factor and percentage probability (Mikaia et al. 2014). The match factor compares the sample's results with spectra stored in the reference library, and scores this from 0-1000. Match factors scores >700 (fair match) were considered for further investigation. The reverse match factor ignores unknown peaks in the sample's results when comparing them to reference spectra. The percentage probability describes the degree of overlapping between the sample results when compared to reference spectra. Scores >50% probability were considered for further investigation. The identified compounds are found in the Appendices of this report and described in Table 2.

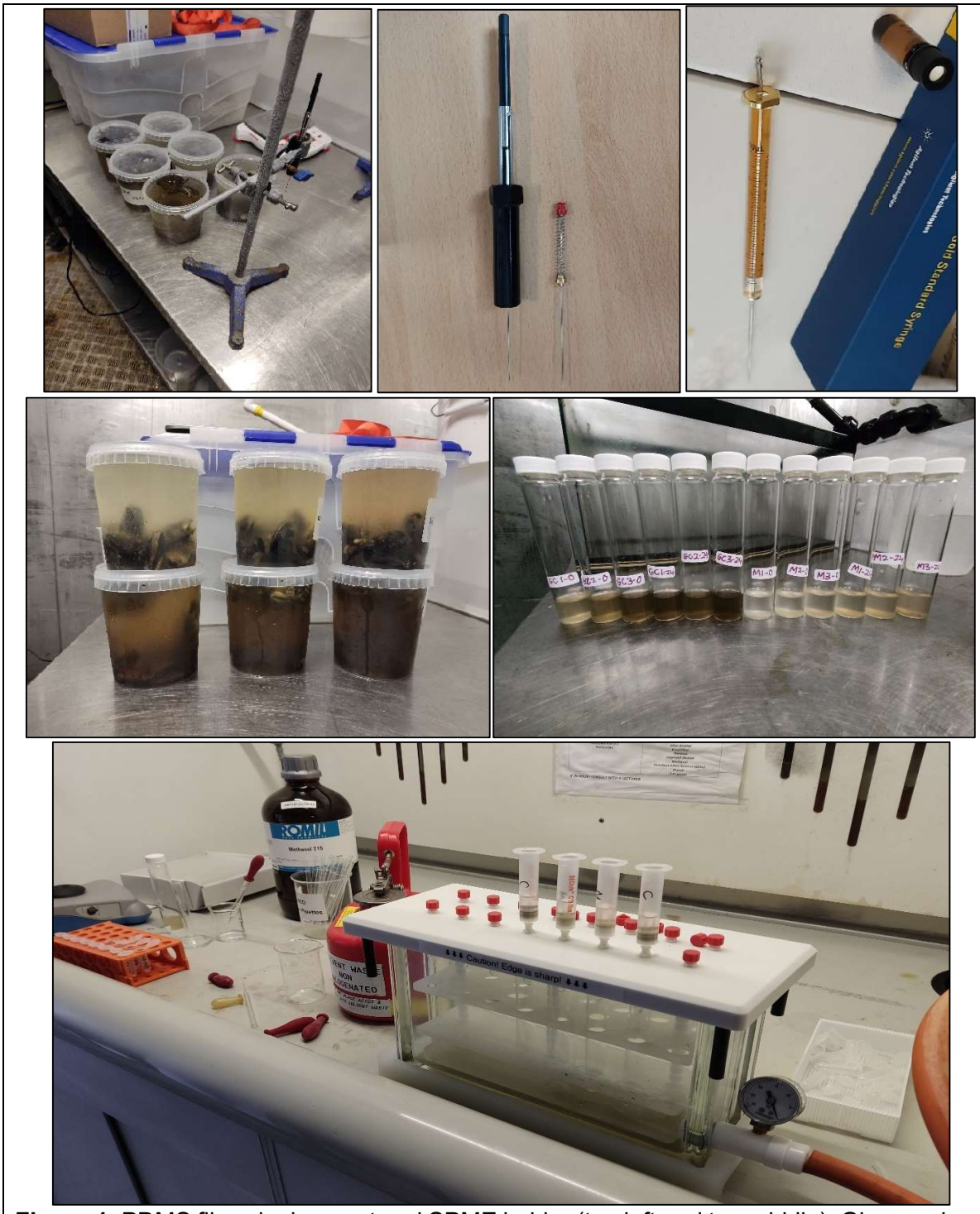

Figure 1. PDMS fibre deployment and SPME holder (top left and top middle). Glass syringe used for DI (top right). Soaking containers with green crab and mussel material stacked above (middle left). Samples collected in universal glass containers for SPE filtration prior to analysis (middle right). SPE filtration set up, with SPE cartridge filled with water sample, drawn down through apparatus using vacuum.

3. RESULTS AND DISCUSSION

Attractant identification

A range of compounds were recognised by the analytical machine (found in Table 2 of the Appendices of this report) and referenced using the NIST MS software. Similarity between compound derivatives detected in the soaking seawater holding the bait materials included:

- Long chain fatty acids, both saturated and unsaturated, including hexadecanoic acid (Palmitic acid), octadecanoic acid (Stearic acid), tetradecanoid acid (Myristic acid) and oleic acid,
- Pheromones and aromatic compounds including hexadecenal (aldehyde),
- Steroids,
- Cholesterols/cholestenoids,
- Amino acid derivatives and
- Laboratory contaminants.

A wider range of organic compounds was recorded when soaking water was directly injected into the machine or injected after solid phase extraction compared to SPME fibre sampling. More contaminants were recorded when using SPME fibre method, including siloxane from the fibre itself. Table 1 below describes the number of compounds recorded between the different sampling methods. Sampling for DI and SPE both showed a greater description of the chemical signatures described within the soaking water, with water directly injected into the analytical instrument showing the greatest variety in chemical compounds identified overall.

Table 1. Compounds recorded relative to sampling method

Sampling method	Samples analysed	Compounds recorded
SPME fibre	18	10
DI	20	25
SPE	20	23

Similar classes of compound derivatives were detected in the four bait materials, including:

Aromatic pheromones such as aldehyde, naturally occurring steroids and sex hormones such as oestradiol and androstanes as well as endogenous metabolites including cholesterols and cholestenoids were identified. Long chain fatty acids can be seen across all the bait material types; however a greater range was seen in the crab material. Some amino acid derivatives were seen, primarily asparagine, and mainly found in green crab and mussel material. Some of the aromatic compounds described by the analysis are products of the metabolism and decomposition of material by bacteria.

General discussion

The methods used in this analysis have sufficient resolution to categorize the chemical constituents released to the surrounding seawater by a bait material type during soaking. A wide range of compounds were detected in the soak water from all baits, including the two crab species which had stimulated a foraging response in the whelk during the behavioural trials and which are recognised by the industry as effective bait materials. This indicates that

the odour plume which is carried from the baits by water currents contains a complex mixture of chemical compounds. The compound or mixture of compounds that elicit the foraging response in *B. undatum* are not known. The foraging response in another carnivorous gastropod scavenger, the mud snail *Nassarius sp.* is stimulated by lactate and glycine. However, the animal shows a stronger foraging response when presented with a crude extract from its shrimp prey (Carr, 1967). This is consistent with observations of olfactory responses in crustaceans and fish (Hara 1982, Carr and Derby 1986, Carr et al. 1996), suggesting that chemoreception may involve the combined or possible synergistic effects of several compounds rather than a single compound acting in isolation.

Ferrari and Targett (2003) successfully identified a carbohydrate-protein complex in the eggs of horseshoe crabs, *Limulus polyphemus,* as the main attractant to the mud snail, *Ilyanassa obsolete*. Such a compound would comprise multiple fatty acids and amino acids as were detected in this analysis, making chemical synthesis unfeasible. Many of the compounds detected in the baits were present in some form of degradation in all of the baits tested. The preferred crab baits may contain a unique complex of these constituents. Alternatively, the crab baits may emit a more potent odour plume to the surrounding seawater. This is supported by the fact that the soaking water from the crab material was visibly more turbid compared to the soaking water from the mussel and sea star material (Figure 1). The chemical signals derived from the decomposition and breakdown of bait material by microorganisms such as bacteria are believed to be key to attractiveness as they indicate to the foraging whelk that the potential predator is dead or damaged and therefore does not pose a threat to the scavenging whelk (Dellinger et al. 2016, Bailey and Laverack 1966, Carr 1967).

The results of this initial screening do not indicate that the attractiveness of crab as a bait for whelk is conferred by any one simple compound (such as a single amino acid or fatty acid). Future efforts to identify and isolate the constituents involved in stimulating the foraging response could focus on identifying the part of the crab carcass which is most attractive to foraging whelk. Subsequently, a more detailed analysis of an extract from that part of the crab could help to isolate complex proteinaceous constituents, similar to the approach taken by Ferrari and Targett (2003). Such a study would require considerable investment and may not yield an ingredient that could be easily synthesised. Alternatively, future behavioural trials could examine the attractiveness to whelk of simple compounds or mixtures of compounds that are present in the odour plumes as potential additives that could increase the attractiveness of a formulated bait.

APPENDICES

Table 2. Alphabetical list of compounds derived from mass spectral analysis of bait materials soaked in seawater

Compound – Instrument ID	Description	Green Crab	Mussel	Brown Crab	Sea star
2-Myristynoyl-glycinamide	Amide derivative of the amino acid glycine				
(E)-13-Docosenoic acid	Long chain fatty acid - High purity Omega-9				
13-Heptadecyn-1-ol	Long chain fatty alcohol - Cosmetic qualities				
17.alfa.,21β-28,30-Bisnorhopane	Sediment - Stratigraphic marker found in oily shales				
1-Hexadecanol, 2-methyl-	Long chain alkane - Lubricative properties				
1-Tetradecanamine, N,N-dimethyl	Organic solvent - Cleaning product surfactant				
3-Ethyl-3-methylnonadecane	Long chain alkane				
4-Oxovaleric acid semi carbazone	Imine used for bonding amino acids and their carbonyl group				
6-Octadecenoic acid	Long chain fatty acid - Plant metabolite				
9-Hexadecenoic acid	Long chain fatty acid - High purity Omega-7				
Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[O-(phenylmethyl) oxime], (3α,5α)-	Naturally occurring steroid and endogenous metabolite - Biosynthesiser found in crab blood				
Behenyl behenate	Long chain fatty alcohol - Cosmetic qualities				
Benzoic acid, 4-ethoxy-, ethyl ester	Aromatic carboxylic acid found in digestion by microorganisms				
Bufa-20,22-dienolide, 3,14-dihydroxy-, (3β,5β)-	Bufalin - Cardiotonic steroid toxin				
Cholest-5-en-3-ol	Cholestenoid - Intermediate metabolite obtained during the synthesis of cholesterol				
Cholesta-3,5-diene	Cholestenoid - Intermediate metabolite obtained				

	during the synthesis of cholesterol				
Cholesterol	Base peak				
Cholesterol	Cholesterol				
Dodecanoic acid, 3-hydroxy	Aromatic saturated fatty acid				
Ergosta-5,22-dien-3-ol, acetate, (3β,22E)-	Cholesterol				
Estra-1,3,5(10)-trien-17β-ol	Naturally occurring steroid and endogenous metabolite - Oestradiol major female sex hormone				
Ethanol, 2-(9-octadecenyloxy)-, (Z)-	Organic alcohol compound				
Glycine, N-[(3α,5β)-24-oxo-3-[(trimethylsilyl)oxy]cholan-24-yl]-, methyl ester	Naturally occurring steroid and endogenous metabolite				
Glycyl-D-asparagine	Non-essential amino acid				
Hexadecanoic acid	Long chain fatty acid - Metabolite				
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Long chain fatty acid - Metabolite				
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (Palmatin - 2 mono)	Saturated fatty acid - Produced by fermentation and oxidation of natural carbohydrates				
Lathosterol	Cholesterol				
n-Hexadecanoic acid	Long chain fatty acid - Metabolite				
Octadecanal	Pheromone - Long chain aldehyde				
Octadecanal, 2-bromo	Pheromone - Long chain aldehyde				
Octadecane, 6-methyl-	Long chain branched alkane				
Octadecanoic acid	Long chain fatty acid - Plant metabolite				
Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester	Long chain fatty acid - Plant metabolite				
Oleic acid	Long chain fatty acid - Plant metabolite				
Oleic acid, 3-hydroxypropyl ester	Long chain unsaturated fatty acid - Metabolite				
Olein, 2-mono	Monoglyceride of oleic acid				
Palmitic acid, trimethylsilyl ester	Long chain fatty acid found in animals and plants				
Palmitoleic acid	Long chain saturated fatty acid				

Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	Metabolite				
Squalene	Produced by plants and animals during steroid synthesis				
Tetradecanal	Pheromone - Myristyl aldehyde found in bacteria associated with raw seafood				
Tetradecane, 2,6,10-trimethyl	Organic solvent - Cleaning product surfactant				
Tetradecanoic acid	Pheromone - Found in bacteria associated with raw seafood				
Tetradecanoic acid, 2,3-dihydroxypropyl ester	Pheromone - Found in bacteria associated with raw seafood				
Thymol	Aromatic pheromone				
trans-13-Octadecenoic acid	Long chain fatty acid - Metabolite also found in mustard seeds				
trans-Traumatic acid	Plant wound healing hormone				
Z,Z-10,12-Hexadecadienal	Pheromone - Aldehyde				
Z-14-Octadecen-1-ol acetate	Metabolic function - Saturated fatty acid acetate				

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