Biological activity screening of isolated freshwater and thermal water cyanobacteria from the Azores

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Rúben Filipe Silva Luz

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Rúben Filipe Silva Luz

Orientadores

Professor Doutor Vítor Manuel da Costa Gonçalves

Professora Doutora Maria do Carmo Barreto

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Abstract

The cyanobacteria, photosynthetic prokaryotes that are common inhabitants of inland waters, are increasingly considered an important and rich source of secondary metabolites with diverse biological activities, such as anticancer, antibacterial, antifungal, anti-inflammatory, etc., leading to an increase of bioprospecting in these organisms. In the Azores, several cyanobacteria species are present in lakes and hot springs but their biological activities were never tested. Due to the environmental conditions and isolation of the archipelago, the cyanobacteria present in the Azores might have unique proprieties and biological activities with great value.

In this work, water and biofilm samples from lakes and hot springs in the Azores where collect for the production of uni-algal cultures of cyanobacteria. The isolation work allowed the establishment of 44 strains of cyanobacteria from thermal, fresh and brackish water habitats. The identification of these strains allowed the enrichment of the basic knowledge of the Azorean cyanobacteria flora, contributing in total with 12 new species, in addition to many others still lacking identification and which are likely new for the Azores.

For the bioprospecting work 20 strains, isolated from thermal and fresh water habitats, were selected to test antioxidant, anti-acetylcholinesterase, antimicrobial, toxicity and cytotoxicity activity. All the strains tested showed activity in at least one of the selected tests, with exception for antimicrobial where none of the strains revealed any activity.

The most relevant result are the identified toxicity and cytotoxicity effects from T07 and L30 strains, that belong to cyanobacteria with no described toxic compounds and also the identified strains as potential sources of anti-acetylcholinesterasic and antioxidant compounds.

Although this work contributes significantly to the increase of knowledge about cyanobacteria in the Azores, our results show that cyanobacteria in the Azores are still understudied, with a lot of taxonomic diversity yet to be described. Also relevant are the important activities hereby identified that in future works might lead to the elucidation of compounds with pharmacological and/or biotechnological activities.

Resumo

As cianobactérias, procariotas fotossintéticos comuns em habitats lacustres, têm sido indicadas como uma importante fonte de metabolitos secundários com diversas atividades biológicas como anticancerígenas, antibacterianas, antifúngicas, anti-inflamatórias, etc., conduzindo a um aumento da bioprospeção nestes organismos. Nos Açores, várias espécies de cianobactérias estão presentes em lagos e fontes termais, mas nenhuma atividade biológica foi testada até agora. Devido às condições ambientais e isolamento do arquipélago, as cianobactérias presentes nos Açores poderão possuir propriedades únicas e atividades biológicas de valor acrescentado.

Neste trabalho foram recolhidas amostras de água e biofilmes de lagos e fontes termais nos Açores para a produção de culturas uni-algais de cianobactérias. O trabalho de isolamento permitiu a produção de 44 estirpes. A identificação destas estirpes permitiu enriquecer o conhecimento da flora de cianobactérias dos Açores, contribuindo no total com 12 novas espécies, para além de muitas por identificar e que provavelmente serão novas para a os Açores.

Para o trabalho de bioprospecção, 20 estirpes foram selecionadas, isoladas de habitats termais e lagos, para os testes de atividade antioxidante, anti-acetilcolinesterase, antimicrobiana, de toxicidade e citotoxicidade. Todas as estirpes selecionadas apresentaram atividade em pelo menos um dos testes selecionados, com exceção da atividade antimicrobiana em que nenhuma apresentou atividade.

Os resultados mais relevantes estão relacionados com os efeitos tóxicos e citotóxicos dos extratos das estirpes T07 e L30, pertencentes a cianobactérias nunca antes descritas com compostos tóxicos, mas também são de realçar as estirpes identificadas como potencial fonte de compostos com efeitos anti-acetilcolinesterásico e antioxidante.

Apesar do contributo significativo deste trabalho para o aumento do conhecimento sobre as cianobactérias nos Açores, os resultados mostram que as cianobactérias dos Açores continuam pouco estudadas, com uma grande diversidade taxonómica ainda por descrever. É também importante de referir as atividades aqui identificadas, que em trabalhos futuros poderão levar à elucidação estrutural de compostos com potenciais atividades na farmacologia e/ou biotecnologia.

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AChE - Acetylcholinesterase

AD - Alzheimer's Disease

DMSO - Dimethyl sulfoxide

DPPH - 2,2-diphenyl-1-picrylhydrazyl

MG - Myasthenia gravis

NRPS - Nonribosomal peptide synthetases

PKS - Polyketide synthases

1. Introduction

1.1. Work context and objectives

Several organisms are able to produce organic compounds that are not involved in the normal growth and development of the organism (Agostini-Costa *et al.*, 2012), but have high and diverse biological activities that can be used for human applications (Mandal & Rath, 2015). These compounds are usually known as secondary metabolites (Vining, 1990). From the various activities they might have some are more notorious as: antibacterial agents, antifungal agents, metal transporting agents, agents of symbiosis, sexual hormones and differentiation effectors (Demain & Fang, 2000).

Cyanobacteria have been revealed as an important source of compounds, recognized as one of the groups of organisms with the widest diversity and production of secondary metabolites with biological activity (Singh *et al.*, 2005; Welker & Von Dõhren, 2006; Arif *et al.*, 2012; Dixit & Suseela, 2013; Vijayakumar & Menakha, 2015; Mazard *et al.*, 2016; Swain *et al.*, 2017; Wang *et al.*, 2017) and/or with biotechnology applications (Abed *et al.*, 2008; Rastogi & Sinha, 2009; Wijffels *et al.*, 2013). For the most important and recognized activities we should emphasize anticancer, antibacterial, antiviral, antifungal and anti-inflammatory. The chemical structure of the most important cyanobacteria secondary metabolites are non-ribosomal peptides, polyketides, alkaloids and terpenes (Mandal & Rath 2015).

The most efficient method for the identification of new compounds with biological activities is by bioprospecting, based in the search of compounds with biological and commercial interests (Cox & King, 2013). Several bioprospecting studies have been realized using different approaches such as genetic methods (*e.g.* Prasanna *et al.* 2010; Brito *et al.*, 2015) or molecular methods (Montalvão *et al.*, 2016).

Cyanobacteria are gram-negative photosynthetic prokaryotes that are common inhabitants of terrestrial and aquatic ecosystems, such as lakes, thermal waters and brackish waters (Whitton & Potts, 2012). Due to their unique abilities they adapt very well to extreme environments of temperature, algae concentration, salinity, etc. (Waterbury, 2006). In the Azores islands, cyanobacteria are widely spread in lakes (Gonçalves, 2008; Cordeiro, 2015), streams (Johansson, 1977), wet soils and rocks (Bourrelly & Manguin, 1946; Johansson, 1977), and were also found in caves (Joahansson, 1977; Aguiar *et al.*, 2010) and thermal waters (Pereira *et al.*, 2010; Moreira *et al.*, 2011).

The great diversity and richness of aquatic ecosystems in the Azores, such as lakes (88, according to Porteiro, 2000) and thermal waters (19, referred by Cruz & França, 2006), creates habitats for the development of microalgae and cyanobacteria, resulting in a high potential to discover biologically active metabolites from the natural inhabitants of these ecosystems.

Therefore, the work proposed in this thesis should be a relevant contribution to (i) the isolation of new cyanobacteria with biological interest, (ii) their culture and identification, taking into advantage their possible unique qualities that might have developed by the influence of the insular isolation and special environmental conditions. With this in focus, the following objectives were set:

- Isolation, purification and maintenance of cultures of cyanobacteria from lakes and thermal waters from the Azores;
- Optimization of an extraction protocol of non-excreted compounds;
- Identification of species that produce compounds with potential biological activity.

1.2. Cyanobacteria

Cyanobacteria are a wide and diverse group of photosynthetic bacteria that are distinguished by their unique set of photosynthetic pigments: chlorophyll *a* and the phycobiliproteins phycocyanin, allophycocyanin and phycoerythrin (Waterbury, 2006). These prokaryotes are part of a phylogenetic group of phototropic bacteria, with significant different morphologies that have a very important impact in ecology, by the production of O_2 and fixation of CO_2 (Garcia-Pichel, 2009).

1.2.1. Morphology

Cyanobacteria are gram-negative prokaryotes with great morphological diversity (Whitton & Potts, 2002). They can go from unicellular forms to filamentous complexes that can have cellular differentiation, going from 0.5 μm to 50-100 μm (Whitton & Potts, 2002; Waterbury, 2006). Cells from unicellular cyanobacteria can present spherical form, bacillus or fusiform, but some species have pleomorphism. Filamentous cyanobacteria can present discoid or barrel shape cells and extend for several millimetres of length. Some species can produce an exopolysaccharide coating, forming a well-defined structure that influences the form of the cells (Garcia-Pichel, 2009).

Several species have heterocysts, specialized cells produced in response to lack of nitrogen in the environment (Garcia-Pichel, 2009). These cells are normally bigger than the others, have a double wall, are yellowish or colourless, and are connected to the adjacent cells by polar nodules. Depending on the species, these cells can acquire different forms, such as cylindrical or rectangular, being this characteristic important in the moment of identification (Sarma, 2013). The main function of the heterocysts is the fixation of atmospheric nitrogen (N_2) and delivery, in amino acids form, to the adjacent cells (Garcia-Pichel, 2009).

The akinetes are specialized cells without motility, differentiated from other cells by their augmented size, thick cellular wall and large nitrogen reservoirs in the form of cyanophycin granules. Due to their great resistance to adverse conditions, the akinetes are considered a latent state of the life cycle of the cyanobacteria and allows their re-growth when conditions become favourable (Garcia-Pichel, 2009).

1.2.2. Ecology

Cyanobacteria normally use water as electron donor during the photosynthesis producing oxygen. However, some strains have the ability to use hydrogen sulphide (H_2S) converting it to sulphur, with the advantage of resisting to low concentrations of O_2 and high concentrations of H_2S that would be toxic to the majority of algae (Vincent, 2009). This capacity can be fundamental to their resistance in eutrophic lakes or in the periphyton (Vincent, 2009).

Common in the periphyton, cyanobacteria may arise forming layers in sand, sediments, rocks, plants or other types of substrates. Depending on the environment, cyanobacteria may accumulate vertically to several centimetres of length. These layers can deposit in the bottom of water masses or arise and create a suspension layer composed mainly of cyanobacteria (Vincent, 2009).

In lakes where the nitrogen amount is high, in the form of nitrate or ammonium, cyanobacteria rarely are the dominant taxa, being competitively suppressed by better adapted algae (Assmy *et al.,* 2009). In the summer, the ratio between nitrogen and phosphorus decreases by the reduction of the amount of available nitrogen, reaching its minimum (Assmy *et al.,* 2009). This favours some species of cyanobacteria, as several of them hold the ability of atmospheric nitrogen fixation in the heterocysts (Whitton & Potts, 2012). Nonetheless, phosphorus is also indicated as a boundary to cyanobacteria growth in the natural environment, being the rise of its concentration, mainly by anthropogenic influences, in the origin of proliferation of cyanobacteria in eutrophic lakes (Whitton & Potts, 2012).

Cyanobacteria can occur in a wide interval of temperature, but prefer warmer climates for optimum growth, normally above 15 ℃ (Vincent, 2009). In *in vitro* culture conditions they show an optimum growth between 20 ℃ and 35 ℃ (Waterbury, 2006). Besides that, cyanobacteria prefer more alkaline water, growing in eutrophic lakes were the pH can reach a value of 9 (Vincent, 2009).

Cyanobacteria possess a high capacity of adaptability, surviving in zones with very low light (Waterbury, 2006). They also possess a great capacity to resist high light intensities, even when the UV radiation is high, capacity that could be an indicator of their resistance in superficial water blooms (Vincent, 2009).

1.2.1.1. Cyanobacteria in extreme environments

Extreme environments are, from an anthropocentric point of view, those inhospitable and abnormal places that some organisms may inhabit (Mandal & Rath, 2015). Cyanobacteria are often the main and/or sole autotrophic organisms inhabiting these environments (Komárek & Johansen, 2015a). Extremophile organisms are classified according to their type of "extreme" habitat, since these can present very low or high values of temperature, pH, salinity, heavy metal concentrations, etc. (Mandal & Rath, 2015).

Thermophilic cyanobacteria are those that grow well or resist temperatures above 45 ℃, with a maximum of 72-73 ℃ (Castenholz, 1988). Different types of cyanobacteria have been identified in high temperature habitats, such as *Oscillatoria* and *Mastigocladus*, but the most common is *Synechococcus,* with a maximum upper temperature of 72-73 ℃ (Mandal & Rath, 2015).

Marine and halophilic cyanobacteria are organisms that grown in salt obligatory environments (Oren, 2012), where cyanobacteria can tolerate salinity levels up to saturation (Mandal & Rath, 2015). The marine cyanobacteria *Coleofasciculus chthonoplastes* is the main mat building of cyanobacteria in littoral areas and can tolerate salinity as high as 200 g/L (Oren, 2012).

1.2.3. Taxonomy

Taxonomic classification is the main method used to evaluate biological diversity and has been in continuous modification since its creation. Although they are considered bacteria, cyanobacteria classification has been traditionally done by algologists following the botanic taxonomic principles and their nomenclature code. The recent interest of bacteriologists in this group, together with the use of new techniques in taxonomy, profoundly changed the cyanobacteria classification. In addition, the great morphological diversity and the long evolutionary history of cyanobacteria caused higher difficulties, contributing to a continuous reconstruction of the classification of this group (Komárek *et al.*, 2014).

The older classification was based exclusively in their morphology, but nowadays new techniques such as electronic microscopy and phylogenetic data contribute to cyanobacteria classification (Richmond & Hu, 2013; Komárek, 2016).

After a phylogenetic revision in cyanobacteria, Komárek *et al.* (2014) suggested a new classification which recognizes eight orders: Gloeobacterales, Synechococcales, Spirulinales, Chroococcales, Pleurocapsales, Oscillatoriales, Chroococcidiopsidales and Nostocales (table 1).

Table 1 – Cyanobacteria orders, main characteristics and genera following Komárek *et al.* (2014).

Order	Characteristics	Exp. Genus
I. Gloeobacterales	Absent thylakoids	*Gloeobacter*
II. Synechococcales	Unicellular or filamentous; Parietal thylakoids	*Pseudanabaena*, *Eucapsis*
III. Spirulinales	Regular screw-like coiled trichomes without sheaths	*Spirulina*
IV. Chroococcales	Coccoids that have a more or less irregular thylakoid arrangement	*Microcystis*, *Aphanothece*
V. Pleurocapsales	Pseudo-filamentous which reproduce by baeocytes; division in multiple planes or irregular	*Xenococcus*, *Chroococcidium*
VI. Oscillatoriales	Radial, fasciculate or irregular thylakoid arrangement	*Oscillatoria*, *Lyngbya*
VII. Chroococcidiopsidales	Division in three or more planes; reproduction by baeocytes	*Chroococcidiopsis*
VIII. Nostocales	Filamentous with diversified thallus and specialized cells (heterocysts and akinetes)	*Anabaena*, *Calothrix*

1.3. Cyanobacteria secondary metabolism

The best characterized compounds of cyanobacteria are their toxins, due to their harmful effects to animals and humans when abundant in contaminated water masses (Dittmann *et al.*, 2012). Nonetheless, many other secondary metabolites are known, reaching 1100 compounds from 39 genera (Dittmann *et al.*, 2015). Most of these compounds, which comprise several different activities (table 2), are related to few genera and confined to specific orders mainly because those species are the ones most studied due to their easier *in vitro* cultivation (Welker & Von Döhren, 2006).

The majority of biologically active metabolites that we can extract from cyanobacteria are either peptides, macrolides, or a combination of both (Welker & Von Döhren, 2006). Many of these metabolites have been characterized and are known to be produced by the Nonribosomal Peptide Synthetases (NRPS) and/or by the Polyketide Synthases (PKS) route (Kehr *et al.*, 2011), as illustrated in figure 1. Nonetheless, other synthetic pathways using cytochrome P450 are being studied and elucidated in the secondary metabolism of cyanobacteria (Mandal & Rath, 2015).

Table 2 – Cyanobacteria biological activity list (adapted from Singh *et al.* 2005).

Order	Biological activities
Chroococcales	Enzyme inhibitor, cytotoxic, tumor promoter, endotoxic, hepatotoxic
Pleurocapsales	Antifungal
Oscillatoriales	Anticancer, antifungal, anti-inflammatory, antimicrobial, antimitotic, antiproliferative, brine shrimp toxicity, cytotoxic, herbicidal, hepatotoxin, molluscidal, neurotoxic, tumor promoter, sunscreen pigment, toxin
Nostocales	Anticancer, antifungal, antimalarial, anti-HIV, hepatotoxic, antimicrobial, antimitotic, anti-inflammatory, cytotoxic, enzyme inhibitor, toxin, neurotoxin
Stigonematales	Antifungal, antibiotic, anticancer, antimitotic, cytotoxic, herbicidal

1.3.1. Biosynthetic pathways of peptides

Peptides in cyanobacteria may be produced by two types of biosynthetic pathways, (i) the NRPS or (ii) the ribosomal synthesis with post-translational modification and processing (Kehr *et al.*, 2011). The NRPS consist in domains that work in a progressive way to incorporate a single amino acid in the target molecule (Kehr *et al.*, 2011). The number of these domains can range from seven to 64 depending on the target molecule (Dittmann *et al.*, 2015). The ribosomal synthesis is limited but also contributes with direct gene products with only a few post-translational modifications (Dittmann *et al.*, 2015).

Macrolides are compounds with a macrocyclic lactone ring of 12 or more elements (Mazzei *et al.*, 1993). In cyanobacteria they are produced by the PKS route, and contrary to NRPS this route performs the activation and assemblage of carboxylic acids (Kehr *et al.*, 2011).

1.3.2. Other compounds

Cyanobacteria also produce a vast array of other compounds such as retinoids, alkaloids, lactones and phospholipids (Elersek *et al.*, 2017). Among the most notorious there is saxitoxin, a neurotoxic alkaloid (Ballot *et al.*, 2017). Several natural compounds found in plants such as terpenes can also be produced by cyanobacteria, e.g. geosmin (Dittmann *et al.*, 2015). Other compounds like hapalindole-type alkaloids are a group of hybrid isoprenoid-indole compounds found, according to Dittmann *et al.* (2015), only in *Stigonemataceae* species, though other species have been reported to produce it, extending the production families to *Hapalosiphonaceae* (Hillwig *et al.*, 2014).

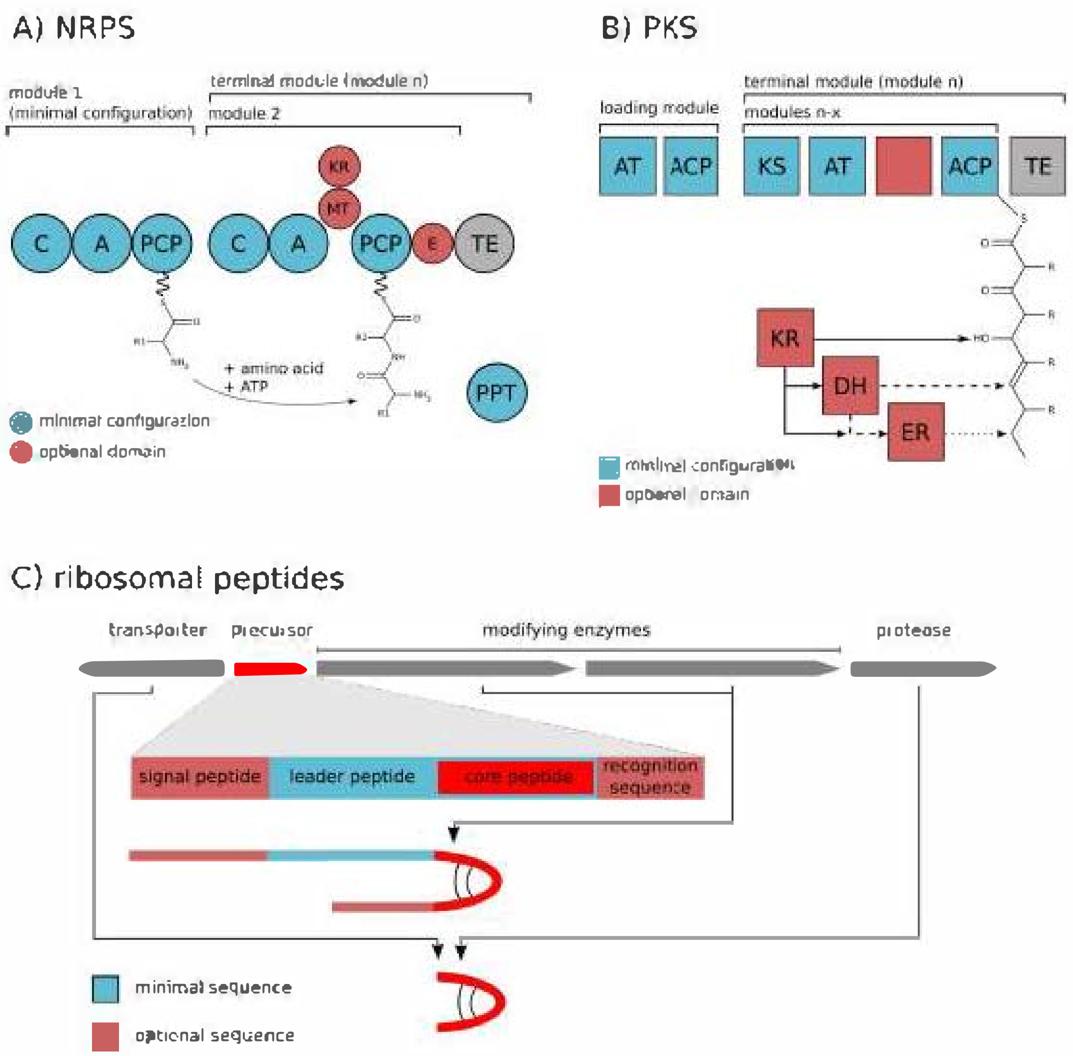

Figure 1 – NRPS, PKS and ribosomal pathways (from Kehr *et al.* 2011).

1.4. Pharmacological and biotechnological applications of secondary metabolites from cyanobacteria.

The wide diversity of secondary metabolites that has been identified in cyanobacteria render these organisms into excellent models for pharmacological and biotechnological studies, considering that a great part of cyanobacteria remains unstudied (Dittmann *et al.*, 2015). The search for applications for the metabolites of cyanobacteria is in focus with several reviews published in recent years with that topic (e.g. Dias *et al.*, 2015; Varshney *et al.*, 2015; Vijayakumar & Menakha, 2015; Mazard *et al.*, 2016; Raja *et al.*, 2016; Singh *et al*., 2016; Haque *et al*., 2017; Rajneesh *et al*., 2017; Singh *et al*., 2017; Swain *et al*., 2017; Wang *et al*., 2017).

Among the investigated activities the pharmaceutical field is the one most researched, with diverse activities such as antimicrobial, anticancer, antifungal, etc. Several works have focused on anticancer activity, as shown by Vijayakumar & Menakha (2015) and Wang *et al*. (2017) reviews, where a wide array of metabolites have been identified, many of which obtained from the marine cyanobacteria *Lyngbya majuscula*. Antimicrobial and antifungal activities have been widely detected in cyanobacteria from several identified and elucidated secondary metabolites, as reported by Swain *et al*. (2017) and Wang *et al*. (2017). Cyanobacteria also produce a wide array of toxins, many already identified and with also several identified activities in the pharmaceutical field (Dias *et al.*, 2015).

Several biotechnological applications have been proposed for cyanobacteria, such as biofertilizers, biofuels, bioremediation agents and food supplements. Among food supplements *Arthrospira platensis* (Spirulina) is already widely used due to its high protein and nutrient content (Raja *et al*., 2016; Haque *et al*., 2017). *Anabaena azollae* has been widely used in Asia as biofertilizer and is recognized as an important species due to its symbiotic relation with *Azolla*, an aquatic fern used to enrich Asian crops, allowing its use as a source of organic N_2 (Watanabe, 1982). Recently several other cyanobacteria have been studied to be used as biofertelizers, with their relation and interaction with the destination crop investigated (Singh *et al*., 2016). Due to their nitrogen fixation ability and lipid content, cyanobacteria is also studied as possible sources of biofuel, which if used might minimize the cost and harmful effects of the presently used fuels (Mazard *et al*., 2016; Haque *et al*., 2017; Rajneesh *et al*., 2017).