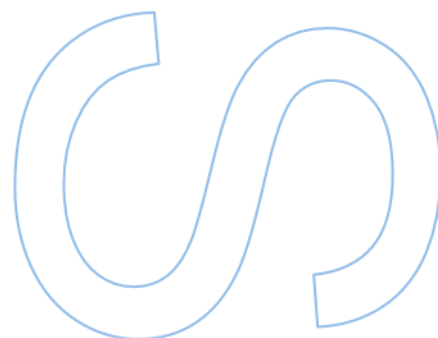
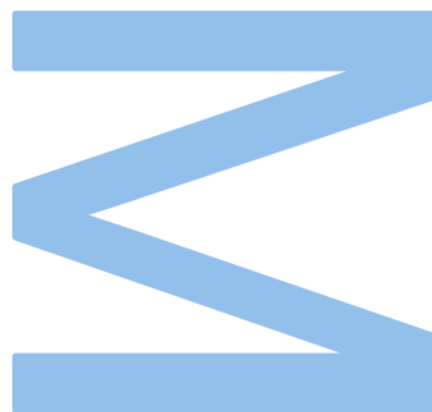


Bioprospecting Cyanobacterial Metallophores for Alzheimer's Disease Therapy



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Master in Applications in Biotechnology and Synthetic Biology

Department of Chemistry and Biochemistry
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RESUMO

As doenças neurodegenerativas (NDs), incluindo a doença de Alzheimer (AD), a doença de Parkinson (PD), a doença de Huntington (HD) e a esclerose lateral amiotrófica (ALS), representam um dos maiores desafios da medicina moderna devido à sua crescente prevalência e à ausência de terapias eficazes capazes de modificar o curso da doença. Entre estas patologias, a AD é a forma mais comum de demência e caracteriza-se por mecanismos patológicos complexos que envolvem a acumulação de beta amiloide (A β), a hiperfosforilação da proteína tau, o stress oxidativo, a neuroinflamação crónica, a disfunção colinérgica e a excitotoxicidade glutamatérgica. Dada a natureza multifatorial da AD, a descoberta de novos compostos bioativos capazes de modular vias patológicas-chave continua a ser uma prioridade. Os produtos naturais têm historicamente constituído uma fonte rica de agentes terapêuticos, e as cianobactérias emergiram como organismos particularmente promissores devido à sua notável diversidade metabólica e capacidade de produzir uma ampla gama de metabolitos bioativos.

O presente estudo teve como objetivo avaliar o potencial neuroprotetor de frações de cianobactérias obtidas a partir de cem estirpes diferentes, com especial ênfase em estirpes que apresentam regiões genómicas associadas à biossíntese de metalóforos. Foram utilizadas um total de oitocentas frações obtidas através de cromatografia líquida de alta eficiência (HPLC) e estas foram avaliadas quanto à sua atividade inibitória da acetilcolinesterase (AChE) utilizando o ensaio de Ellman. Em paralelo, frações selecionadas foram avaliadas quanto à atividade anti-inflamatória em macrófagos RAW 264.7 estimulados com LPS. Os efeitos anti-inflamatórios foram avaliados através do método de Griess, enquanto a viabilidade celular foi determinada pelo ensaio de determinação da viabilidade celular através do brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT).

Nenhuma das frações avaliadas apresentou uma inibição significativa da atividade da AChE. No entanto, o rastreio anti-inflamatório revelou um número substancial de frações capazes de reduzir a inflamação induzida por LPS sem comprometer a viabilidade celular. Entre as placas avaliadas, as frações da WP12 apresentaram o perfil mais promissor, exibindo uma atividade anti-inflamatória consideravelmente superior à observada nas WP5 e WP11. A fração mais ativa, LEGE_08334_H, reduziu a inflamação relativa para 12,30% do controlo, correspondendo a uma redução de aproximadamente dez vezes na produção de óxido nítrico. Destaca-se que nove dos

dez melhores resultados anti-inflamatórios foram obtidos com a estirpe *Sphaerospermopsis* sp. LEGE 08334.

Os ensaios de citotoxicidade demonstraram que a maioria das frações apresentou baixa toxicidade, mantendo frequentemente níveis de viabilidade celular iguais ou superiores aos do controlo. Algumas frações chegaram mesmo a aumentar a viabilidade celular relativa para valores superiores a 200%, sugerindo potenciais efeitos imunomoduladores ou proliferativos sobre macrófagos. Para identificar as frações mais relevantes, foi aplicado um índice bioativo integrado que combina atividade anti-inflamatória e viabilidade celular. As frações derivadas de *Sphaerospermopsis* sp. LEGE 08334 apresentaram consistentemente as pontuações mais elevadas nomeadamente as frações LEGE_08334_A, LEGE_08334_C e LEGE_08334_B. Adicionalmente, frações da estirpe *Lusitaniella coriacea* LEGE 07167 demonstraram também um potencial bioativo relevante.

Globalmente, os resultados demonstram que as cianobactérias constituem uma fonte valiosa e ainda pouco explorada de metabolitos bioativos com potencial aplicação na investigação de NDs.

Palavras-chave: doenças neurodegenerativas, doença de Alzheimer, cianobactérias, produtos naturais, atividade anti-inflamatória, atividade antiacetilcolinesterase

ABSTRACT

Neurodegenerative diseases (NDs), including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), represent one of the greatest challenges facing modern medicine due to their increasing prevalence and the absence of effective disease-modifying therapies. Among these disorders, AD is the most common form of dementia and is characterized by complex pathological mechanisms involving beta-amyloid (A β) accumulation, tau hyperphosphorylation, oxidative stress, chronic neuroinflammation, cholinergic dysfunction, and glutamatergic excitotoxicity. Given the multifactorial nature of AD, the discovery of novel bioactive compounds capable of modulating key pathological pathways remains a priority. Natural products have historically provided a rich source of therapeutic agents, and cyanobacteria have emerged as particularly promising organisms due to their remarkable metabolic diversity and capacity to produce a wide range of bioactive metabolites.

The present study aimed to evaluate the neuroprotective potential of cyanobacterial fractions obtained from one hundred different strains, with special emphasis on strains harboring genomic regions associated with metallophore biosynthesis. A total of eight hundred fractions generated through high-performance liquid chromatography (HPLC) fractionation and screened for acetylcholinesterase (AChE) inhibitory activity using the Ellman assay. In parallel, selected fractions were evaluated for anti-inflammatory activity using LPS-stimulated RAW 264.7 macrophages. Anti-inflammatory effects were assessed through the Griess reaction and cell viability determined using a cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

None of the evaluated fractions displayed significant inhibition of AChE activity. However, the anti-inflammatory screening revealed a substantial number of fractions capable of reducing LPS-induced inflammation without compromising cell viability. Among the evaluated plates, fractions from WP12 exhibited the most promising profile, showing markedly stronger anti-inflammatory activity than those from WP5 and WP11. The most active fraction, LEGE_08334_H, reduced relative inflammation to 12.30% of the control, corresponding to an approximately tenfold reduction in NO production. Notably, nine of the ten best anti-inflammatory results originated from WP12, and six belonged to the same cyanobacterial strain, *Sphaerospermopsis* sp. LEGE 08334.

Cytotoxicity assays demonstrated that most fractions exhibited low toxicity, with many maintaining cell viability at levels equal to or greater than those of the control. Several fractions even increased relative cell viability beyond 200%, suggesting potential immunomodulatory or proliferative effects on macrophages. To identify the most relevant candidates, an integrated bioactivity score combining anti-inflammatory activity and cell viability was applied. Fractions derived from *Sphaerospermopsis* sp. LEGE 08334 consistently achieved the highest scores, with LEGE_08334_A, LEGE_08334_C, and LEGE_08334_B ranking as the top-performing fractions. Additionally, fractions from strain *Lusitaniella coriacea* LEGE 07167 displayed notable bioactive potential.

Overall, the results demonstrate that cyanobacteria constitute a valuable and underexplored source of bioactive metabolites with potential applications in neurodegenerative disease research.

Keywords: Neurodegenerative diseases, Alzheimer's disease, cyanobacteria, natural products, anti-inflammatory activity, anti-acetylcholinesterase activity.

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LIST OF ABBREVIATIONS & ACRONYMS

A β : β -amyloid peptide

Ach: Acetylcholine

AChE: Acetylcholinesterase

ACH: Amiloid cascade hypothesis

AD: Alzheimer's disease

ALS: Amyotrophic lateral sclerosis

AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate

APP: Amyloid precursor protein

ATCh: Acetylthiocholine iodide

BACE1: β -secretase

BBB: Blood–brain barrier

BChE: Butyrylcholinesterase

BOGA: Bioterium of Aquatic Organisms

CBD: Cannabidiol

ChAT: Choline acetyltransferase

ChE: Cholinesterase

CH: Cholinergic hypothesis

CNS: Central nervous system

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)

EFSA: European Food Safety Authority

EGCG: Epigallocatechin gallate

FBS: Fetal bovine serum

FDA: Food and Drug Administration

GH: Glutaminergic hypothesis

HD: Huntington's disease

HPLC: High-performance liquid chromatography

IOSH: Inflammation and oxidative stress hypothesis

LPS: Lipopolysaccharide

MP: Mother Plate

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NDs: Neurodegenerative diseases

NF- κ B: Nuclear factor κ B

NGF: Nerve growth factor

NMDA: *N*-methyl-D-aspartate

NO: Nitric oxide

NFTs: Neurofibrillary tangles

NPs: Natural products

NRP: Non-ribosomal peptide

PART: Primary Age-Related Tauopathy

PCB: Phycocyanobilin

PD: Parkinson's disease

PNS: Peripheral nervous system

RiPP: Ribosomally synthesized and Post-translationally modified Peptides

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SOD: Superoxide dismutase

TH: Tau hypothesis

WHO: World Health Organization

WP: Working Plate

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1. INTRODUCTION

1.1. NEURODEGENERATION

Neurodegeneration is commonly defined in the scientific literature as the progressive process of neuronal dysfunction and death that affects both the central nervous system (CNS) and the peripheral nervous system (PNS) ¹. Although it is difficult to attribute a single and definitive cause to this phenomenon, several mechanisms have been identified that, independently or in an interrelated manner, contribute to neuronal degeneration. Among the most relevant are the accumulation of misfolded proteins, mitochondrial dysfunction, synaptic loss, chronic inflammation, and the increased presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) ^{1,2}.

Neurodegeneration constitutes the hallmark of a broad group of neurodegenerative diseases (NDs), which, although they may share common clinical manifestations such as disorientation, motor disorders, or memory deficits, differ fundamentally in the specific patterns of brain involvement, the proteins implicated, and the associated genes. Among the most prevalent NDs are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) ².

It should be noted that most patients do not present a "pure" NDs, but rather mixed forms characterized by the coexistence of neuropathological alterations and overlapping symptomatology, in which the clinical phenotype associated with one of the pathologies usually predominates ³. This often hinders early diagnosis and the identification of the most appropriate treatment for each case.

Numerous NDs show a close association with aging, and given that global life expectancy has shown a sustained increase since the mid-20th century ⁴, the prevalence and frequency of these pathologies have also increased significantly. In 2021, more than one million deaths in Asia were attributed to AD and other forms of dementia, together with approximately five hundred thousand in Europe and nearly two million worldwide ⁵.

Despite the high incidence of NDs and projections indicating a continuous increase in their impact on the population, there is currently no effective therapy capable of halting or significantly reversing their progression. Although multiple therapeutic strategies have been developed, no available drug has demonstrated decisive efficacy against these disorders ⁶, this makes the search for effective treatments one of the greatest contemporary challenges for the pharmaceutical industry.

1.2. ALZHEIMER'S DISEASE

As previously noted, AD is a NDs and represents the most common form of dementia, accounting for approximately 60 to 70% of cases worldwide. It is estimated that in 2015, this pathology affected nearly 47 million people. However, based on current demographic trends, global prevalence is expected to increase substantially, reaching approximately 75 million people by 2030 and up to 132 million cases by 2050 ⁷.

Although the incidence of dementia associated with AD tends to increase and consolidate with aging, this pathology should not be considered exclusive to older ages. Several studies suggest that certain unhealthy lifestyle habits, such as physical inactivity, a high-calorie diet associated with obesity, tobacco use, and excessive alcohol consumption, may increase the risk of developing dementia ⁷⁻⁹.

However, susceptibility to this disease also involves a significant genetic component. In general terms, the population risk of an individual developing dementia when there is a family history is approximately 10%. This percentage can increase to around 20% in families with certain genetic configurations, in which multiple low-effect gene variants interact to elevate disease risk. However, a small proportion of the population presents a family history of early-onset dementia with autosomal dominant inheritance. In these cases, each offspring has a 50% probability of inheriting the causal mutations, conferring a lifetime risk of developing dementia exceeding 95% ¹⁰.

Despite the extensive effort that has been devoted and continues to be devoted to understanding AD, its origin, development, and progression remain an enigma for the scientific community. However, several hypotheses have been developed that may provide an explanation for this disease (Figure 1).

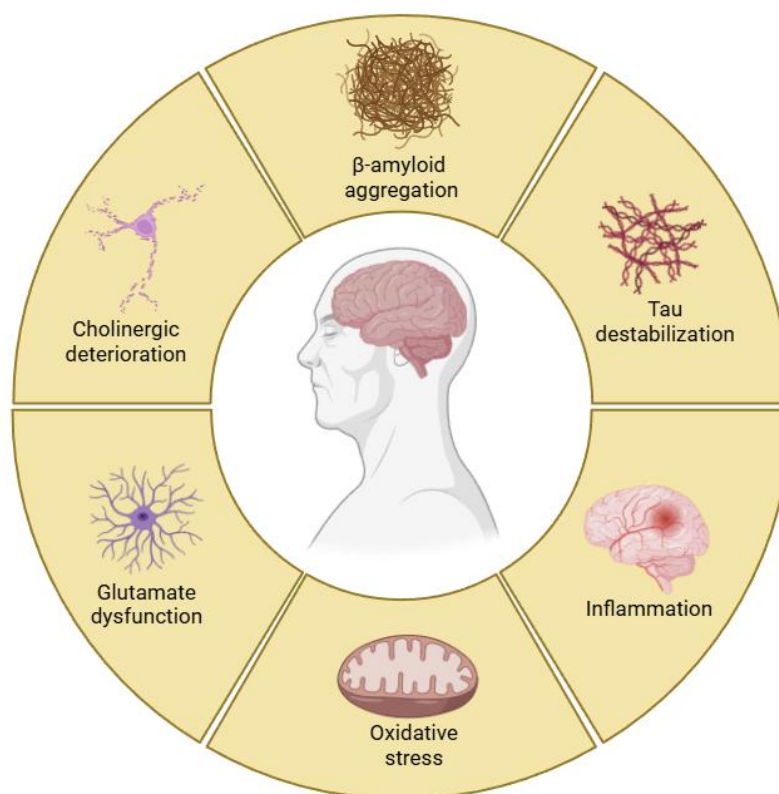


Figure 1: Schematic representation of what are believed to be the main proposed causes for the development of Alzheimer's disease. Created in <https://BioRender.com>.

1.2.1. AMILOID CASCADE HYPOTHESIS (ACH)

The ACH was first defined by John Hardy and David Allsop in 1991¹¹. The authors concluded that, although there may be many causes leading to AD, it is highly likely that a common sequence of pathological events occurs in all cases. In their hypothesis, they identify amyloid plaques, which are characteristically present in AD patients, as the primary driver of the disease's onset and the associated progressive neurodegeneration¹¹.

The amyloid precursor protein (APP) is a transmembrane protein that is naturally expressed in neuronal membranes and participates in fundamental processes such as cell adhesion, intracellular signaling, and the maintenance of synaptic integrity. This protein can be processed through two main proteolytic pathways. The first corresponds to the so-called non-amyloidogenic pathway, in which APP is predominantly cleaved by the enzyme α -secretase, resulting in the release of a soluble fragment known as sAPP α . This fragment possesses neuroprotective properties and has been associated with the promotion of neuronal survival as well as axonal and dendritic growth^{12,13}.

The second pathway, known as the amyloidogenic pathway, involves the initial cleavage of APP by β -secretase (BACE1), generating the soluble fragment sAPP β and a membrane-bound C-terminal fragment known as C99. Subsequently, γ -secretase processes the C99 fragment, producing the beta-amyloid peptide (A β). The A β peptide tends to aggregate when it is produced in excess or when its clearance mechanisms are compromised, forming the basis for the characteristic amyloid plaques observed in AD ^{6,11–13}.

The accumulation and aggregation of the A β peptide can induce synaptic dysfunction, oxidative stress, alterations in neurotransmission, and metabolic imbalances, contributing early to memory impairment even before neuronal death occurs. Additionally, the body responds to these amyloid aggregates as if they were a threat, activating glial cells such as microglia and astrocytes, which leads to the release of proinflammatory cytokines. Over time, this inflammatory response can become chronic and promote progressive neuronal damage ^{6,14,15}.

For numerous authors, this hypothesis also constitutes the conceptual framework that precedes and supports the tau protein hypothesis in the pathophysiology of AD ^{9,14,16}.

1.2.2. TAU HYPOTHESIS (TH)

Tau protein is commonly associated with microtubules in neuronal axons and plays a fundamental role in cytoplasmic transport, which is essential for maintaining synaptic signaling, neuronal structure, and neuronal signaling ^{17,18}. In the healthy brain, tau undergoes dynamic cycles of phosphorylation and dephosphorylation that regulate its affinity for microtubules, allowing it to adapt to the functional needs of the neuron. In the characteristic pathophysiology of AD, tau undergoes aberrant hyperphosphorylation, resulting in abnormal conformations and its pathological detachment from the neuronal axon ^{19,20}. Consequently, tau loses its ability to promote tubulin polymerization and dissociates from microtubules, leading to their destabilization. This phenomenon causes disruption of axonal transport and neuronal metabolism, fostering a state of energy and functional deficit that progressively contributes to neuronal deterioration and dysfunction ^{21,22}.

Because of axonal destabilization, tau also tends to redistribute to dendritic compartments, where it interferes with the function of *N*-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors,

compromising synaptic transmission. This phenomenon is closely associated with cognitive decline ²³.

Additionally, hyperphosphorylated tau exhibits a high propensity for self-assembly, leading to the formation of oligomeric aggregates and, subsequently, the so-called neurofibrillary tangles (NFTs), which are considered highly neurotoxic ^{19,20}.

Furthermore, the loss of tau's association with axonal microtubules promotes its propagation throughout brain tissue via a prion-like mechanism. This process contributes to the destabilization of initially healthy neurons and has significant implications for the temporal and spatial progression of neurodegeneration ^{24,25}.

Tau protein hyperphosphorylation is typically associated with an increase in the activity of the kinases responsible for regulating its physiological phosphorylation cycles ^{19,20}. This increase in kinase activity may be mediated by processes of cellular stress or by chronic inflammation, both phenomena characteristic of the ACH in the context of the pathological accumulation of A β . In this sense, ACH has been proposed as a possible trigger of tau pathology and, consequently, of the development of AD ^{9,14,16}.

However, during the natural physiological process of human aging, a progressive increase in the basal activity of various kinases is observed, accompanied by a decrease in the activity of the phosphatases responsible for the dephosphorylation of tau ²⁶. This imbalance in the regulation of the protein could explain the development of tau pathology in the absence of significant A β accumulation, in a process known as Primary Age-Related Tauopathy (PART) ²⁷.

1.2.3. INFLAMMATION AND OXIDATIVE STRESS HYPOTHESIS (IOSH)

The IOSH proposes that a chronic state of brain inflammation, together with a sustained increase in oxidative damage, constitutes a key factor in the origin and progression of AD.

During aging, the brain undergoes a series of physiological changes, including a reduction in antioxidant systems, an increase in oxidative stress, and greater microglial reactivity ^{28,29}. Although these modifications do not constitute a pathology in themselves, they do contribute to the generation of a more vulnerable cerebral environment, characterized by a reduced capacity for repair and greater susceptibility to external and internal insults. Taken together, these changes promote a disproportionate and

persistent inflammatory response, which could facilitate the development of neurodegenerative processes such as those observed in AD ¹⁵.

The decrease in cerebral antioxidant capacity leads to an increase in the production of ROS, generated mainly as by-products of neuronal metabolism and enhanced by the presence of transition metals with redox activity, such as iron (Fe^{2+}) and copper (Cu^{2+}) ³⁰. The resulting oxidative stress has adverse effects on various cellular components: it induces peroxidation of membrane lipids, promotes protein oxidation with the consequent alteration of their folding and functionality, and causes damage to nuclear DNA and mitochondrial DNA ^{6,31}. Neurons are particularly vulnerable to these processes due to their high content of polyunsaturated fatty acids, their critical dependence on mitochondrial metabolism for energy production, and their limited capacity for regeneration. Taken together, these alterations can culminate in accelerated degeneration and, eventually, neuronal death ³².

Neuronal damage triggers an exaggerated activation of glial cells, which release greater amounts of proinflammatory cytokines and ROS ^{15,29}. This response amplifies oxidative stress and inflammation, establishing a vicious cycle that promotes a progressive and persistent neurodegenerative process, laying the foundations for the development of AD.

Additionally, oxidative stress favors the amyloidogenic processing of APP, promoting the generation and accumulation of A β plaques. The presence of these deposits induces an even stronger glial response, reinforcing the inflammatory loop and contributing to the worsening of the pathology ^{33,34}.

ROS can also inhibit the activity of phosphatases, enzymes essential for the physiological dephosphorylation of the tau protein. This inhibition promotes tau hyperphosphorylation and, consequently, the development of tauopathies associated with AD ²⁶. Taken together, this hypothesis proposes a cascade-like progression of AD originating from inflammatory and oxidative processes, modulated by the physiological changes inherent to brain aging. This model is consistent with the mechanisms described in PART ²⁷.

1.2.4. GLUTAMINERGIC HYPOTHESIS (GH)

The GH postulates that the pathological accumulation of glutamate in the brain constitutes a determining factor in synaptic dysfunction and the development of AD.

Glutamate is the principal excitatory neurotransmitter of the CNS, responsible for approximately 90% of excitatory synapses. It plays an essential role in processes such as learning, memory, cognition, and synaptic plasticity^{35–37}. In addition to its neurotransmitter function, glutamate participates in various metabolic pathways, including its role as an intermediate in the Krebs cycle; however, its relevance in the CNS is especially critical³⁸.

It is important to note that glutamate is also naturally present in numerous foods and is widely used in the food industry as a flavor enhancer, associated with the so-called umami taste. However, dietary glutamate consumption has not been linked to the development of neurodegenerative diseases, since this amino acid does not cross the blood–brain barrier (BBB) to a significant extent^{39,40}. This barrier strictly regulates the passage of substances into brain tissue, ensuring that glutamate involved in neurotransmission is synthesized locally in the brain⁴⁸. Accordingly, national and international organizations such as the European Food Safety Authority (EFSA), the U.S. Food and Drug Administration (FDA), and the World Health Organization (WHO) consider glutamate safe for human consumption at usual dietary doses^{41–43}.

Glutamate is released by presynaptic neurons during synaptic transmission and binds to specific receptors located on the postsynaptic membrane, triggering excitatory signaling. Its concentration in the synaptic space is subsequently strictly regulated through uptake by glial cells, particularly astrocytes. In these cells, glutamate is converted into glutamine by the enzyme glutamine synthetase. The resulting glutamine is transported back to neurons, where it is reconverted into glutamate, thereby closing the so-called glutamate–glutamine cycle³⁸. This mechanism is essential for maintaining glutamate homeostasis and ensuring proper synaptic signaling.

Glutamate exerts its action by binding to ionotropic glutamate receptors, primarily AMPA and NMDA receptors. AMPA receptors are the main mediators of fast excitatory transmission, as their activation allows the entry of sodium ions (Na^+), generating the initial depolarization of the postsynaptic membrane. This depolarization expels the magnesium ion (Mg^{2+}) that, under resting conditions, blocks the NMDA receptor channel in a voltage-dependent manner. Once this block is removed, the NMDA receptor is activated in the presence of glutamate and allows the entry of calcium ions (Ca^{2+}) into the neuron. The intracellular increase of Ca^{2+} acts as a secondary messenger, activating various sensor proteins and intracellular signaling cascades that regulate synaptic plasticity and support the continuation of neuronal transmission³⁶.

In this process, astrocytes rapidly and efficiently degrade glutamate, since despite its fundamental role in excitatory neurotransmission, its accumulation in the extracellular space is highly neurotoxic. During aging, glial cell efficiency tends to decline, which may lead to reduced glutamate uptake and degradation, favoring its extracellular accumulation ⁴⁴.

Pathological presence of glutamate in the extracellular space causes overstimulation of postsynaptic glutamate receptors, generating uncontrolled Ca^{2+} entry into neurons. This excess intracellular Ca^{2+} induces mitochondrial dysfunction, damage to cellular membranes, and cytoskeletal alterations, in addition to activating pro-apoptotic signaling pathways, inevitably leading to neuronal death ³⁶.

Furthermore, the increase in intracellular calcium can promote the activation of various kinases and the inhibition of certain phosphatases, fostering tau protein hyperphosphorylation. Additionally, excess intracellular Ca^{2+} enhances BACE1 activity, increasing the amyloidogenic processing of APP and promoting $\text{A}\beta$ accumulation. Simultaneously, mitochondrial dysfunction associated with Ca^{2+} overload contributes to an increase in ROS, intensifying oxidative stress and the inflammatory response ^{6,45,46}.

Clinically, some drugs have been developed to counteract these mechanisms. Memantine acts as a non-competitive antagonist of NMDA receptors, blocking their pathological activation induced by excessive extracellular glutamate accumulation while preserving the physiological glutamatergic signaling necessary for proper synaptic function ⁴⁷.

1.2.5. CHOLINERGIC HYPOTHESIS (CH)

The cholinergic hypothesis (CH) emerged in the late 1970s and constitutes one of the earliest proposals aimed at explaining the origin and progression of AD. This hypothesis postulates that dysfunction of the cerebral cholinergic system represents a central factor in the cognitive decline characteristic of this pathology ⁴⁸.

Acetylcholine (ACh) is a fundamental neurotransmitter and neuromodulator of the CNS, synthesized and released mainly by the so-called cholinergic neurons. These neurons play an essential role in various cognitive processes, including memory, attention, learning, and the regulation of the sleep–wake cycle ^{6,49}.

In patients with AD, an abnormal degeneration of cholinergic neurons has been consistently described, especially in regions such as the hippocampus and the neocortex

⁶. The hippocampus, a key structure of the limbic system, plays a determining role in the formation and early consolidation of episodic memories and is essential for the acquisition of new information and the generation of detailed memories. The neocortex, in turn, is responsible for long-term memory storage and is involved in higher cognitive functions such as language, reasoning, and sensory integration ⁵⁰⁻⁵².

During the progression of AD, a gradual deterioration of cholinergic neurons becomes evident, accompanied by a decrease in the activity of choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of ACh. In parallel, a significant increase in the activity of butyrylcholinesterase (BChE) and acetylcholinesterase (AChE), the enzymes responsible for ACh degradation, is observed. In the healthy brain, these degradative enzymes perform essential functions in regulating cholinergic signaling, preventing receptor saturation and ensuring the precision of neurotransmission. Additionally, they facilitate the recycling of molecular components by hydrolyzing ACh into acetate and choline, allowing the latter to be reused by cholinergic neurons in the synthesis of new ACh (Figure 2) ^{6,49}.

Additionally, certain cholinergic receptors facilitate the preferential processing of APP through the non-amyloidogenic pathway, thereby reducing A β generation. These receptors also participate in the modulation of kinases and phosphatases involved in the regulation of tau protein phosphorylation. Consequently, a dysfunctional cholinergic system could be closely related to the development of AD ^{19,26,53}.

Since CH was one of the first proposals to explain the pathophysiology of AD, it has also been extensively investigated. Currently, numerous drugs intended for the symptomatic treatment of the disease focus on the inhibition of AChE. Among the drugs approved for use in human patients are galantamine, donepezil, and rivastigmine, which have been shown to partially improve cognitive functions by prolonging the availability of ACh in the synapse ⁵⁴.

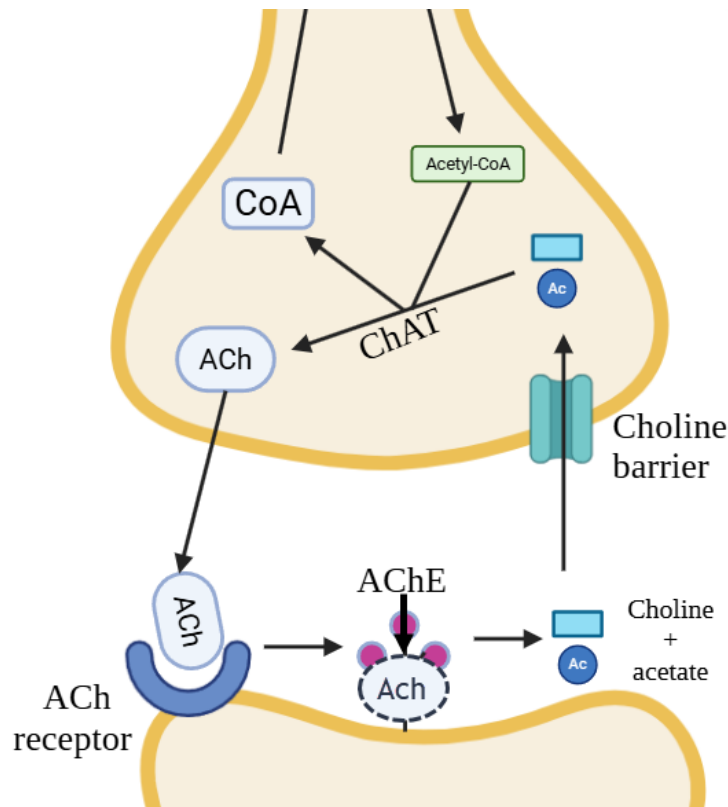


Figure 2: Schematic representation of neurotransmission via the cholinergic system of a neuron. Created in <https://BioRender.com>.

1.2.6. CRITIQUE OF THE HYPOTHESES

Regarding the ACH, various studies have repeatedly confirmed the presence of A β deposits in the brains of cognitively healthy individuals, without dementia symptoms and who were not even in preclinical stages of AD. Consequently, although A β aggregation cannot be ruled out as a possible initiating event in the development of this pathology, it is not conclusive to assert that every individual with cerebral A β accumulation will develop, or is necessarily susceptible to AD^{55,56}. This idea is reinforced by the fact that ACH is not strictly required for the development of other hypotheses, such as the TH²⁷.

On the other hand, attributing age as the sole cause of TH development is also not fully satisfactory. While the increased activity of certain kinases and the decreased activity of phosphatases during aging represent valid and consistent processes, these changes usually occur gradually and with limited spatial distribution in the brain, which is insufficient to explain the early onset and accelerated progression of AD⁵⁷.

Regarding the IOSH, the formation of A β plaques or NFTs characteristic of tauopathies can trigger a glial cell-mediated immune response, resulting in the release of proinflammatory cytokines and the establishment of a chronic neuroinflammatory state,

generating the inflammatory loop described in this hypothesis^{15,58,59}. In this context, IOSH could be interpreted not so much as a primary triggering factor but rather as a secondary consequence of other pathological processes. Furthermore, this hypothesis does not provide a satisfactory explanation for the early onset of AD, as it is based on physiological changes associated with aging that precede the clinical development of the disease. In this sense, IOSH is more appropriately considered as part of the development of a PART²⁷, suggesting that, when considered in isolation, it constitutes a limited hypothesis for explaining the origin of AD.

Despite the relevance of CH in the development of pharmacological strategies for AD, it does not provide a satisfactory explanation of the underlying origin of the disease. While treatments exist that are primarily aimed at inhibiting AChE and are widely used in human patients, their action is limited to symptom mitigation without modifying disease progression or addressing its fundamental causes. These drugs prolong the synaptic availability of acetylcholine by delaying its degradation, but they do not influence its synthesis, so although they may attenuate the severity of cognitive deficits, they do not prevent their eventual manifestation^{60,61}.

GH focuses on glutamate-mediated neurotoxicity and Ca^{2+} dysregulation as a factor in neuronal damage, but, like CH, it fails to explain the underlying origin of AD, providing only an explanation for its development and symptomatology. Furthermore, excitotoxicity can amplify other pathological pathways (tau, $\text{A}\beta$, inflammation), acting more as an amplifying factor than as a primary trigger of the disease. Existing treatments, such as memantine, mitigate symptoms and slow cognitive decline but do not alter disease progression or act on the primary cause. For these reasons, current literature considers GH complementary to other hypotheses^{40,62}.

Some authors have also dismissed demonic possession or the influence of curses as plausible causes for the development of AD⁶³. However, to date, no conclusive empirical evidence has been presented to definitively confirm or refute this hypothesis, and the arguments put forward are mainly based on unverified inferences and uncritically accepted assumptions. Consequently, more thorough research would be necessary to definitively rule out a possible demonic involvement in the development of dementia associated with AD.

As can be observed, the proposed underlying mechanisms for the origin of AD are multiple and heterogeneous. In many cases, these processes interact in a complex and non-

linear manner, establishing feedback and amplification networks that complicate the delineation of direct causal relationships. This interconnection limits our ability to accurately identify the primary triggering event and, consequently, to determine the ultimate etiological factor responsible for disease onset.

1.3. NATURAL PRODUCTS

Although the human body endogenously produces certain compounds with neuroprotective potential, such as melatonin or interleukin-10^{64,65}, historically most natural-origin drugs have been isolated from fungi, algae, marine invertebrates, and microorganisms^{64,66,67}. These biological resources continue to represent an essential source of bioactive molecules with potential application in the treatment of NDs.

Natural products (NPs), mostly secondary metabolites, are organic molecules produced by living organisms that exhibit great structural diversity and a wide range of biological activities, many of which have been effectively harnessed in fields such as pharmacology, agriculture, and biotechnology^{1,66,67}. These metabolites are not essential for the primary growth or development of the organism that synthesizes them; however, they are generated as a result of adaptive mechanisms that enhance survival and competitiveness in a given environment⁶⁷. For example, fungi of the genus *Penicillium* produce penicillin, an antibiotic that inhibits the growth of competing bacteria within the same ecological niche⁶⁸. Similarly, the tobacco plant (*Nicotiana tabacum*) synthesizes nicotine, an alkaloid produced in the roots and transported to the leaves, which acts as a defensive compound against herbivores by interfering with the insect nervous system⁶⁹.

Since ancient times, NPs have been used to treat diseases worldwide, with some of the earliest written records coming from ancient Mesopotamia, where extracts from various plants were used to treat fevers, coughs, and even parasitic diseases around 2600 BCE⁷⁰. Today, it is estimated that approximately 64.9% of medications are derived directly from NPs or have been inspired by them, either through synthetic derivatives or drugs designed to mimic their biological function⁷¹.

1.3.1. NATURAL PRODUCTS AGAINST NDs

With regard to NDs, it is well established that several natural products are either already used or show significant potential for the pharmacological treatment of these disorders⁷² (Table 1).

Table 1: Sum up of some natural products that could have relevant effects regarding the treatment of NDs and their origins.

COMPOUND	ORIGIN	EFFECT RELEVANT TO NDS	KEY REFERENCES
Resveratrol	Nuts, grapes and other plants	Anti-inflammatory, glutathione regulation and inhibits A β aggregation	73–77
Curcumin	<i>Curcuma longa</i>	Anti-inflammatory, antioxidant and regulation of NF- κ B pathways	78,79
Epigallocatechin gallate (EGCG)	<i>Camellia sinensis</i>	Inhibits A β aggregation and improves cognitive function	80,81
Berberine	<i>Coptidis rhizoma</i> , <i>Berberis vulgaris</i> L., and <i>Scutellaria baicalensis</i>	Anti-inflammatory, reduction of ChE and regulation of NF- κ B pathways	82,83
Huperzine A	<i>Huperzia serrata</i>	Inhibits AChE, maintains ACh levels, Anti-inflammatory and antioxidant	84

Ginkgolides	<i>Ginkgo biloba</i>	Inhibits A β aggregation	85
Cannabidiol (CBD)	<i>Cannabis sativa</i>	Anti-inflammatory and antioxidant	86,87
Omega-3	Fish and seafood	Anti-inflammatory, antioxidant and maintains neuronal membrane fluidity	88
Astaxanthin	<i>Haematococcus pluvialis</i> , crustaceans and some fish species	Anti-inflammatory and antioxidant	89,90
Phlorotannins	Brown algae	Anti-inflammatory, antioxidant and improves cognitive function	91

Resveratrol is a polyphenol found in plants such as grapes and nuts. Numerous studies have highlighted its anti-inflammatory potential, as well as its ability to interfere with A β aggregation ⁷⁶. In experimental animal models, resveratrol administration has been associated with increased brain levels of glutathione and evidence of neuroprotection, particularly in the hippocampal region. Furthermore, in transgenic mouse models supplemented with red wine rich in resveratrol a reduction in amyloid pathology has been observed ^{73–75,77}.

Curcumin is a secondary metabolite produced by turmeric (*Curcuma longa*) and is widely used as a spice due to its coloring and organoleptic properties ⁷⁸. Several studies indicate that curcumin interacts with multiple factors involved in inflammatory processes, including kinases, cytokines, glutathione, and ROS. In addition, it has been shown to inhibit activation of the nuclear factor κ B (NF- κ B) pathway, reduce microglial and astrocyte activation, decrease tau tangle formation, and improve cognitive function in experimental models of AD ^{78,79}.

Epigallocatechin gallate (EGCG), a polyphenol present in green tea (*Camellia sinensis*), has demonstrated the ability to inhibit A β aggregation and reduce its cytotoxicity. Both in vitro and in vivo studies have associated this compound with neuroprotective effects and improvements in cognitive function ^{80,81}.

Berberine is an alkaloid widely distributed in the roots, rhizomes, and stems of various plant species. It is particularly abundant in *Coptidis rhizoma*, *Berberis vulgaris* L., and *Scutellaria baicalensis*, plants traditionally used in Asian medicine ⁸². This compound has demonstrated antioxidant and anti-inflammatory activity, as well as the ability to modulate the NF- κ B signaling pathway. Additionally, it has been reported to reduce cholinesterase (ChE) activity in PD models ⁸³.

Huperzine A is an alkaloid isolated from *Huperzia serrata* that exhibits notable neuroprotective potential. Its effects include inhibition of AChE, maintenance of ACh levels, and modulation of oxidative and inflammatory processes. Some studies suggest that its inhibitory potency against AChE may exceed that of drugs such as galantamine, in addition to showing a high capacity to cross the BBB ⁸⁴.

Ginkgolides, terpenoid compounds derived from *Ginkgo biloba*, have shown neuroprotective effects in various in vitro and in vivo studies. Among their properties is the ability to interfere with A β aggregation, suggesting potential therapeutic value in AD ⁸⁵.

Cannabidiol (CBD), a non-psychoactive phytocannabinoid produced by *Cannabis sativa*, has a lipophilic nature that facilitates its systemic distribution, including passage across the BBB. CBD has been reported to possess antioxidant and anti-inflammatory properties, and in animal models it has demonstrated the ability to prevent neuronal loss in regions such as the hippocampus, generating growing interest in its potential application in NDs^{86,87}.

Omega-3 fatty acids, abundant in fish and seafood, are widely used as nutritional supplements. They have been attributed neuroprotective, antioxidant, and anti-inflammatory properties, as well as a relevant role in maintaining neuronal membrane fluidity⁸⁸.

Astaxanthin is a carotenoid found mainly in crustaceans, although it can also be synthesized by some fish species, giving them their characteristic reddish color. *Haematococcus pluvialis* is a green microalga considered the primary natural producer of this compound. It has shown strong antioxidant activity, the ability to modulate inflammatory responses through cytokine regulation, and beneficial effects on amyloid pathology, making it a subject of study as a potential therapeutic agent in NDs^{89,90}.

Fungi of the genus *Hericium*, such as *Hericium erinaceus* (commonly known as “lion’s mane”), have traditionally been used for their medicinal properties. Current scientific evidence supports these uses and attributes neuroprotective capabilities to them, including the prevention of neuronal death and the promotion of new synapse formation through a protein known as nerve growth factor (NGF), thereby contributing to improvements in cognitive functions such as memory^{92,93}.

Phlorotannins, phenolic compounds produced by brown algae, exhibit antioxidant and anti-inflammatory properties, as well as beneficial effects on cognitive function, making them promising candidates for the development of therapies targeting AD^{72,91}.

Overall, these examples highlight the broad potential of NP’s as a source of bioactive compounds for the treatment of NDs. Numerous secondary metabolites exhibit favorable pharmacological properties, and the continuous discovery of new molecules in this field suggests that there remains considerable scope for the identification and development of innovative therapeutic agents.

1.3.2. METALLOPHORES

Within the broad group represented by NPs, there are certain molecules of particular relevance that, depending on environmental conditions, can mean the difference between life and death for an organism, placing them at the conceptual frontier between classical secondary metabolites and molecules essential for survival⁹⁴⁻⁹⁶. These are the so-called metallophores, molecules that bind metal ions to either facilitate their transport to cells that require them or to expel excess metals via extracellular chelation^{94,97}.

They are widely recognized for their role as transporters of the iron ferric form (Fe^{3+}), a micronutrient essential for fundamental metabolic processes such as photosynthesis, cellular respiration, and DNA synthesis^{94,96,98}. Despite the high abundance of iron in the Earth's crust, in aerobic environments this element is predominantly in the ferric form (Fe^{3+}), which is highly insoluble and therefore poorly bioavailable. This limited availability highlights the importance of metallophores as specialized uptake systems for the organisms that synthesize them⁹⁴. While their role in iron acquisition is the most studied, literature has shown that various metallophores can also exhibit affinity for other metals, including copper, zinc, cadmium, lead, and even uranium⁹⁹. Depending on their predominant specificity or affinity, some authors use particular terms such as siderophores (iron), chalkophores or cuprophores (copper), and zincophores (zinc). However, since many of these molecules can chelate more than one type of metal depending on the chemical and environmental context, it is not strictly correct to attribute exclusivity to a single element^{100,101}.

In cyanobacteria, metallophore-mediated metal homeostasis plays a particularly relevant role due to their oxygenic photosynthetic lifestyle and their high dependence on trace metals such as iron and copper for essential electron transport processes within photosystems and respiratory chains. As photoautotrophic organisms inhabiting environments often characterized by fluctuating metal availability and redox conditions, cyanobacteria have evolved efficient metal acquisition and detoxification strategies, including the production of siderophore-like compounds and other metal-binding metabolites that contribute to maintaining intracellular metal balance and protecting cellular machinery from metal-induced oxidative stress¹⁰².

These molecules are being intensively evaluated for multiple biotechnological applications, many related to bioremediation and environmental health, showing promising results⁹⁹. Several bacteria recognized for their ability to produce metallophores have been employed in the development of biofilters for wastewater treatment, achieving reductions of up to 96% in nickel concentrations¹⁰³. Likewise, uranium extraction from liquid media using synthetic metallophores has been reported

¹⁰⁴. Similarly, the application of siderophores has allowed for the mobilization and extraction of ferric ions present in asbestos fibers, significantly reducing their toxicity ¹⁰⁵. Overall, metallophores constitute a molecular group with remarkable biotechnological potential and represent a research field with broad development prospects.

Increasingly, the scientific literature recognizes that mechanisms of metal acquisition, transport, and homeostasis play a critical role in human pathophysiological processes ^{106–110}. In particular, metal imbalance has been widely implicated in the progression of neurodegenerative diseases such as AD ^{108–110}.

Although the influence of metal ions on NDs is not yet fully characterized, evidence from clinical and preclinical studies suggests a significant pathological role (Table 2). While therapeutic trials aimed at modulating metal homeostasis have not yielded completely conclusive results, several approaches have shown very promising outcomes, supporting the relevance of metals as biological targets ¹⁰⁸. In this context, metallophores emerge as a molecular group with therapeutic potential, either by reducing toxic metal load, restoring homeostasis of essential metals, or through the development of synthetic analogs capable of selectively sequestering cations in pathological microenvironments without interfering with physiological functions associated with normal metabolism.

Table 2: Summary table of known metallophores that may have relevant effects for the treatment of NDs, the metals they chelate, and the stage of the studies supporting them.

<i>Compound</i>	<i>Type</i>	<i>Target metal(s)</i>	<i>Mechanism relevant to ND</i>	<i>Evidence</i>	<i>Key references</i>
<i>Clioquinol (PBT1)</i>	Hydroxyquinoline	Fe, Cu, Zn	Metal redistribution, inhibits A β aggregation	Clinical studies	111,112
<i>PBT2</i>	Hydroxyquinoline derivative	Fe, Cu, Zn	Reduces soluble A β and improves cognition	Clinical studies	111,113
<i>Desferrioxamine (DFOA)</i>	Siderophore	Fe, Al	Reduces iron-mediated oxidative stress	Clinical studies	114,115
<i>Deferiprone (DFP)</i>	Siderophore	Fe	Decreases brain iron accumulation	Clinical trials	116
<i>Deferasirox (DFX)</i>	Siderophore	Fe	Decreases brain iron accumulation	Clinical studies	117
<i>8-hydroxyquinoline</i>	Chalkophore	Cu, Zn	Metal redistribution, inhibits A β aggregation	In vitro	118
<i>Curcumin</i>	Polyphenolic chelator	Fe, Cu	Chelation, antioxidant and anti-inflammatory response	In vitro, in vivo	79,119

<i>Epigallocatechin gallate (EGCG)</i>	Polyphenolic chelator	Fe, Cu	Chelation, inhibits A β aggregation	In vitro, in vivo	80,81,120
<i>Resveratrol</i>	Polyphenolic chelator	Fe, Cu	Redox modulation, inhibits A β aggregation and weak chelation	In vitro, in vivo	74,121
<i>α-lipoic acid</i>	Endogenous chelator	Fe, Cu, Al	Antioxidant, glutathione regulation	Clinical studies	122,123
<i>Lactoferrin</i>	Metalloprotein	Fe, Al	Iron sequestration, anti-inflammatory	In vivo	124
<i>Ferritin</i>	Metalloprotein	Fe	Iron storage, protection against ROS	Observational	125
<i>Metallothioneins</i>	Cysteine-rich proteins	Cu, Zn	Anti-inflammatory and antioxidant response	In vitro, in vivo	126

1.4. CYANOBACTERIA

Cyanobacteria constitute an ancient and phylogenetically diverse phylum of oxygenic photoautotrophic bacteria, widely recognized for their role in the early evolution of the biosphere and in the primordial oxygenation of the Earth's atmosphere ¹²⁷. The main characteristic shared by this group, which differentiates it from other bacterial phyla, is the ability to perform oxygenic photosynthesis, a process mediated by pigment complexes in which chlorophyll-a acts as the primary photosynthetic pigment, while phycobiliproteins function as pigments responsible for light capture ^{128–130}.

Cyanobacteria exhibit remarkable morphological and ecological diversity, encompassing free-living unicellular forms, colonial and filamentous forms, as well as specialized structures such as heterocysts, akinetes, and hormogonia, which allow them to adapt to different environmental conditions and perform essential ecological functions in aquatic and terrestrial ecosystems ^{128,131,132}. This notable phylogenetic and structural diversity of cyanobacteria is reflected in high metabolic plasticity, characterized by their ability to synthesize a wide variety of primary and secondary metabolites. This biochemical versatility positions the phylum as a biotechnologically valuable source for the prospecting and discovery of new bioactive compounds with pharmacological and therapeutic potential ¹⁰⁰.

Since the 1970s, more than 1,630 unique cyanobacterial compounds have been described, grouped into 260 metabolite families. Most of these compounds are considered to be produced either through non-ribosomal peptide (NRP) or hybrid polyketide–NRP biosynthetic pathways, or through ribosomal synthesis of propeptides that are post-translationally modified (RiPP) ¹³³. Other metabolites, such as pigments, polysaccharides, or fatty acids, have also demonstrated significant potential for application in the development of new drugs ¹³⁴.

1.4.1. CYANOBACTERIA AND NDs

Several studies highlight cyanobacteria, particularly those of the genus *Arthrospira*, promising agents with neuroprotective activity in the context of NDs (Table 3). These effects are largely attributed to their rich composition of bioactive compounds, including phycobiliproteins, carotenoids, phenolic compounds, and essential fatty acids ^{1,6}.

Table 3: Summary table of known cyanobacterial compounds that may have relevant effects for the treatment of NDs, their source, their chemical class, their mechanism relevant to the treatment of NDs and the stage of the studies supporting them.

<i>Compound / compound family</i>	<i>Cyanobacterial source</i>	<i>Chemical class</i>	<i>Mechanism relevant to neurodegeneration</i>	<i>Evidence</i>	<i>References</i>
<i>C-phycocyanin</i>	<i>Arthrospira</i> spp.	Phycobiliprotein	Antioxidant activity; reduction of oxidative stress and neuroinflammatory pathways	<i>In vitro, in vivo</i>	135–137
<i>Phycocyanobilin</i>	<i>Arthrospira</i> spp.	Tetrapyrrole pigment	ROS scavenging and inhibition of oxidative damage pathways	<i>In vitro, in vivo</i>	138,139
<i>Mycosporine-like amino acids (MAAs)</i>	Marine cyanobacteria	Amino acid derivatives	Antioxidant activity and protection against oxidative stress	<i>In vitro</i>	133,140
<i>Cyanobactins</i>	<i>Nostoc</i> spp. and other cyanobacteria	RiPP peptides	Modulation of cellular signalling pathways and receptor interactions	<i>In vitro</i>	141,142
<i>Aeruginosins</i>	<i>Microcystis</i> spp., <i>Planktothrix</i> spp.	Linear peptides	Protease inhibition and possible modulation of inflammatory pathways	<i>In vitro</i>	143
<i>Cyanopeptolins</i>	Freshwater cyanobacteria	Cyclic depsipeptides	Serine protease inhibition and regulation of inflammation-associated pathways	<i>In vitro</i>	143,144
<i>Microginins</i>	Freshwater cyanobacteria	Lipopeptides	Enzyme inhibition and potential anti-inflammatory effects	<i>In vitro</i>	145–147

<i>Nostocyclopeptides</i>	<i>Nostoc</i> spp.	Cyclic peptides	Protease inhibition and modulation of cellular responses	<i>In vitro</i>	148
<i>Largazole</i>	<i>Symploca</i> spp.	Cyclic depsipeptide	HDAC inhibition and modulation of inflammatory signalling	<i>In vitro, in vivo</i>	149–151
<i>Curacin A</i>	<i>Lyngbya/Moorea</i> spp.	Polyketide	Interaction with cytoskeleton and microtubule dynamics	<i>In vitro</i>	152,153
<i>Apratoxin A</i>	<i>Lyngbya/Moorea</i> spp.	Cyclic depsipeptide	Modulation of ER stress and cellular signalling pathways	<i>In vitro</i>	154–156
<i>Scytonemin</i>	<i>Scytonema</i> spp.	Indolic alkaloid	Anti-inflammatory activity through inhibition of inflammatory signalling	<i>In vitro</i>	157–159
<i>Carotenoids</i>	Cyanobacteria broadly	Terpenoids	Antioxidant activity and protection against oxidative damage	<i>In vitro, in vivo</i>	89,90,160,161
<i>Alkylresorcinols</i>	Freshwater cyanobacteria	Phenolic lipids	Antioxidant activity and membrane protection	<i>In vitro</i>	162,163
<i>Cyanobacterial fatty acid derivatives</i>	Various cyanobacteria	Lipids	Modulation of lipid signalling and inflammatory responses	<i>In vitro. In vivo</i>	164–166

The main chromophore, phycocyanobilin (PCB), covalently bound to C-phycocyanin, is structurally similar to bilirubin and potently inhibits Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase. Through this mechanism, PCB reduces the production of ROS, which are key mediators of oxidative damage in neurodegeneration. In vivo studies have demonstrated that diets enriched with *Arthrospira* increase cerebellar glutathione levels and suppress proinflammatory cytokines such as TNF- α and IL-1 β . These combined effects attenuate oxidative stress and neuroinflammation in aged rodent models^{135,136,138,167}.

In experimental models of PD and AD, orally administered C-phycocyanin has been shown to improve motor coordination and spatial learning abilities. These functional improvements are accompanied by preservation of dopaminergic neurons in the substantia nigra, reduced microglial activation, and decreased accumulation of neurotoxic protein aggregates. Furthermore, it enhances the activity of endogenous antioxidant enzymes, including superoxide dismutase (SOD) and catalase, thereby protecting neurons from ROS-induced apoptosis^{1,136–138}.

Across multiple experimental systems, *Arthrospira* consistently reduces microglial activation, limits α -synuclein aggregation, and mitigates A β deposition, supporting its potential use as a natural adjuvant in the management of PD, AD and ALS. Additionally, its immunomodulatory effects may contribute to restoring homeostasis in the central nervous system by regulating the balance between pro- and anti-inflammatory signaling pathways^{1,137,138}.

Beyond *Arthrospira*, other cyanobacterial genera have also shown promising neuroprotective potential. Extracts from the genus *Lyngbya* contain bioactive metabolites with antioxidant and anti-inflammatory activity. Some of these compounds have demonstrated the ability to interact with key enzymes involved in AD pathogenesis, such as BACE1, which plays a central role in A β production. Inhibition of BACE1 represents a major therapeutic strategy aimed at reducing amyloid plaque formation^{6,168}.

Similarly, phycobiliproteins isolated from the genus *Leptolyngbya* have emerged as promising candidates. In studies using the model organism *Caenorhabditis elegans*, these compounds have shown the capacity to modulate BACE1 activity and reduce proteotoxic stress associated with amyloid aggregation, suggesting potential translational relevance^{6,169}.

Moreover, extracts from the genera *Oscillatoria* and *Lyngbya* are characterized by a high content of phenolic compounds, flavonoids, and other phytochemicals with strong antioxidant capacity. These molecules contribute to scavenging free radicals, reducing lipid peroxidation, and modulating signaling pathways involved in inflammation, such as NF- κ B. Given that oxidative stress and chronic neuroinflammation are central features in the pathophysiology of NDs, these properties further support the therapeutic potential of cyanobacteria-derived compounds ^{1,170}.

Although the freshwater cyanobacterial genus *Sphaerospermopsis* remains relatively underexplored, it has been reported to exhibit antibacterial and antifungal activities and to produce a diverse repertoire of secondary metabolites, including cyanobactins, chlorinated fatty acid-derived lactylates, and NRPs. The structural diversity and pharmacological potential of these metabolites highlight *Sphaerospermopsis* as a promising source of novel bioactive compounds targeting NDs ^{171–173}.

Lusitaniella coriacea is a cyanobacterial species known to produce lusichelins A–E, a recently identified family of bioactive metallophores. Some members of this compound class have exhibited promising anticancer activity, highlighting their potential as lead molecules for the development of novel therapeutic agents ¹⁷⁴.

Overall, the growing body of evidence suggests that cyanobacteria represent a valuable and underexplored source of neuroprotective agents. Their multitarget mechanisms, including antioxidant, anti-inflammatory, and antiamyloidogenic effects, make them particularly attractive candidates for the development of novel therapeutic strategies against NDs.

1.5. OBJECTIVES

The aim of this study was to screen cyanobacteria, namely those signalized with the presence of potential metallophones, for AD therapies.

The cyanobacterial strains included in the study were obtained from the LEGE-CC (Blue Biotechnology and Ecotoxicology Culture Collection) of the Interdisciplinary Centre of Marine and Environmental Research, University of Porto (CIIMAR I UP), a reference collection dedicated to the preservation and biotechnological exploration of cyanobacteria and microalgae diversity. This collection represents a valuable resource for the discovery of bioactive secondary metabolites with potential applications in fields such as blue biotechnology and drug discovery ^{175,176}. Associated with this collection, the LEGE-

Natural Products Library (LEGE-NPL) is also hosted at BBEH facilities. This integrated NPs platform comprises a solid library of methanolic (MeOH) extracts and a liquid library of fractions, both generated from cyanobacterial strains deposited in the LEGE-CC. These resources are available to the scientific community and provide a repository of cyanobacterial chemical diversity, facilitating access to a wide range of potentially bio-active natural compounds. Together, the LEGE-CC and the LEGE-NPL constitute a strategic infrastructure for the biotechnological exploration of cyanobacteria, enabling the development of biological activity screening studies, chemical characterization, and the discovery of molecules with pharmaceutical, industrial, and environmental relevance¹⁷⁷.

During this study, four cyanobacterial strains were cultivated and subjected to extraction and fractionation processes for their inclusion in the LEGE-NPL collection. Alongside a comprehensive screening of fractions already available in the LEGE-NPL collection derived from up to one hundred different cyanobacterial strains was performed. The screening focused particularly on their ability to inhibit AChE activity and their anti-inflammatory properties. Although additional cyanobacterial strains were also included in the present study, special attention was given to those whose genomes contain regions associated with metallophore biosynthesis. This focus was based on the hypothesis that these molecules may exhibit neuroprotective effects and contribute to the regulation of intracellular metal homeostasis (Figure 3).

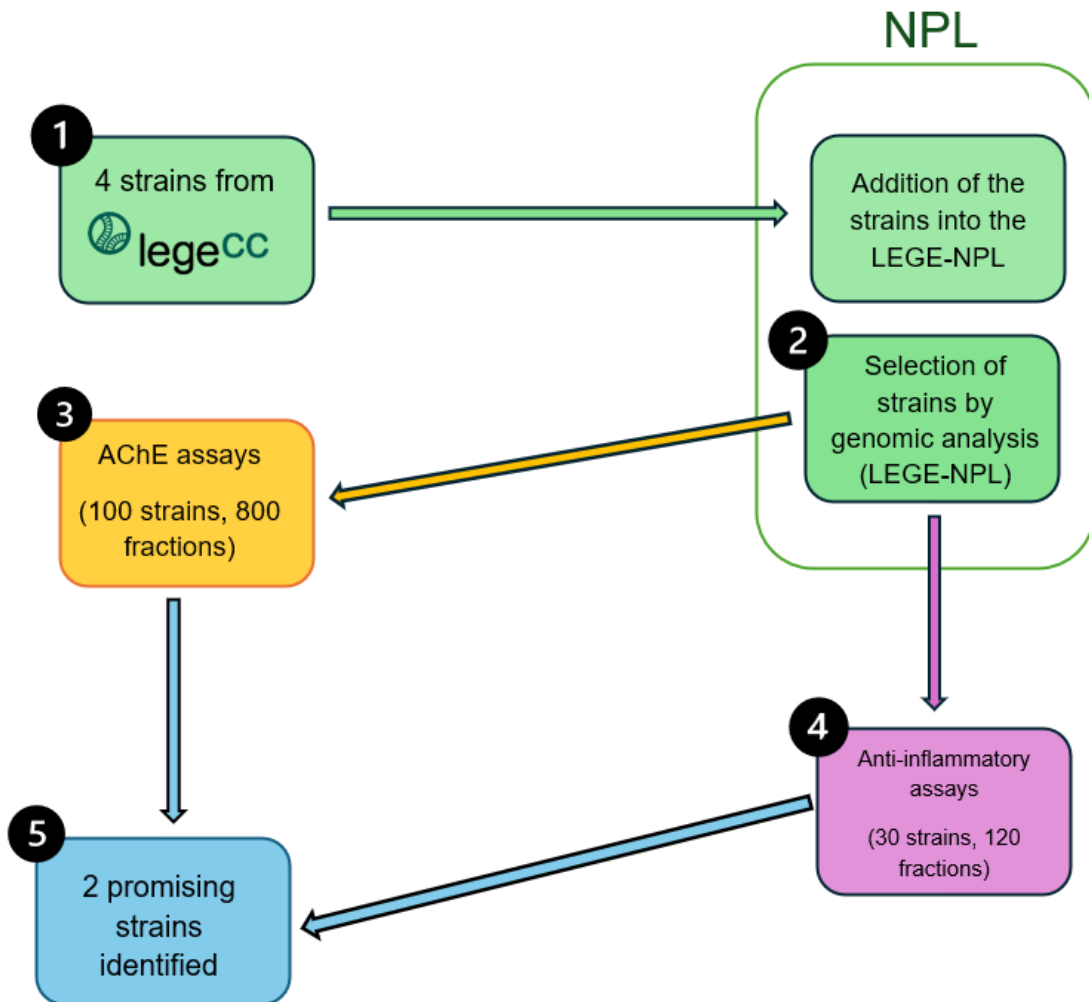


Figure 3: Workflow followed during the development of this study.

2. MATERIALS AND METHODS

The cyanobacterial fractions contained in the LEGE-NPL and generated from strains belonging to the LEGE-CC are stored at -20°C in 96-well plates, referred to as Mother Plates (MP). Each MP contains fractions derived from a maximum of ten different cyanobacterial strains, with eight fractions preserved per strain.

To avoid potential degradation or loss of sample integrity associated with repeated freeze–thaw cycles, transportation, or possible contamination events, the fractions stored in the MPs are not directly used for experimental procedures. Instead, a small aliquot of each fraction was transferred into a new 96-well plate, designated as Working Plate (WP), which was used for all subsequent analyses. Therefore, WPs represent working replicas of the original MPs, preserving the same fraction distribution while minimizing manipulation of the original library.

Each WP was assigned a specific identification number corresponding to the MP from which it was generated. Detailed information regarding the WPs, including the cyanobacterial strains and fractions contained in each plate, is provided in Table I in the annex. The WPs were subsequently used for the evaluation of the bioactive potential of cyanobacterial fractions through acetylcholinesterase (AChE) inhibition assays, Griess assay to assess anti-inflammatory activity.

2.1. CYANOBACTERIA CULTURE

The cultivation of cyanobacterial strains was carried out at the Biotarium of Aquatic Organisms (BOGA), located at the CIIMAR facilities, under a photoperiod of 14 h of light conditions and 10 h dark and constant aeration. The strains were cultured in liquid Z8 medium, described by Kotai¹⁷⁸, commonly used for cyanobacterial cultivation (Table II of the annex). The Z8 medium was prepared using ultrapure water as the main component, to which the following solutions were added: solution A (10 mL/L), solution B (10 mL/L), Fe-EDTA solution (10 mL/L), and a micronutrient solution (1 mL/L). Additionally, in the case of strains isolated from marine environments, the medium was supplemented with commercial sea salt (Tropic Marin®) at a concentration of 25 g/L and vitamin B₁₂ (1 mL/L). This modified culture medium was designated as Z8+25.

Prior to medium preparation, the required volume of ultrapure water was transferred to a glass volumetric flask and sterilized by autoclaving. The addition of the solutions was carried out under aseptic conditions, within a laminar flow cabinet previously disinfected with 70% (v/v) ethanol and subjected to germicidal ultraviolet irradiation for 20 minutes,

The culture was initiated with a volume of 40 mL of liquid medium in cell culture flasks. Subsequently, a progressive scale-up was performed by transferring the cultures to an intermediate volume of 400 mL, again in cell culture flasks, until reaching a final volume of 4 L in a glass flask.

The change of medium and container was carried out under aseptic conditions, as previously described. The criterion used to perform each scale-up phase was based on the visual assessment of cell density, considering that the culture had reached its maximum growth capacity for the available volume when high turbidity and cell concentration were observed.

During the course of this study, the strains *Nostoc* sp. LEGE 221427, *Drouetiella* sp. LEGE 221434, *Scytonema* sp. LEGE 221431, and *Tolypothrix* sp. LEGE 221382 were cultured.

2.2. BIOMASS COLLECTION

Biomass from cyanobacteria cultures was separated from the culture medium and collected using different procedures, depending on the physiological characteristics of each strain. In most cases, recovery was performed by centrifugation using a Sorvall™ BIOS 16 bioprocessing centrifuge (Thermo Scientific™) at 15,000 rpm for 15 min, allowing the biomass to sediment as a pellet at the bottom of the centrifugation vessel. The supernatant was discarded into a diluted sodium hypochlorite solution in order to prevent the release of viable biological material into the waste system.

In the case of strains with filamentous morphology, biomass was recovered by filtration, using in each case the most appropriate pore size according to the characteristics of the culture. The filtrate was likewise discarded into a diluted sodium hypochlorite solution.

The collected biomass was transferred to previously weighed sterile 100 mL plastic flasks and frozen at -20°C . It was subsequently subjected to lyophilization for one week to ensure complete removal of moisture. After the drying process, the samples were weighed in the same container, and the dry biomass weight was determined by the difference between the final weight of the set (flask + dry biomass) and the initial weight of the empty flask.

The flasks were stored in a dry place under dark conditions until the extraction process could be carried out.

2.3. EXTRACTION

Freeze-dried cyanobacterial biomass was initially homogenized in small portions using a porcelain mortar and pestle. The grounded biomass was then transferred to a flat-bottom flask for solvent extraction. The primary extraction was performed by adding 50 mL of analytical grade methanol to the flask. The mixture was subjected to ultrasound-assisted extraction in an ultrasonic bath for 5 min, maintaining the temperature below 30°C . Following sonication, the particulate matter was allowed to settle for several minutes. The supernatant was subsequently decanted and filtered under vacuum through a Büchner funnel equipped with a vacuum adapter, standard filter paper, and a

hydrophobic cotton layer. This extraction and filtration sequence was repeated twice using 25 mL of methanol in each cycle.

The combined methanolic filtrates collected in a 250 mL round-bottom flask were concentrated under reduced pressure using a rotary evaporator at 30 °C. The concentrated extract was then transferred into a pre-weighed 20 mL vial by passing it through a Pasteur pipette packed with cotton to remove any remaining insoluble particulate matter. The solvent was further evaporated via rotary evaporation, and the vial was subjected to high vacuum until thoroughly dry to determine the final mass of the collected extract. Obtained extracts were cataloged in the institutional library database and stored at -80 °C within the repository boxes for subsequent fractionation.

2.4. FRACTIONATION

A 2 mL glass vial was previously tared on a precision balance. Using a metal spatula previously disinfected with acetone and distilled water, approximately 40 mg of frozen extract were weighed into the vial. The extract was then dissolved in methanol (MeOH; LC-MS grade) and homogenized by vortexing and sonication until reaching a final concentration of 40 mg/mL.

The resulting solution was filtered through 0.2 µm syringe filters and transferred to glass vials compatible with high-performance liquid chromatography (HPLC). Separation of the methanolic extract fractions was carried out by HPLC using a Waters Alliance e2695 system. During the process, 500 µL of each extract were injected into a 1 mL loop and then separated on an ACE 10 C8 column (50 × 10 mm, ACE, Reading, UK), using an H₂O:MeCN gradient (10% Acetonitrile, 90% Ultra-pure Water) (Table 4). Each extract was fractionated into eight fractions, which were automatically collected using the Alliance system collector module.

Table 4: HPLC fraction collection parameters

Fractions	Time (min)	Flow (mL/min)	MeCN (%)	H ₂ O (%)	collection time (min)
A	0.0	3.0	10	90	1.00-2.30
B	2.0	3.0	80	20	2.30-3.60
C	3.0	3.0	80	20	3.60-4.90
D	4.0	3.0	100	0	4.90-6.20
E	8.9	3.0	100	0	6.20-7.50
F	9.2	3.5	100	0	7.50-8.80
G	12.0	3.5	100	0	8.80-10.26
H	12.3	3.0	100	0	10.26-11.50
	14.0	3.0	100	0	
	15.0	3.0	10	90	
	18.0	3.0	10	90	

The fractions were collected in 48-well plates. The HPLC system used 500 µL from each vial for the fractionation process, corresponding to approximately 20 mg of the initial extract distributed among the eight obtained fractions.

Once fractionation was completed, the solvent was removed by a drying process using a SpeedVac SPD300DDA vacuum concentrator system. Subsequently, the samples were resuspended with DMSO and stored at -20 °C in the MPs until use in subsequent assays (Figure 4).

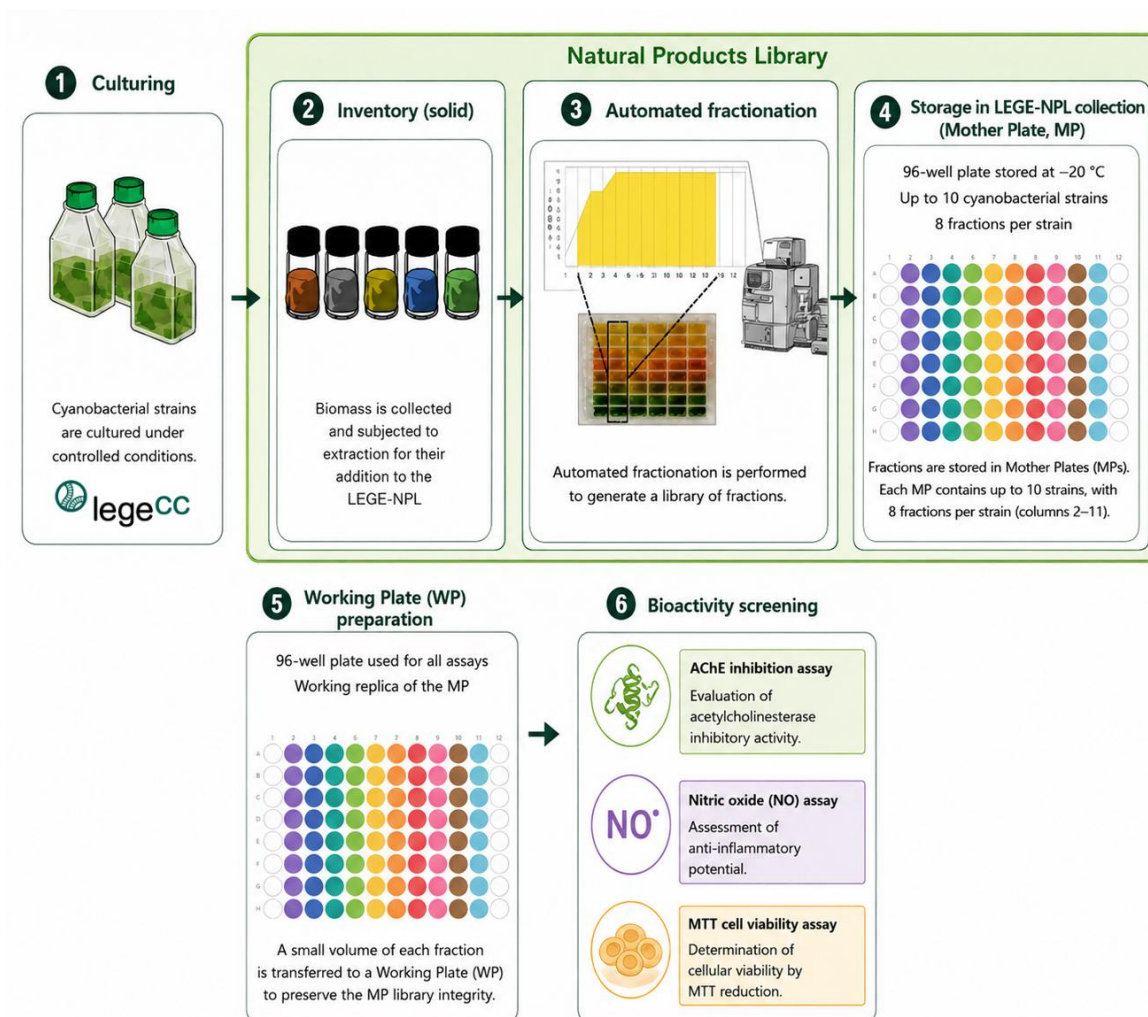


Figure 4: Schematic representation of the workflow employed in this study for cyanobacterial processing and analysis.

2.5. ACETYLCHOLINESTERASE ENZYMATIC ASSAY

To evaluate the effect of the fractions on AChE activity, the colorimetric method described by Ellman et al.¹⁷⁹, widely used for the determination of this enzyme's activity, was employed.

The assay is based on the use of three main components: (i) an artificial substrate, acetylthiocholine iodide (ATCh), used in the present study; (ii) the enzyme whose activity is to be evaluated, in this case AChE; and (iii) Ellman's reagent, also known as DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), which enables the colorimetric detection of the enzymatic reaction product.

The theoretical basis of the method relies on the ability of AChE to catalyze the hydrolysis of the ATCh substrate, generating thiocholine and acetate as reaction products (Figure 2). The released thiocholine contains a free thiol group ($-\text{SH}$), which reacts with DTNB. This reaction produces a mixed disulfide and releases the TNB^- anion (5-thio-2-nitrobenzoate), a yellow-colored compound.

TNB^- has a maximum absorbance at 412 nm, allowing its spectrophotometric quantification. Thus, the increase in absorbance at this wavelength is proportional to the amount of thiocholine formed and, consequently, to the enzymatic activity of AChE present in the sample. The comparison of absorbance values between treated samples and controls allows estimation of the relative enzyme activity.

In the present study, AChE from electric eel (*Electrophorus electricus*)¹⁸⁰ (Sigma Aldrich - Ref: D8130-5G) was used at a final concentration of 0.25 U/mL. As substrate, a solution of acetylthiocholine iodide ($\text{C}_7\text{H}_{16}\text{INOS}$)¹⁷⁹ (Merck) at a concentration of 0.075 M was used, while Ellman's reagent (Sigma Aldrich)¹⁷⁹ was prepared at a concentration of 10 mM. All dilutions and concentration adjustments were performed using a 0.1 M phosphate buffer solution (pH 7.2).

The assay was carried out in 96-well flat-bottom microtiter plates (Figure 5), compatible with an Agilent BioTek Cytation 5 microplate reader. The fractions to be evaluated, contained in the mother plate, were resuspended in 0.5 mL of dimethyl sulfoxide (DMSO), obtaining an initial concentration of 5 mg/mL. Subsequently, these solutions were diluted in phosphate buffer using an intermediate plate until reaching a concentration of 0.5 mg/mL. Finally, 6 μL of each diluted fraction were transferred to the corresponding wells of the assay plate, resulting in a final concentration of 10 $\mu\text{g/mL}$ of the fractions in each well.

Due to the use of DMSO as a solvent, a blank solvent consisting of 6 μL of 10% DMSO was included. Due to the intense intrinsic coloration of some of the evaluated fractions, a sample blank control was included in each plate. This control consisted of the corresponding fraction in the absence of enzyme and was used to correct the absorbance values obtained, thereby minimizing potential interferences arising from the inherent color of the extracts. As a negative control, 6 μL of 10% DMSO were also used, while as a positive control, 6 μL of galantamine (Merck - Ref. Y0001279) at a concentration of 60 $\mu\text{g/mL}$ were employed. Additionally, a plate blank consisting exclusively of phosphate buffer was included.

To the wells containing the fractions, as well as to the positive and negative controls, 50 μL of the AChE solution were added in order to allow the enzymatic reaction and evaluate potential inhibition of enzyme activity. In the wells corresponding to the blanks (plate blank, DMSO blank, and fraction blanks), the enzyme was replaced with 50 μL of phosphate buffer, thus maintaining constant final volumes and concentrations without enzymatic reaction occurring.

Finally, 244 μL of the reaction mixture, composed of phosphate buffer, DTNB, and ATCh, freshly prepared immediately prior to addition, were added to all wells (Table 5).

Table 5: Table showing the reagents and their respective quantities present in each well of the plate.

	Buffer	DMSO 10%	Galantamine	Fractions	Sol. Reaction	AChE
<i>Control (-)</i>		6 μL			244 μL	50 μL
<i>Control (+)</i>			6 μL		244 μL	50 μL
<i>Blank DMSO</i>	50 μL	6 μL			244 μL	
<i>Blank Plate</i>	56 μL				244 μL	
<i>Blank Fractions</i>	50 μL			6 μL	244 μL	
<i>Fractions</i>				6 μL	244 μL	50 μL

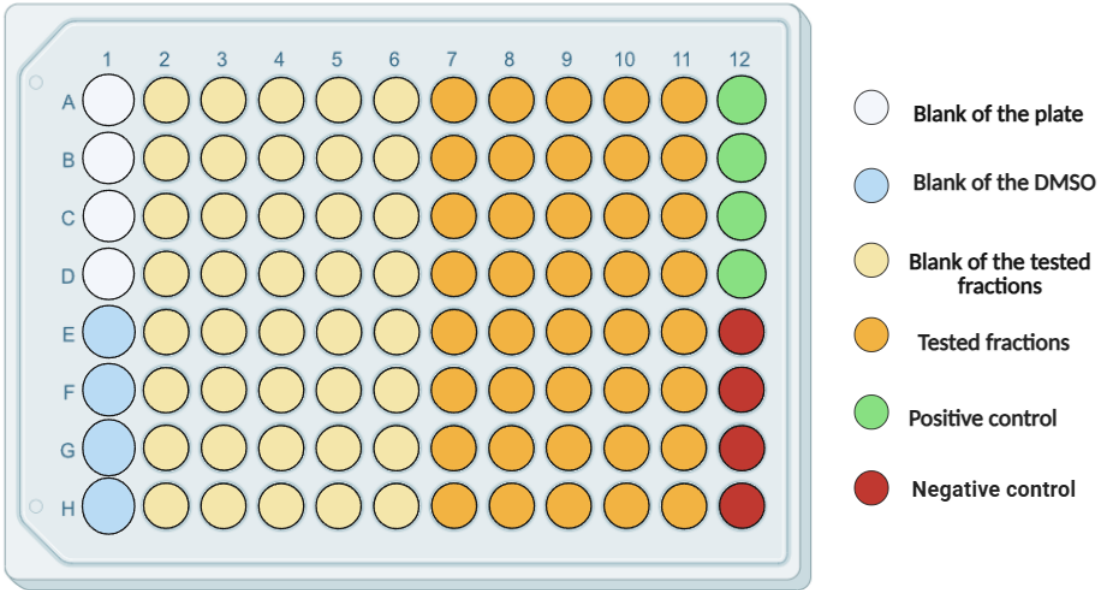


Figure 5: Diagram illustrating the plate layout tested in the AChE assay. Created in <https://BioRender.com>

After the addition of the reaction solution, the plates were subjected to gentle agitation for 10 s at 700 rpm in order to homogenize the contents of the wells. Immediately afterward, the plates were placed in the microplate reader for colorimetric measurement.

Absorbance was recorded at 412 nm over a total period of 15 min, with readings taken at 15 s intervals while maintaining a constant temperature of 25 °C.

It should be noted that all reagents, with the exception of the buffer solution, were kept under refrigerated conditions in an ice box until the moment of use, in order to preserve enzyme stability, as it is particularly sensitive to temperature variations.

The percentage of enzymatic inhibition was calculated from raw spectrophotometric data following initial normalization of the negative control. Briefly, the mean absorbance of DMSO control replicates was calculated for each plate and subtracted from the mean absorbance of the corresponding negative control replicates, yielding a plate-specific adjusted negative control value used as the reference baseline in this study.

$$A_{NC, adj} = \bar{A}_{NC} - \bar{A}_{DMSO}$$

For each fraction, raw absorbance values were corrected by subtracting the corresponding sample blank to account for potential interference due to intrinsic coloration of the extracts.

$$A_{fraction, corr} = A_{fraction} - A_{blank}$$

The resulting corrected values were then compared to the adjusted negative control using the following equation to determine the percentage of AChE activity:

$$AChE \text{ activity (\%)} = \left(\frac{A_{fraction, corr}}{A_{NC, adj}} \right) \times 100$$

Each fraction was evaluated in at least two independent experiments, and final values reported in this study correspond to the mean of all independent assays performed for each sample.

2.6. ANTI-INFLAMMATORY AND CELL VIABILITY ASSAYS

2.6.1. ANTI-INFLAMMATORY

The anti-inflammatory capacity of the fractions was tested following the methodology described by Barbosa et al.¹⁸¹. The assay is based on the determination of nitrite accumulation in the medium as an indicator of nitric oxide (NO) production, which in turn is a marker of cellular inflammation¹⁸². Nitrite quantification was based on the Griess

colorimetric method. The assay was carried out in 96-well flat-bottom microtiter plates (Figure 6). Optical densities were measured in a Varioskan LUX Multimode Microplate Reader (Thermo Scientific™).

The anti-inflammatory potential of fractions was tested using the macrophage cell line RAW 264.7 previously stimulated with LPS (Merck). To assess a possible intrinsic capacity of fractions to induce an inflammatory response the assay was also performed without LPS stimulation.

The RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glutamine and pyruvate, 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin (penicillin 100 IU/mL and streptomycin 100 µg/mL). Cultures were incubated at 37 °C, 5% CO₂, in a humidified atmosphere. Prior to the experiment, cell status was assessed by observation under an inverted microscope to confirm adhesion and morphology. Culture medium was changed every two days.

At 70% confluency cells were detached by mechanical action using a scraper and transferred, to 15 mL conical Falcon tube. The cell suspensions were centrifuged at 3000 rpm for 3 min, after which the supernatant was discarded, and the cell pellet resuspended in 1–2 mL of fresh medium. For cell quantification and determination of viability, 20 µL of cell suspension was mixed with the same volume of a 4% trypan blue solution. Cell quantification was performed using a Neubauer chamber.

For anti-inflammatory assays cells were seeded in 96-well plates at a density of 3.5×10^5 cells per mL, in a final volume to 100 µL culture medium. The plates were incubated for 24 h under the previously described conditions to allow cell adhesion.

After 24 h, the medium was replaced with fresh medium and 0.5 µL of the fractions was added to be evaluated at a final concentration of 5 mg/mL. After 2 h a group of wells was treated with 5 µL of the LPS working solution to a final concentration of 20 µg/mL. The cells were incubated again for 24 h under the same culture conditions. Cell controls consisted in cells treated and no treated with LPS.

After incubation 75 µL of the supernatant from each well were transferred to a new 96-well plate. Subsequently, 75 µL of Griess reagent were added to each well, and the plates were left to stand for 10 min in the dark prior to spectrophotometric measurement of absorbance at 562 nm using the microplate reader.

The Griess reagent consisted in a mixture of equal volumes of a sulfanilamide solution (10 mg/mL in ultrapure water) and N-(1-naphthyl)ethylenediamine (1 mg/mL in a 2% aqueous phosphoric acid). The resulting solution was protected from light by covering

with aluminum foil and homogenized by vortexing to ensure complete mixture of the reagents.

NO production, expressed as a percentage of the inflammatory response relative to the control condition, was calculated using the following equation, where the negative control was defined as 100% NO production:

$$NO\ production\ (\%) = \left(\frac{A\ fraction}{\bar{A}\ NC} \right) \times 100$$

2.6.2. RAW 264.7 CELL VIABILITY ASSAY

To infer the cytotoxicity of the fractions on the RAW 264.7 cells, the cell viability assay based on the reduction MTT was performed. In this method, functional mitochondria reduce the MTT, a yellow compound, into purple formazan crystals. This colorimetric change is quantified by spectrophotometry, providing an estimate of the number of metabolically active cells present in the sample¹⁸³.

As in the anti-inflammatory assays, the potential cytotoxicity of fractions was evaluated in the presence of inflammation induced by LPS and in the absence of inflammatory induction.

For the MTT assay cells were seeded in 96-well plates at a density of 2.5×10^5 cells per mL (Figure 6). The seeding methodology and culture conditions were identical to those used in the anti-inflammatory assays, with both experiments conducted in parallel.

After 24 h of treatment with the fractions, 20 μ L of MTT solution were added to each well under dark conditions. The plates were incubated for 3 h under standard culture conditions, allowing substrate reduction by viable cells. After the incubation period, the supernatant was carefully removed, retaining the adherent cells containing the formazan crystals. Subsequently, 100 μ L of DMSO were added to each well to solubilize the formed formazan. Finally, absorbance was measured at 550 nm using a microplate reader.

Cell viability was determined using the following equation, where the negative control was defined as 100% cell viability under the experimental conditions:

$$Cell\ viability\ (\%) = \left(\frac{A\ fraction}{\bar{A}\ NC} \right) \times 100$$

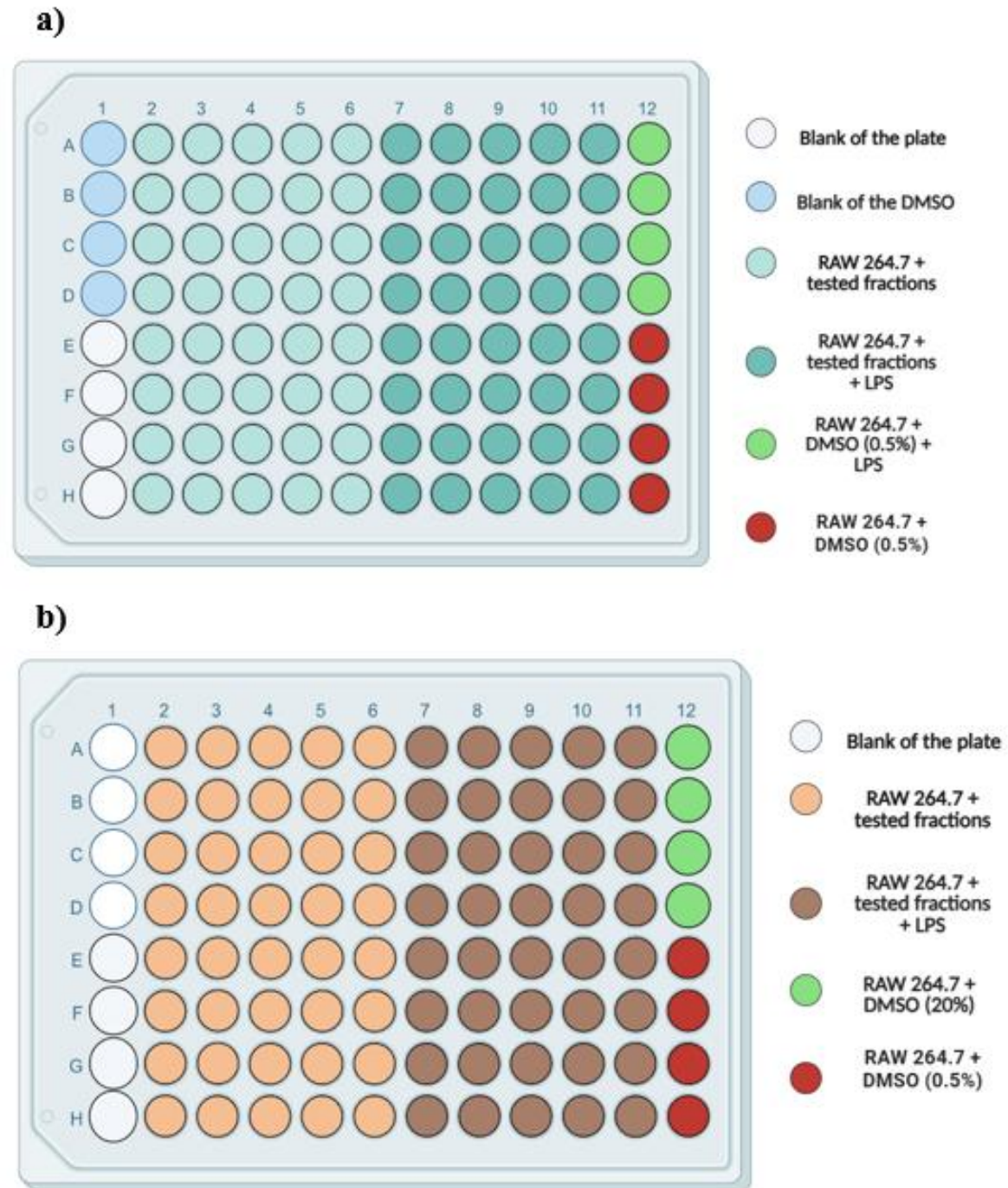


Figure 6: Diagram representing the plate layouts of the anti-inflammatory assays (a) and the cell viability assays (b). Created in <https://BioRender.com>

2.6.3. DATA PROCESSING AND OUTLIER HANDLING

During the timeframe of this study, between three and four independent assays for NO production and cell viability corresponding to plates WP5, WP11 and WP12 (Table I) were performed.

During data analysis, potential outliers were identified that affected the reliability of the calculated mean values and standard deviations, particularly given that the experiments were performed in triplicate. To address this, a simple median-based criterion was applied to assess extreme deviations between independent experiments. This approach was selected due to the limited sample size ($n = 3$), for which classical statistical outlier detection methods have reduced reliability, as previously discussed by Yang et al. (1998) and Hofer et al. (2000)^{184,185}.

Values deviating more than three times above or below the median were considered potential outliers and were excluded from subsequent analyses.

$$|x - \text{median}| > 3 \times \text{median}$$

All statistical analyses were performed using Microsoft Excel, and the resulting data were plotted using RStudio.

3. RESULTS AND DISCUSSION

3.1. CYANOBACTERIA CULTURE AND MASS COLLECTION

After one month of cultivation, strain *Drouetiella* sp. LEGE_221434 reached the required biomass yield, and the biomass was harvested, obtaining a dry weight of 2.7 g.

After two and a half months of cultivation, the biomass from strain *Scytonema* sp. LEGE_221431 was harvested, resulting in a dry weight of 1.3 g.

After four months of cultivation, the biomass from strains *Tolypothrix* sp. LEGE_221382 and *Nostoc* sp. LEGE_221427 was collected, obtaining 0.9 and 1 g of dry biomass respectively. Since this amount was considered insufficient to proceed with the subsequent steps of the workflow, the biomass samples were stored in a dark and dry environment, and the strains were re-cultivated until a higher biomass yield was achieved.

The slower growth rate and lower biomass production observed for strains *Tolypothrix* sp. LEGE_221382 and *Nostoc* sp. LEGE_221427 may be attributed to their specific growth patterns. Strain *Tolypothrix* sp. LEGE_221382 grew exclusively attached to the walls of the glass flask, meaning that most of the 4 L flask volume was occupied only by culture medium, substantially limiting the available space for cyanobacterial growth. In contrast, strain *Nostoc* sp. LEGE_221427 grew mainly as floating aggregates on the

surface of the medium, with a small fraction attached to the flask walls, which also restricted biomass accumulation. Additionally, this strain exhibited particularly slow growth.

Strains *Drouetiella* sp. LEGE_221434 and *Scytonema* sp. LEGE_221431 grew throughout the entire volume of the flask, maximizing the available space for biomass production and resulting in higher biomass yields.

Alternative cultivation strategies are recommended for scaling up the cultivation of strains *Tolypothrix* sp. LEGE_221382 and *Nostoc* sp. LEGE_221427 in order to optimize growth conditions and maximize biomass production.

3.2. ACETYLCHOLINESTERASE ENZYMATIC ASSAY

The ability of the fractions obtained from cyanobacterial strains to inhibit AChE activity was evaluated using the Ellman method ¹⁴¹. In total, eight fractions from each of the 100 selected strains were analyzed, resulting in the evaluation of a total of 800 fractions.

Due to the intrinsic coloration of some fractions, which could interfere with spectrophotometric measurements, absorbance values were corrected using the corresponding blanks for each fraction (Figure 5). The adjusted results were then compared with the negative control and expressed as percentage of relative enzymatic activity.

The negative control, in the absence of any inhibitory compound, was defined as 100% enzymatic activity. Accordingly, fractions exhibiting values close to or above this threshold were considered to lack meaningful inhibitory activity against AChE (Figure 7).

Under the experimental conditions employed, none of the analyzed fractions exhibited substantial inhibitory activity against AChE (Figure 6). Although several fractions, including LEGE_08334_C, LEGE_06150_B, LEGE_07177_A, LEGE_17966_F, LEGE_06100_A, LEGE_06105_A, LEGE_06108_A, LEGE_06108_B, LEGE_19967_E, and LEGE_02266_F, showed slightly reduced enzymatic activity relative to the control, the observed effects were minimal. In most cases, inhibition values no higher than 10%, indicating weak or negligible biological activity (Figure 8).

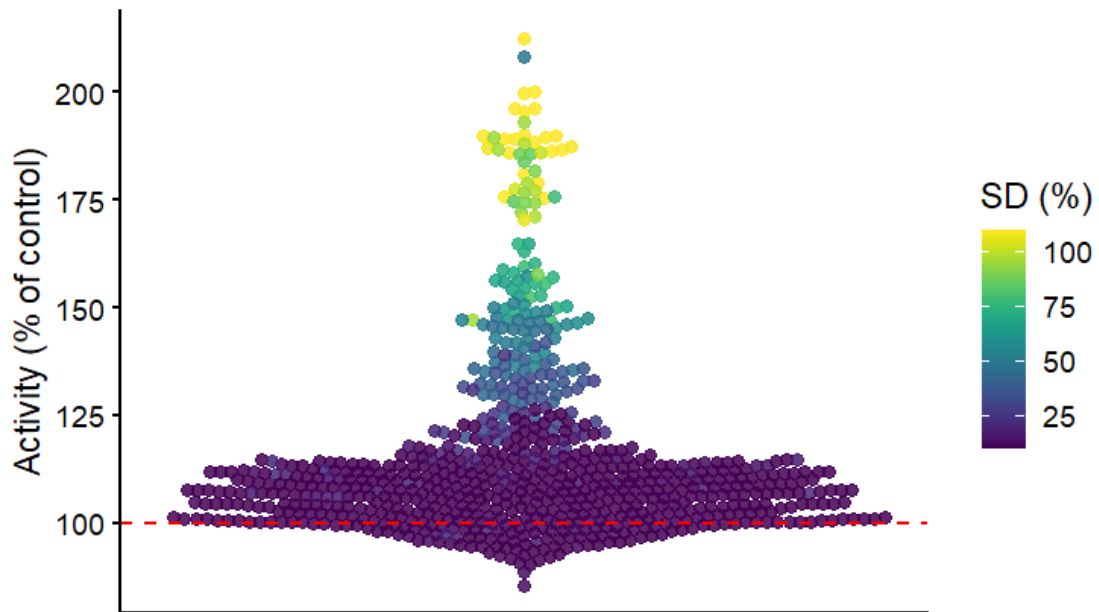


Figure 7: Scatter plot representing the percentage of relative enzymatic activity for each of the evaluated fractions, as well as their corresponding standard deviation. The Y-axis shows enzymatic activity expressed as a percentage, where 100% corresponds to the negative control value. Standard deviation is represented using a color scale.

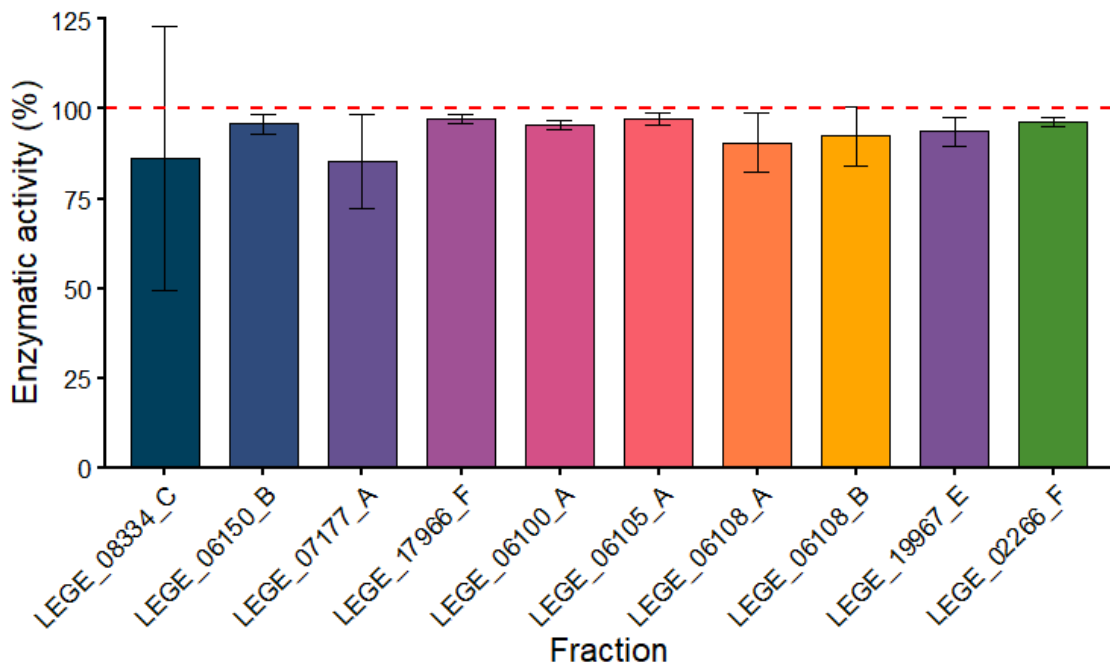


Figure 8: Graph showing the results obtained for the best performing fractions in the enzymatic AChE assays. The X-axis represents the identification of the evaluated fractions, while the Y-axis shows the percentage of relative enzymatic activity compared to the control.

In the specific case of fractions LEGE_08334_C and LEGE_07177_A, the enzymatic assay results indicate a reduction in activity exceeding 10%. Nevertheless, the relatively high variability observed among replicates for these fractions further limits the robustness of these findings and complicates their biological interpretation (Table 6).

Table 6: Table showing the raw results obtained for the best-performing fractions in the enzymatic AChE assays.

FRACTION	% ENZYMATIC ACTIVITY	STANDART DEVIATION
LEGE_08334_C	86,10	36,76
LEGE_06150_B	95,75	2,62
LEGE_07177_A	85,41	12,94
LEGE_17966_F	97,31	1,27
LEGE_06100_A	95,57	1,43
LEGE_06105_A	97,17	1,63
LEGE_06108_A	90,52	8,31
LEGE_06108_B	92,39	8,26
LEGE_19967_E	93,71	4,07
LEGE_02266_F	96,41	1,42

Overall, these results suggest that the cyanobacterial strains evaluated under the present experimental conditions do not represent a particularly promising source of AChE inhibitors targeting the cholinergic system. Nevertheless, the slight activity observed in a subset of fractions may warrant further investigation using more sensitive assays or fractionation approaches to identify potential bioactive metabolites.

These results contrast with those previously reported by Almeida et al. ¹⁸⁶, who, using an equivalent biochemical approach, evaluated crude organic and aqueous extracts from 18 cyanobacterial strains isolated from Portuguese estuarine environments, intertidal rocky shores, and freshwater systems against commercially available pure AChE. In that study, several extracts showed significant inhibition of enzymatic activity compared with the control (80%-100% inhibition), both in their organic form (*Synechocystis* sp. LEGE 06079 and *Microcoleus* sp. LEGE 07076) and aqueous form (*Synechocystis* sp. LEGE 06079, LEGE 06083, and LEGE 07073; *Leptolyngbya* sp. LEGE 07075; *Microcoleus* sp. LEGE 07076; and *Cylindrospermopsis raciborskii* LEGE 99043), with statistically significant differences relative to the control observed in most cases.

The discrepancy between these findings and the results obtained in the present study may be explained by several non-mutually exclusive factors. Firstly, while Almeida et al. employed crude extracts at a final concentration of 30 µg/mL, the fractions evaluated

herein were obtained through a fractionation process that, although reducing sample chemical complexity, may separate the bioactive metabolite from other compounds with which it could act synergistically in the original crude extract. Consequently, the inhibitory effect detectable in the crude extract may be attenuated when individual fractions are evaluated separately.

Secondly, the lack of overlap between the strains investigated in both studies does not allow excluding the possibility that the absence of activity observed in the present work simply reflects the non-inclusion, within the panel of 100 strains analysed, of cyanobacterial lineages producing the previously identified AChE-inhibitory metabolites.

3.3. ANTI-INFLAMMATORY ASSAY

3.3.1. ANTI-INFLAMMATORY assay

The anti-inflammatory capacity of the fractions derived from the strains corresponding to plates WP5, WP11 and WP12 was evaluated using the colorimetric method described by Barbosa et al.¹⁸¹. The cellular response to the Griess reagent was assessed under two experimental conditions: LPS-induced inflammation and a non-inflammatory condition. This dual approach allowed not only the evaluation of the potential anti-inflammatory activity of the fractions, but also the identification of possible pro-inflammatory effects, which would be undesirable in the context of therapeutic applications targeting NDs.

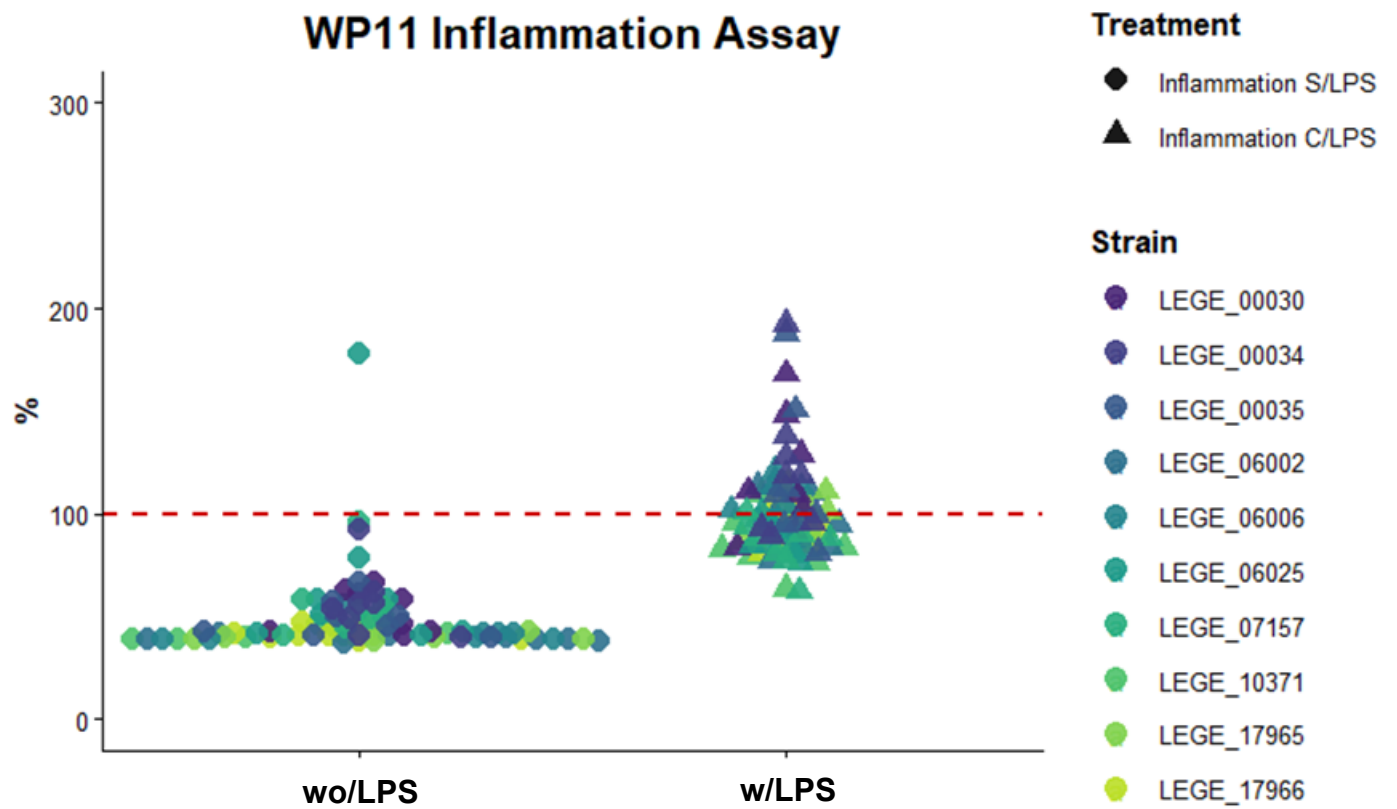
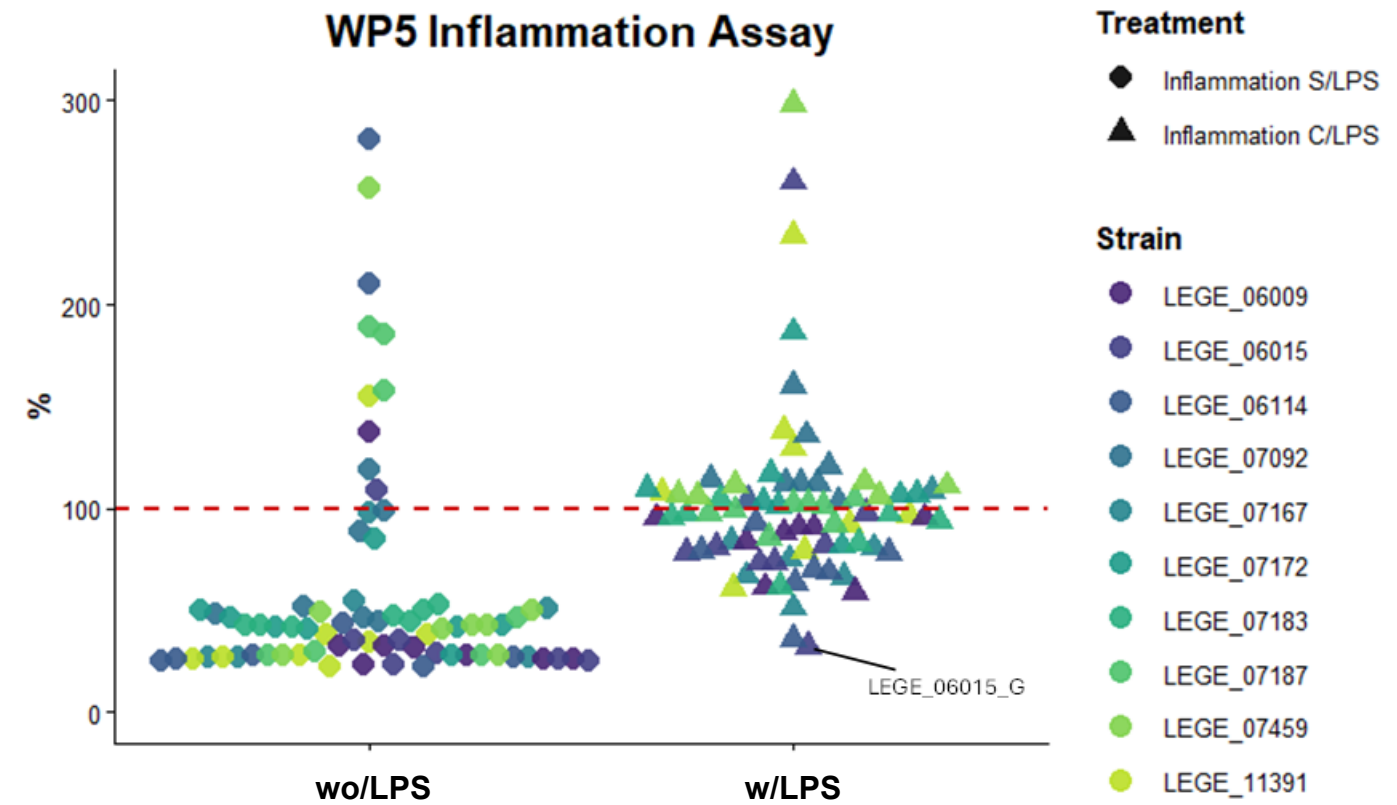
Overall, most fractions evaluated under non-inflammatory conditions did not exhibit inflammation levels higher than those observed in the negative control, with some fractions showing values below 30% relative to control. However, certain fractions displayed inflammation levels comparable to or higher than the control. These results suggest that some of the analyzed fractions may induce pro-inflammatory responses in the cellular system employed and should therefore be interpreted with caution, particularly when considering their potential use in other biological contexts or assays related to neuroprotection, such as glutamatergic modulation or other neuroprotective mechanisms.

Under LPS-induced inflammatory conditions, a general increase in inflammatory levels was observed. Across all plates, a subset of fractions exhibited inflammation values equal to or higher than those of the control compared with the non-stimulated condition. However, a relevant number of fractions still displayed values below the control level.

In WP5, minimum inflammation values close to 32.3% relative to control were recorded, whereas in WP11 the lowest observed value was approximately 62.14%. These results indicate a higher heterogeneity in the response of WP5 fractions compared to WP11. Notably, the most pronounced anti-inflammatory activity was observed in WP12, where the lowest recorded value was 12.30% relative to control. This corresponds to a strong inhibition of the LPS-induced inflammatory response.

Of the ten most active anti-inflammatory fractions, nine originated from WP12, and six corresponded to the same strain (*Sphaerospermopsis* sp. LEGE 08334) (Table III).

Overall, these findings indicate that, although not all fractions exhibit anti-inflammatory activity and some may even induce pro-inflammatory responses, a considerable number display significant anti-inflammatory potential. This reinforces their interest in the context of drug discovery and further supports cyanobacteria as a promising source of bioactive compounds for the investigation of NDs (Figure 9).



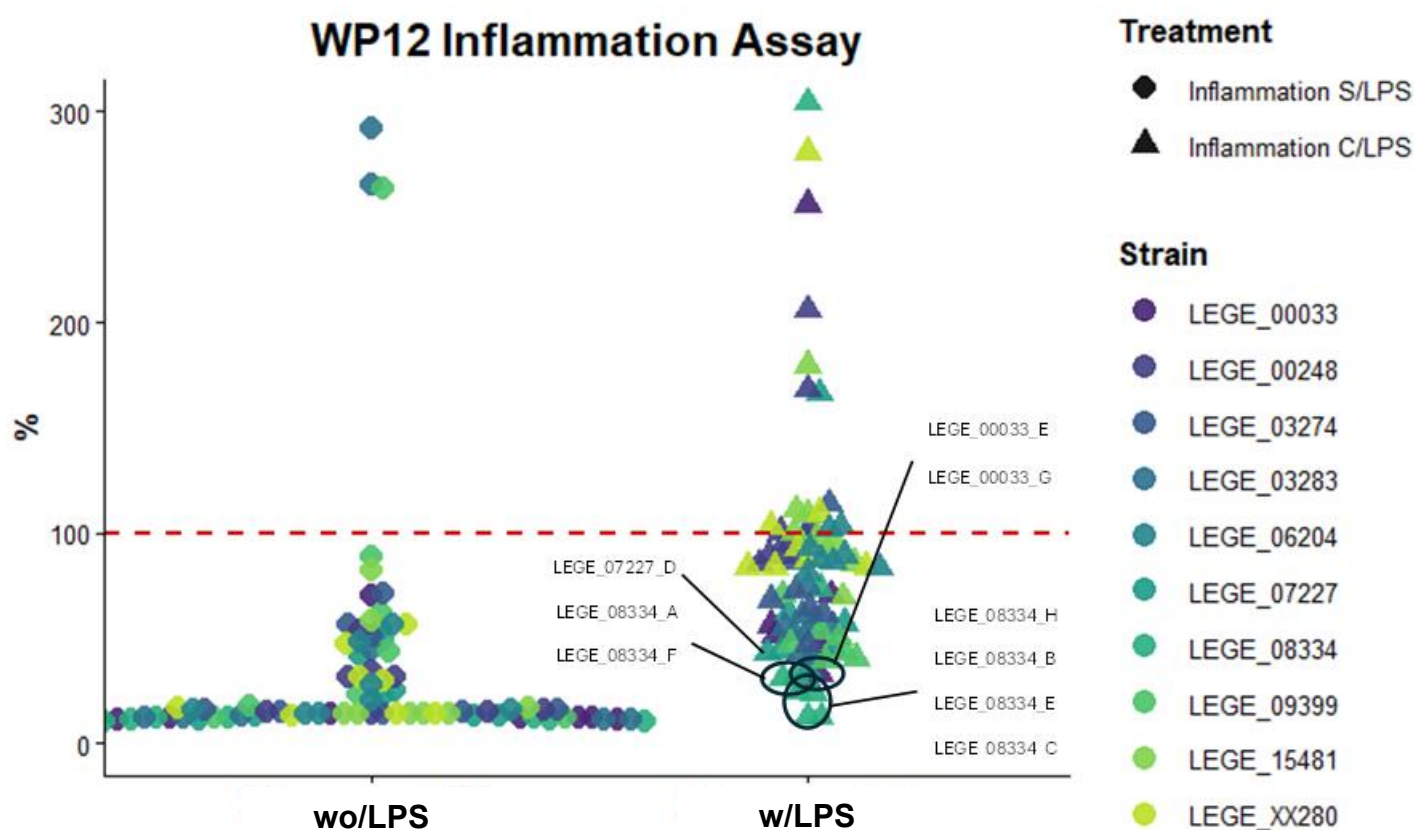


Figure 9: Scatter plots globally representing the anti-inflammatory effect of the fractions corresponding to the strains included in plates WP5, WP11 and WP12. Results obtained in the absence of LPS (wo/LPS) are represented by circles on the left side of each graph, whereas those corresponding to treatments with LPS-induced inflammation (w/LPS) are shown by triangles on the right side. Each color corresponds to a different strain, as indicated in the legend. The Y-axis represents the percentage of relative inflammation with respect to the negative control. The value corresponding to the negative control was established as 100% inflammation and is indicated by a red dashed horizontal line. Values above this line represent inflammation levels higher than those observed in the control. The most relevant fractions cited in the text are highlighted.

3.3.2. CELL VIABILITY

To validate inflammatory results the potential cytotoxicity of the fractions derived from the strains corresponding to plates WP5, WP11 and WP12 was evaluated using the MTT assay¹⁸³. These cell viability assays were performed in parallel with the anti-inflammatory assays to ensure that fractions exhibiting anti-inflammatory activity did not simultaneously display cytotoxic effects capable of inducing cell death.

As previously described, anti-inflammatory activity was assessed through the Griess reaction that detects nitrites, and indirectly NO levels in the culture medium, which is used as a marker of inflammatory response¹⁸¹. In this context, cell death induced by compounds present in the evaluated fractions could prevent NO production, thereby generating false-positive results in the anti-inflammatory assays by artificially mimicking a reduction in inflammatory activity¹⁸⁷. Therefore, the combined assessment of

cytotoxicity and anti-inflammatory activity is essential to ensure the validity and correct interpretation of the biological results.

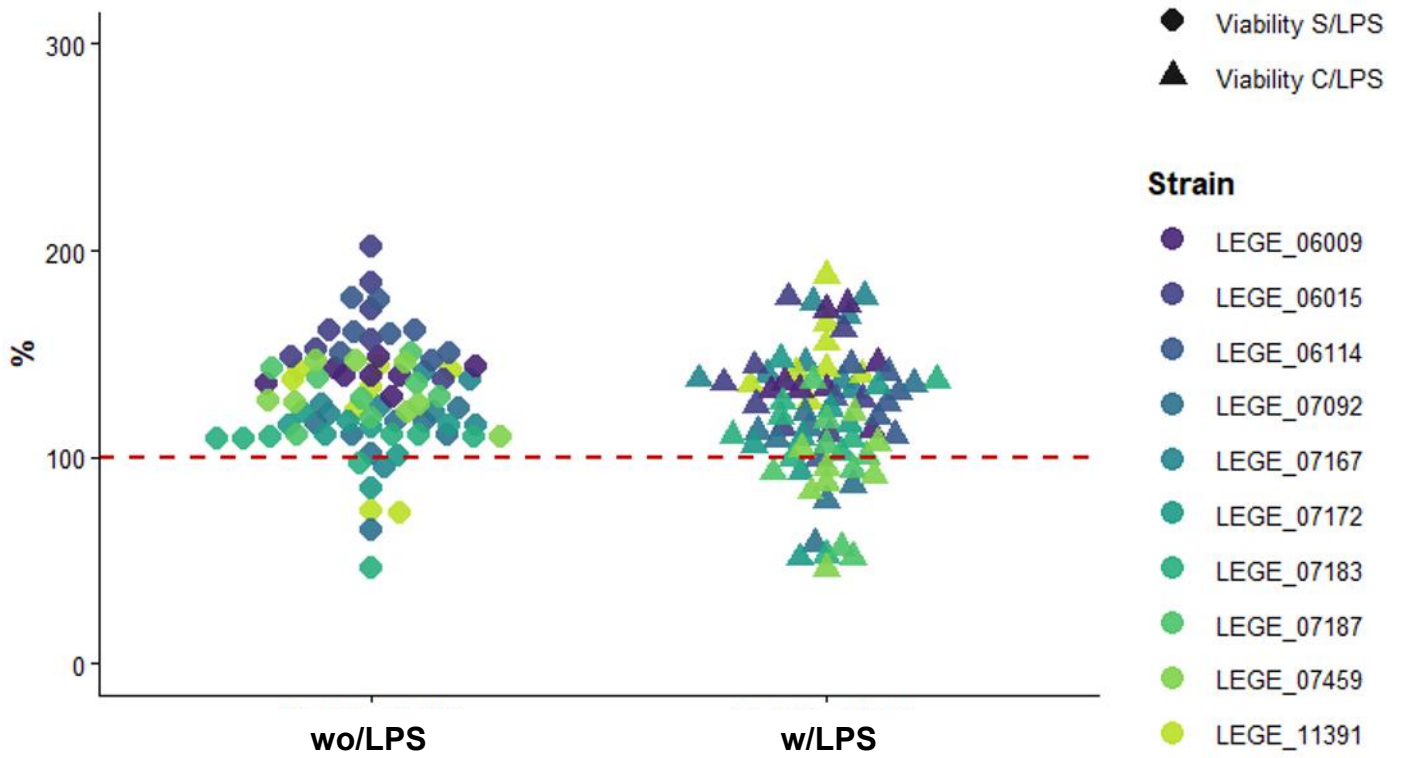
Under non-inflammatory conditions, the results obtained for plate WP5 (Figure 10) show that most fractions exhibited cell viability levels close to or above 100% relative to the control, with only a limited number of fractions ($n = 7$) displaying values below this reference level. These results indicate that the majority of the evaluated fractions did not exert cytotoxic effects on the cell line and, in some cases, may even promote increased metabolic activity, as values exceeding 200% were observed for LEGE_06015_C (201.43%), corresponding to more than a twofold increase in cell viability compared with the negative control.

Under LPS-induced inflammatory conditions, a subset of fractions ($n = 17$) showed viability values below 100% relative to the control, a higher proportion than that observed under non-inflammatory conditions. This suggests that certain fractions are less capable of maintaining cell viability under inflammatory stress. Nevertheless, the majority of fractions ($n = 63$) preserved viability levels comparable to those observed under non-inflammatory conditions, remaining close to or above control levels even in the presence of LPS.

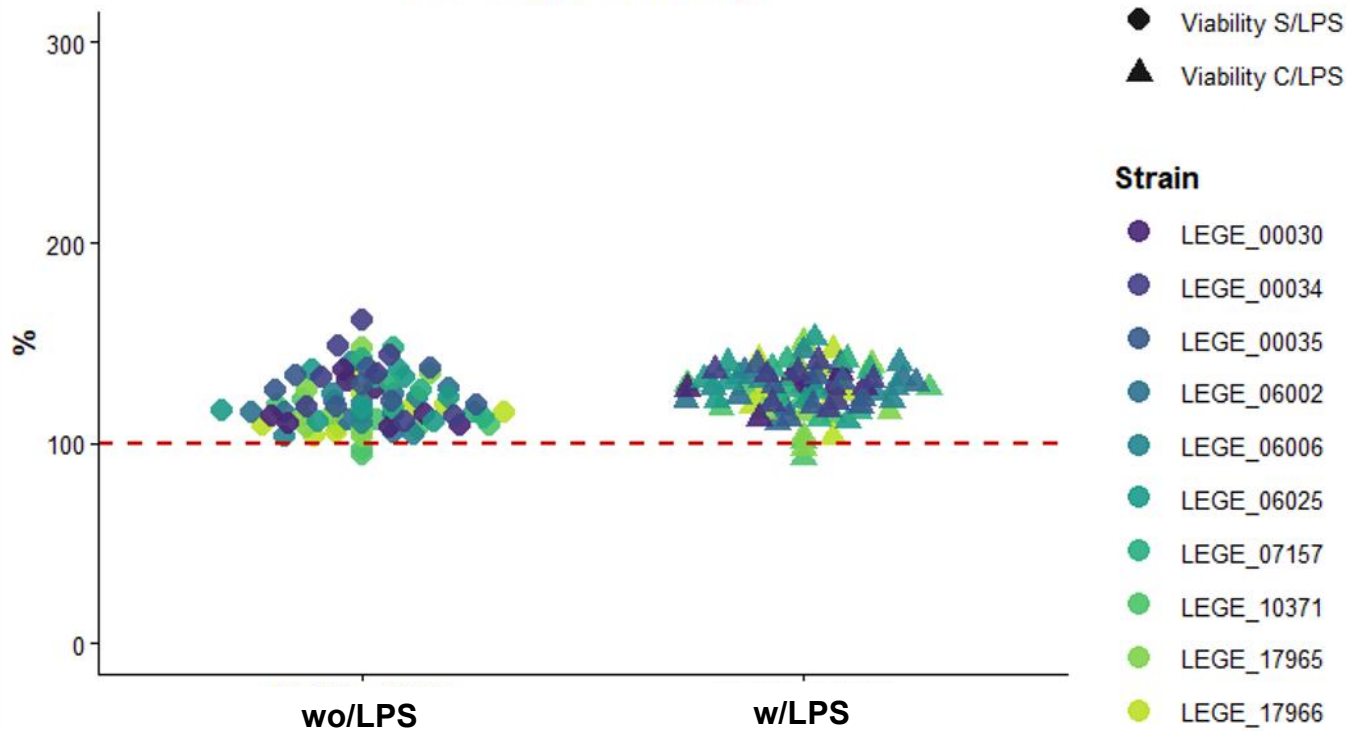
In plate WP11 (Figure 10), all evaluated fractions exhibited cell viability values equal to or higher than the reference control under both non-inflammatory and LPS-induced conditions, indicating a consistent absence of cytotoxic effects. A similar trend was observed in WP12, where all fractions displayed viability values equal to or higher than the control under both conditions, with low variability among treatments. Notably, this plate showed the most pronounced effects, with cell viability values exceeding 230% under both experimental conditions.

Taken together, these results demonstrate the overall low cytotoxicity of the evaluated fractions, supporting their potential biological interest for future pharmacological and biotechnological applications. The results obtained from the anti-inflammatory activity and cytotoxicity assays provide, in themselves, valuable information regarding the pharmacological potential of the evaluated cyanobacterial extracts. Nevertheless, the main objective of this study was to identify fractions with the highest bioactive relevance for potential application in the context of NDs. In this regard, particular interest was given to fractions displaying, under LPS-induced conditions, relative inflammation values below 100% combined with cell viability values equal to or higher than 100%.

WP5 MTT Assay



WP11 MTT Assay



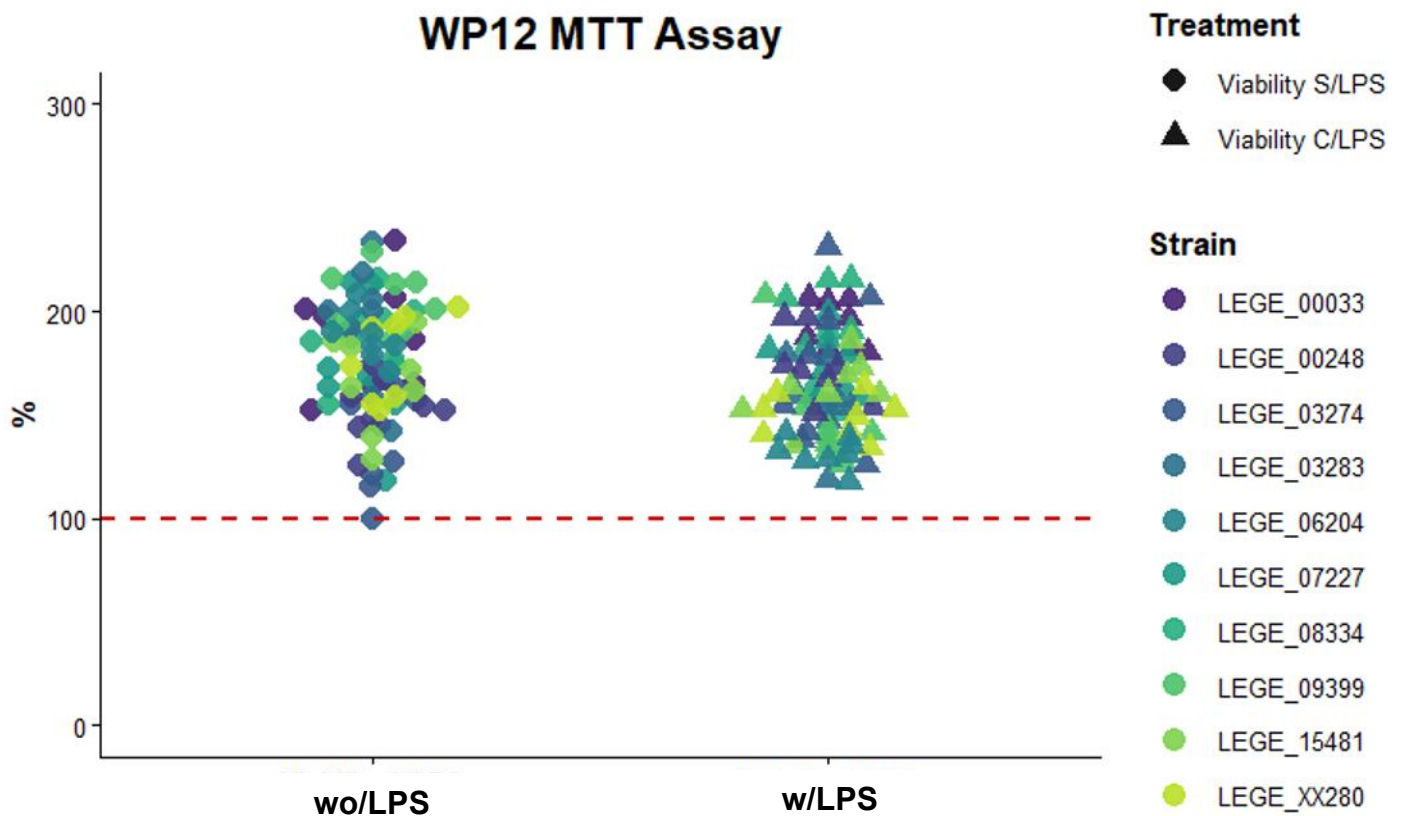


Figure 10: Scatter plots globally representing the cytotoxic effect of the fractions corresponding to the strains included in plates WP5, WP11 and WP12. The Y-axis represents the percentage of relative cell viability with respect to the negative control. The Results obtained in the absence of LPS (wo/LPS) are represented by circles on the left side of each graph, whereas those corresponding to treatments with LPS-induced inflammation (w/LPS) are shown by triangles on the right side. Each color corresponds to a different strain, as indicated in the legend. value corresponding to the negative control was established as 100% cell viability and is indicated by a red dashed horizontal line. Values below this line represent lower levels of cell viability than those observed in the control.

3.3.3. IDENTIFICATION OF BIOACTIVE FRACTIONS THROUGH INTEGRATED DATA ANALYSIS

The combined analysis of both assays (Figure 11) revealed that a considerable number of fractions met both established criteria simultaneously, demonstrating the ability to reduce inflammation without compromising cell viability. Notably, WP5 exhibited greater heterogeneity in its response compared to WP11 and WP12. In WP5, the three most active fractions, considering both parameters, were LEGE_06015_G (inflammation: 32.30%; viability: 114.18%), LEGE_06114_E (35.95%; 128.55%), and LEGE_07167_C (51.19%; 168.42%). In WP11, the best performing fractions were LEGE_07157_C (62.15%; 141.24%), LEGE_10371_F (63.66%; 117.91%), and LEGE_10371_D (76.17%; 127.43%) (Table IV and Table V). In contrast, WP12 showed markedly stronger anti-inflammatory activity while maintaining high viability, with fractions LEGE_08334_H

(12.30%; 182.23%), LEGE_08334_B (12.52%; 190.73%), and LEGE_08334_E (24.11%; 162.85%) (Table 7).

Table 7: Table showing the three highest relative inflammatory inhibition results from each plate, ranked in ascending order, together with their corresponding associated cell viability values.

FRACTION	INFLAMMATION (%)	CELL VIABILITY (%)	WORKING PLATE
LEGE_08334_H	12.30	182.23	WP12
LEGE_08334_B	12.52	190.73	WP12
LEGE_08334_E	24.11	162.85	WP12
LEGE_06015_G	32.30	114.18	WP5
LEGE_06114_E	35.95	128.55	WP5
LEGE_07167_C	51.19	168.42	WP5
LEGE_07157_C	62.15	141.24	WP11
LEGE_10371_F	63.66	117.91	WP11
LEGE_10371_D	76.17	127.43	WP11

Overall, these results indicate a consistent trend toward higher anti-inflammatory activity in fractions derived from WP12 compared to WP5 and WP11, highlighting strain *Sphaerospermopsis* sp. LEGE 08334 as particularly relevant for the identification of anti-inflammatory compounds with potential relevance in NDs (Table VI).

Although the assays performed in this study were primarily focused on anti-inflammatory activity, cytotoxicity data were used as a biological validation parameter. Interestingly, fractions displaying cell viability values above 100% (Table VII) warrant further investigation, as this behavior may suggest potential effects on cellular growth, maintenance, or proliferation. This observation is particularly relevant given that the cell line used corresponds to macrophages, key effector cells of the innate immune system¹⁸². In the context of dose response biology, such an increase in viability may be indicative of a hormetic response, whereby low concentrations of bioactive compounds induce a mild cellular stress that activates adaptive and compensatory mechanisms, ultimately enhancing macrophage metabolic activity or survival¹⁸⁸. Mild stimulatory effects in these cells may reflect modulation of redox balance, energy metabolism, or pro-survival signaling pathways, which are commonly associated with hormetic effects. These findings may therefore indicate that certain compounds present in the evaluated fractions are capable not only of modulating macrophage function but also of enhancing cellular fitness through adaptive stress responses, thereby opening new perspectives for the study of cyanobacteria-derived immunomodulatory compounds.

In this context, the absence of cytotoxic effects observed in this study for most of *Lusitaniella coriacea* LEGE 07167 fractions in RAW 264.7 cells may reflect an inherent cell-specific tolerance associated with the metabolic and functional characteristics of macrophages, which differ substantially from those of rapidly proliferating tumour cell lines commonly used in cytotoxicity screening. Conversely, the previously reported cytotoxic activity of compounds derived from this strain in other cellular systems suggests that its secondary metabolites may exert a selective and context-dependent bioactivity spectrum, influenced by cellular phenotype and proliferation rate. Taken together, these findings highlight *Lusitaniella coriacea* LEGE 07167 as a promising source of marine natural products combining anti-inflammatory activity with a favourable macrophage safety profile, while also reinforcing the importance of multi-cell-line screening strategies to fully elucidate the biological potential and selectivity of cyanobacterial metabolites.

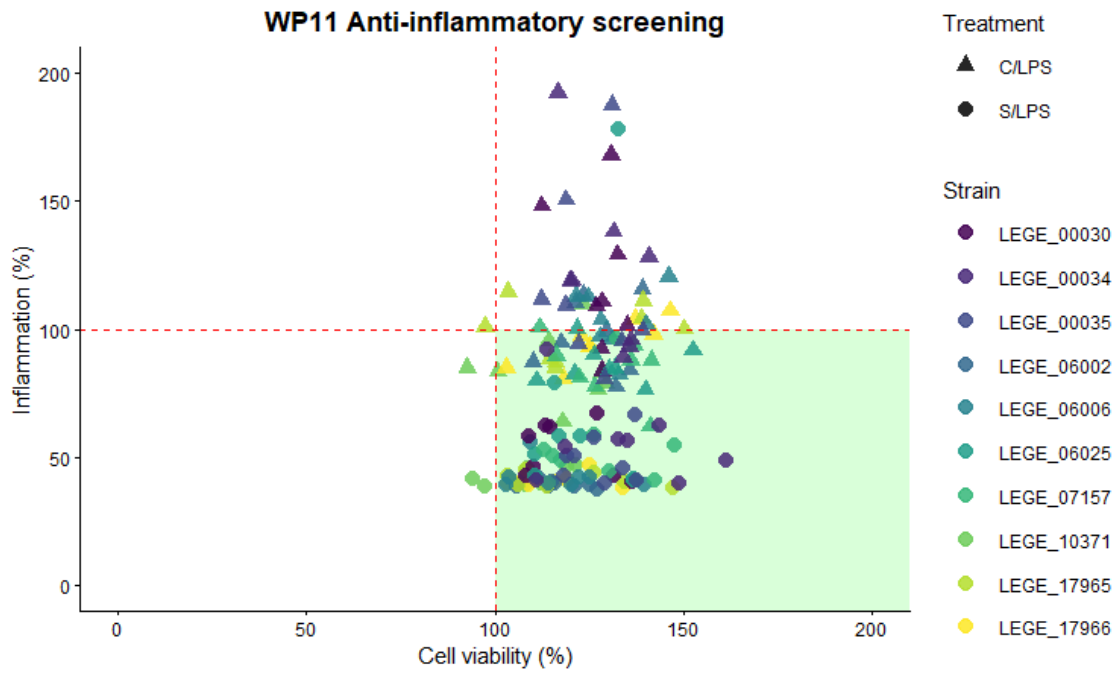
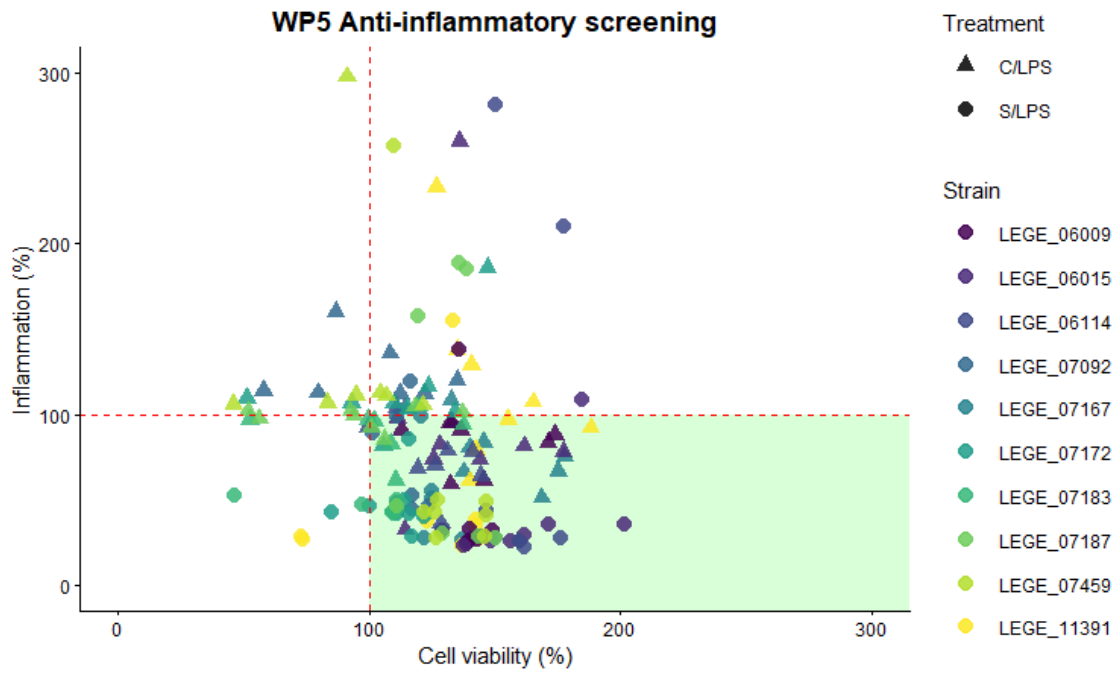
To integrate both biological parameters into a single quantitative selection criterion, a Score was calculated as the difference between the cell viability and inflammation values obtained under LPS-induced conditions ($\text{Score} = \% \text{ viability} - \% \text{ inflammation}$, both measured under LPS stimulation). Because both variables are expressed on the same control-relative scale, this difference provides a direct measure of the balance between reduced inflammatory response and preserved or enhanced cell viability, with higher values indicating a more favourable bioactive profile. Unlike the selection criterion based on a binary threshold (inflammation $< 100\%$ and viability $\geq 100\%$), which identifies the fractions with the lowest inflammation among those exceeding the viability threshold, the Score offers a continuous ranking that weighs both variables simultaneously. Consequently, the Score-based ranking may differ, in part, from the ranking obtained when considering anti-inflammatory activity alone, as it can favour fractions with a more balanced combination of moderate inflammation reduction and particularly high viability over fractions with the lowest inflammation values but comparatively lower viability.

An additional trend was observed when comparing Score values across WP5, WP11, and WP12 (Tables IV–VI), where WP12 fractions consistently exhibited higher values. In WP12, the three highest Score values corresponded to LEGE_08334_A (184.68), LEGE_08334_C (181.22), and LEGE_08334_B (178.21), further supporting the strong bioactive potential of strain LEGE_08334; notably, only LEGE_08334_B appears among both the top three fractions ranked by inflammation alone and the top three ranked by Score, illustrating how the inclusion of the viability term can reprioritise fractions with a more favourable overall balance even when their inflammation values are not the absolute lowest. In WP5, only three fractions exceeded a Score of 100: LEGE_07167_C (117.24), LEGE_07167_A (108.46), and LEGE_07167_B (102.42), suggesting moderate

but notable activity associated with strain LEGE_07167. In WP11, the highest Score values were observed for LEGE_07157_C (79.09), LEGE_06025_E (63.70), and LEGE_06025_C (60.64), all of which were lower than those observed in WP5 and substantially lower than those in WP12 (Table 8).

Table 8: Table showing the three highest Score values from each plate, ranked in ascending order.

FRACTIONS	SCORE	WORKING PLATE
LEGE_08334_A	184.68	WP12
LEGE_08334_C	181.22	WP12
LEGE_08334_B	178.21	WP12
LEGE_07167_C	117.24	WP5
LEGE_07167_A	108.46	WP5
LEGE_07167_B	102.42	WP5
LEGE_07157_C	79.09	WP11
LEGE_06025_E	63.70	WP11
LEGE_06025_C	60.64	WP11



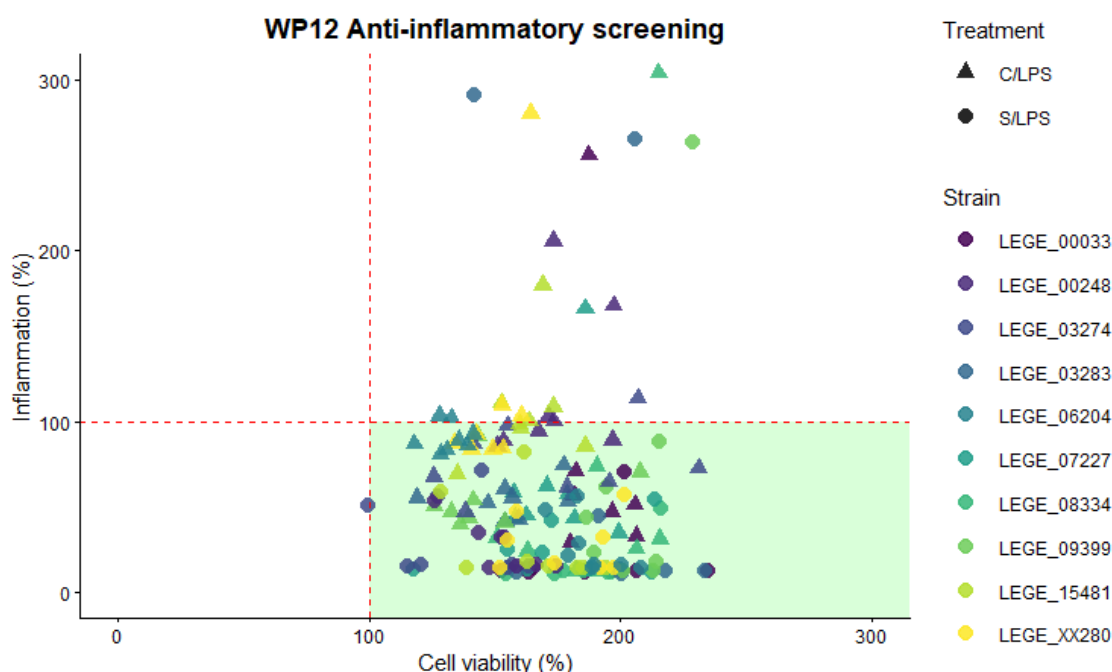


Figure 11: Scatter plots representing a comparative analysis of inflammation and cell viability values of the fractions evaluated in plate WP5, WP11 and WP12, expressed as a percentage relative to their respective controls. Results obtained in the absence of LPS are represented by circles, whereas those corresponding to LPS-induced inflammatory conditions are shown as triangles. Each color corresponds to a different strain, as indicated in the legend. The X-axis represents relative inflammation values, with the negative control set at 100%, indicated by a red dashed horizontal line. The Y-axis represents relative cell viability values, with the reference value (negative control) also set at 100% and indicated by a red dashed vertical line. The green shaded area corresponds to the region of the plot in which fractions simultaneously exhibit reduced inflammation and increased cell viability, suggesting a potentially favorable biological profile.

At this point, it is relevant to highlight that the WP12 plate, which showed significantly more promising results in the anti-inflammatory assays compared to the other evaluated plates, is composed almost exclusively of cyanobacterial strains originating from freshwater environments (Table I).

The existing literature indicates that both marine and freshwater environments harbor cyanobacteria with high chemical diversity. Comparative studies have demonstrated that marine and freshwater cyanobacteria constitute distinct reservoirs of bioactive secondary metabolites. Although marine cyanobacteria tend to exhibit greater structural diversity of chemical compounds, freshwater cyanobacteria contain a comparable richness of bioactive metabolite classes and remain a relatively underexplored source of compounds with pharmacological relevance^{189,190}.

Furthermore, the most relevant strain within this plate (*Sphaerospermopsis* sp. LEGE 08334) belongs to the genus *Sphaerospermopsis* (Table I), a freshwater cyanobacterial genus reported to produce diverse secondary metabolites, including cyanobactins,

chlorinated fatty acid-derived lactylates, and NRPs. Some of these compounds have demonstrated antimicrobial and antibiofilm activities, suggesting potential pharmacological interest^{171–173}.

To date, the anti-inflammatory activity of metabolites associated with the genus *Sphaerospermopsis* has been scarcely investigated, and no systematic studies have comprehensively evaluated this biological effect. Consequently, the chemical and biological characterization of metabolites produced by this genus remains limited, and further studies are required to better elucidate their therapeutic potential and possible applicability in the development of novel bioactive agents.

It is also relevant to highlight that the anti-AChE active strains identified by Almeida et al.¹⁸⁶ were not restricted to freshwater environments but included representatives from estuarine, intertidal, and freshwater habitats, suggesting that ecological origin alone is not a reliable predictor of anti-AChE activity. This contrasts with the trend observed in the present study for anti-inflammatory activity, where freshwater strains from WP12 displayed a markedly higher bioactivity profile. Therefore, caution should be exercised when generalizing the role of the ecological origin of cyanobacterial strains as a predictive criterion for bioactivity, as such relationships may be highly dependent on the specific biological target evaluated.

The cyanobacterial strain *Lusitaniella coriacea* LEGE 07167 exhibited a particularly interesting bioactivity profile in several of its fractions, characterized by a clear anti-inflammatory effect in LPS-stimulated RAW 264.7 macrophages, together with a lack of cytotoxicity, as reflected by cell viability values exceeding 100%. In contrast, previous studies have reported that the metallophore compounds lusichelins, isolated from *Lusitaniella coriacea* LEGE 07167, can display significant cytotoxic activity in tumour-derived cell lines^{174,177}. Indeed, these compounds were reported to be present in the LEGE_07167_C fractions. The differences in cell viability results obtained in the present study may be due to the use of a different cell line or to the degradation of the compounds in the tested fractions. Mass spectrometry experiments could be used to investigate their presence and relative abundance.

Importantly, several marine and terrestrial natural products have been reported to be non-cytotoxic in RAW 264.7 macrophages while exhibiting selective cytotoxicity in other mammalian cell lines, particularly tumour-derived models such as A549, HepG2, or MCF-7¹⁹¹. For instance, anti-inflammatory metabolites isolated from marine algae and other natural sources have been shown to inhibit NO production and inflammatory mediators in LPS-activated RAW 264.7 cells without affecting macrophage viability,

while still retaining biological activity¹⁹². Similarly, plant-derived flavonoids and alkaloids have demonstrated minimal or no cytotoxicity in RAW 264.7 macrophages, further supporting the concept of cell-type dependent sensitivity to NDs¹⁹³.

4. CONCLUSION

Neurodegenerative diseases, particularly AD, involve a complex interplay between multiple pathological mechanisms, including neuroinflammation, oxidative stress, impaired neurotransmission, and protein aggregation. Therefore, the identification of multi-target bioactive compounds capable of modulating these processes represents a promising strategy for therapeutic development. However, the discovery of natural products with pharmacological potential remains a long, complex, and costly process, requiring the evaluation of a large number of compounds before identifying those with the desired properties. Within this framework, a predominance of inactive or weakly active samples in initial screening efforts should not be interpreted as a failure of the approach, but rather as an expected and informative outcome of a progressive selection strategy, in which each round of screening serves to narrow down the chemical space and prioritize the most promising candidates for subsequent, more resource-intensive investigation.

In this study, such a first step was undertaken through the screening of 800 fractions obtained from 100 cyanobacterial strains, targeting two complementary biological endpoints relevant to neurodegenerative diseases: AChE inhibition and modulation of the inflammatory response, the latter further validated through parallel cytotoxicity assays. Although none of the evaluated fractions exhibited relevant AChE inhibitory activity under the experimental conditions employed, the screening revealed a remarkable anti-inflammatory potential among several cyanobacterial-derived fractions, highlighting the value of evaluating multiple, distinct targets within the same pipeline rather than relying on a single bioactivity readout.

The evaluation using LPS-stimulated RAW 264.7 macrophages demonstrated that numerous fractions were able to significantly reduce inflammatory responses while maintaining or enhancing cell viability. Among the analyzed samples, fractions derived from the WP12 plate showed the most promising biological profile, with strain *Sphaerospermopsis* sp. LEGE 08334 standing out as the most relevant candidate: several of its fractions achieved strong reductions in inflammation, with LEGE_08334_H decreasing relative inflammation to 12.30% of the control value, while LEGE_08334-derived fractions consistently achieved the highest integrated bioactivity (Score) values,

reflecting a favorable balance between inflammation reduction and preserved cell viability.

Strain *Lusitaniella coriacea* LEGE 07167 also displayed a promising profile, with several fractions combining relevant anti-inflammatory activity with favorable viability values, supporting its consideration as a secondary candidate for further investigation. Additionally, lusichelin C, isolated from the same strain, had previously been evaluated for its inhibitory activity against AChE and BuChE enzymes, yielding rather unpromising results ¹⁹⁴.

The low cytotoxicity observed in the majority of fractions reinforces the biological relevance of these effects and highlights the potential of cyanobacteria as a valuable source of immunomodulatory metabolites. Notably, the most promising plate (WP12) was composed almost exclusively of freshwater strains, in contrast to the broader taxonomic origin of the strains evaluated for AChE inhibition, suggesting that the relationship between strain habitat and bioactivity may be specific to the biological target under investigation rather than a general predictive trait. In particular, the activity observed in *Sphaerospermopsis* sp. LEGE 08334, belonging to the freshwater genus *Sphaerospermopsis*, is noteworthy given the scarce characterization of secondary metabolites from this genus to date, particularly regarding anti-inflammatory effects, suggesting that underexplored cyanobacterial taxa may represent important reservoirs of novel bioactive compounds.

Overall, this work provides a solid foundation for the identification of cyanobacteria-derived molecules with potential applications in NDs research. The results support the need for further studies focused on the purification and structural characterization of the active metabolites, the elucidation of their mechanisms of action, and the evaluation of their pharmacological properties beyond the endpoints addressed here. In particular, a more in-depth chemical and metabolomic characterization, mass spectrometric analysis, and biological investigation of strain *Sphaerospermopsis* sp. LEGE 08334 and *Lusitaniella coriacea* LEGE 07167 is recommended, as they constitute priority candidates capable of contributing novel insight into the still largely unexplored bioactive potential cyanobacteria.

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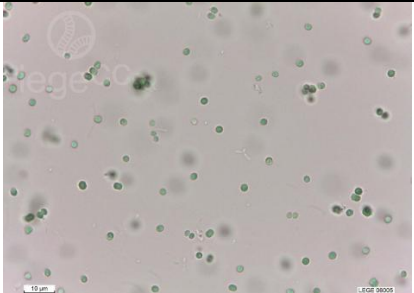
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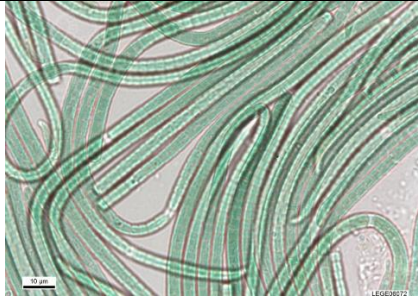
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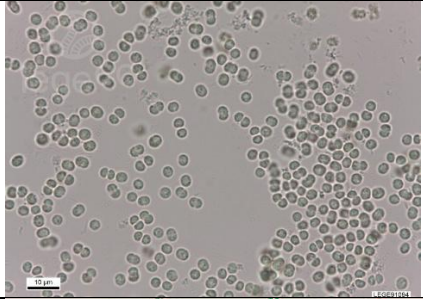
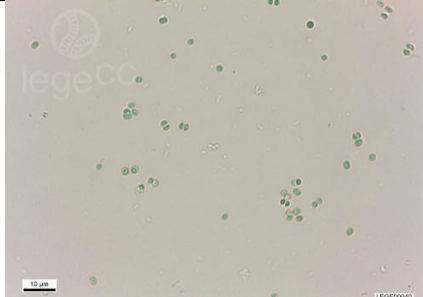
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6.ANNEX

Table I: Available information on the cyanobacterial strains used in this study. The cyanobacteria are presented grouped according to the working plates employed. All information compiled in this table was retrieved from the LEGE-CC.

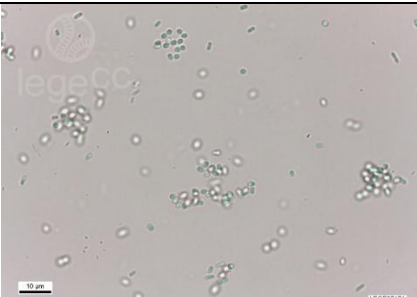
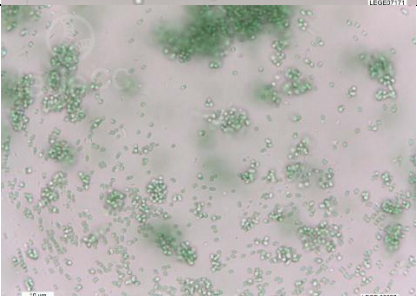
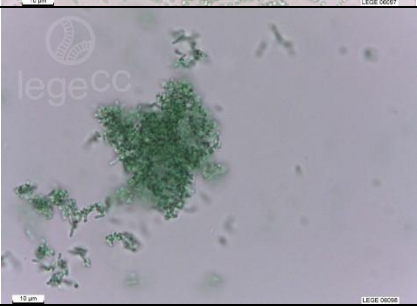
WP1				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
<i>Synechocystis</i> sp. LEGE 06005	<i>Synechocystis</i> sp.	São Pedro de Moel (Portugal)	Aquatic – Marine Coastal	
<i>cf. Romeria</i> sp. LEGE 06013	<i>cf. Romeria</i> sp.	Praia da Foz do Arelho, Caldas da Rainha (Portugal)	Aquatic – Marine Intertidal zone	

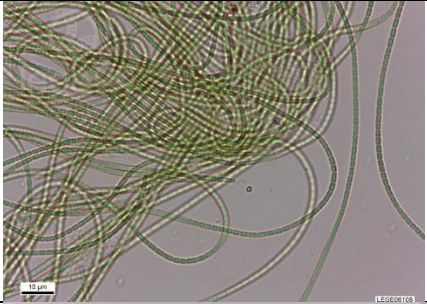
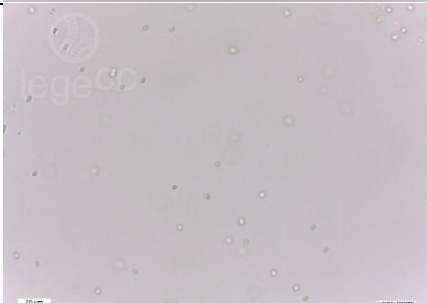
<i>cf. Oxynema acuminatum</i> LEGE 06072	<i>cf. Oxynema acuminatum</i>	Vouga estuary, Ria de Aveiro, São Jacinto (Portugal)	Aquatic – Brackish Mesotidal zone	
<i>cf. Oxynema acuminatum</i> LEGE 06078	<i>cf. Oxynema acuminatum</i>	Douro estuary, Vila Nova de Gaia (Portugal)	Aquatic – Brackish Mesotidal zone	
<i>Nodosilinea</i> sp. LEGE 07085	<i>Nodosilinea</i> sp.	Douro estuary, Porto (Portugal)	Aquatic – Brackish Mesotidal zone	

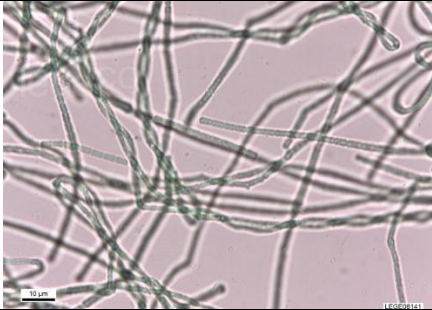
<i>Microcystis aeruginosa</i> LEGE 91094	<i>Microcystis aeruginosa</i>	Lagoa de Mira (Portugal)	Aquatic – Freshwater Pond	
<i>Synechocystis salina</i> LEGE 00040	<i>Synechocystis salina</i>	Praia de Vila Praia de Âncora, Caminha (Portugal)	Aquatic – Marine Intertidal zone	
<i>Phormidium cf. irriguum</i> LEGE 00055	<i>Phormidium cf. irriguum</i>	Imfout reservoir, Oum Rabiaa basin (Morocco)	Aquatic - Freshwater	

<i>Sphaerospermopsis</i> sp LEGE 02266	<i>Sphaerospermopsis</i> sp	Maranhão dam reservoir, Benavila, Avis (Portugal)	Aquatic - Freshwater	
<i>Rivularia</i> sp. LEGE 07159	<i>Rivularia</i> sp.	Praia de Burgau, Budens (Portugal)	Aquatic – Marine Tide pool	

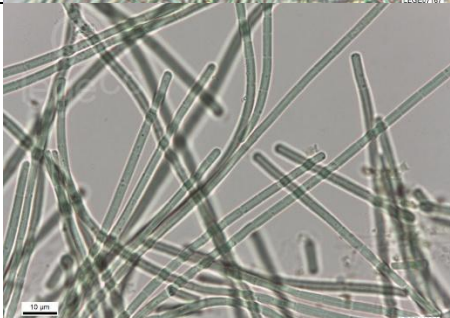
WP2				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
<i>Phormidium</i> sp LEGE 05292	<i>Phormidium</i> sp	Indoor aquarium wall, at Porto (Portugal)	Aquatic – Freshwater Biofilm	

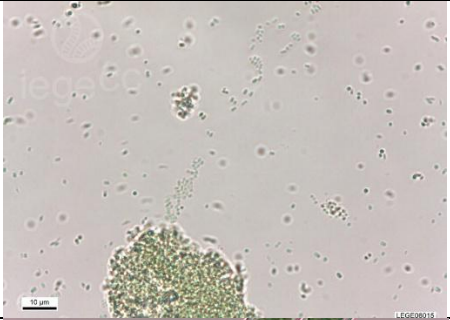
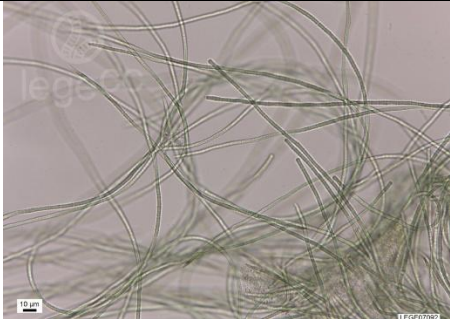
<i>Synechococcus nidulans</i> LEGE 07171	<i>Synechococcus nidulans</i>	Praia de Burgau, Budens (Portugal)	Aquatic – Marine Tide pool	
<i>Cyanobium</i> sp. LEGE 06097	<i>Cyanobium</i> sp.	Praia do Martinhal, Vila do Bispo (Portugal)	Aquatic – Marine Intertidal zone	
<i>Cyanobium</i> sp. LEGE 06098	<i>Cyanobium</i> sp.	Praia do Martinhal, Vila do Bispo (Portugal)	Aquatic – Marine Intertidal zone	

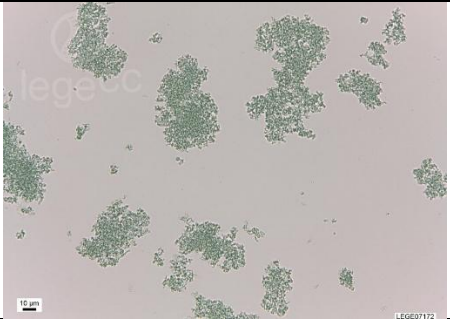
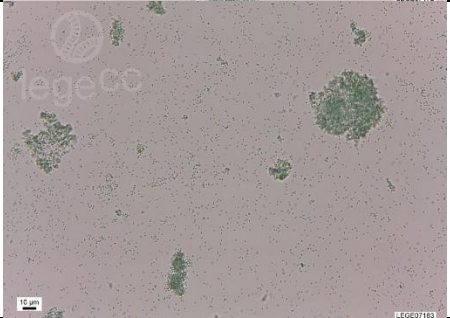
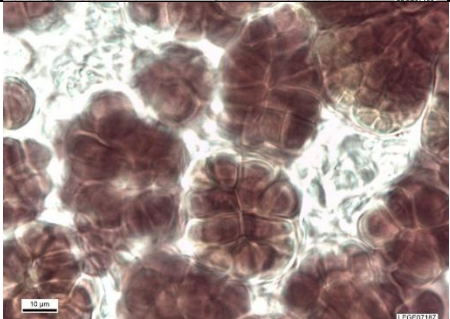
<i>Toxifilum mysidocida</i> LEGE 06108	<i>Toxifilum mysidocida</i>	Praia da Luz, Lagos (Portugal)	Aquatic – Marine Tide pool	
<i>Sphaerospermopsis</i> sp. LEGE 00249	<i>Sphaerospermopsis</i> sp.	Maranhão dam reservoir, campsite at Montargil (Portugal)	Aquatic – Freshwater	
Unidentified unicellular Synechococcales LEGE 08333	Unidentified unicellular <i>Synechococcales</i>	Lake Zumpango (Mexico)	Aquatic - Freshwater	

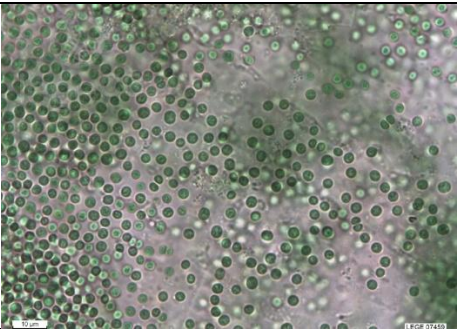
cf. <i>Oculatella</i> sp. LEGE 06141	cf. <i>Oculatella</i> sp.	Praia da Luz, Lagos (Portugal)	Aquatic – Marine Intertidal zone	
<i>Dolichospermum</i> sp. LEGE 00263	<i>Dolichospermum</i> sp.	Maranhão dam reservoir, Benavila (Portugal)	Aquatic - Freshwater	

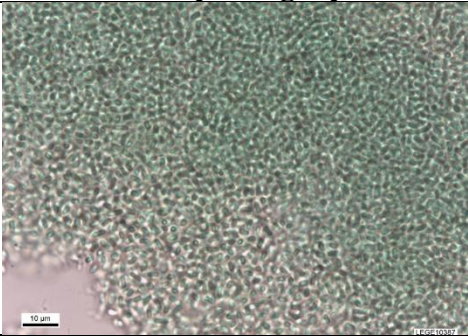
WP5				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph

<i>Lusitaniella coriacea</i> LEGE 07167	<i>Lusitaniella coriacea</i>	Praia de Lavadores, Canidelo (Portugal)	Aquatic – Marine Tide pool	
<i>Geitlerinema</i> sp. LEGE 11391	<i>Geitlerinema</i> sp.	Araruama lagoon, in the state of Rio de Janeiro (Brazil)	Aquatic - Hypersaline	
<i>Nodosilinea</i> sp. LEGE 06009	<i>Nodosilinea</i> sp.	Praia da Foz do Arelho, Caldas da Rainha (Portugal)	Aquatic – Marine Intertidal zone	

<i>Cyanobium</i> sp. LEGE 06015	<i>Cyanobium</i> sp.	Baleal Norte, Ferrel (Portugal)	Aquatic – Marine Intertidal zone	
<i>Plectonema</i> cf. <i>radiosum</i> LEGE 06114	<i>Plectonema</i> cf. <i>radiosum</i>	Praia da Luz, Lagos (Portugal)	Aquatic – Marine Intertidal zone	
<i>Coleofasciculus</i> sp. LEGE 07092	<i>Coleofasciculus</i> sp.	Vouga estuary, Ria de Aveiro, São Jacinto (Portugal)	Aquatic – Brackish Mesotidal zone	

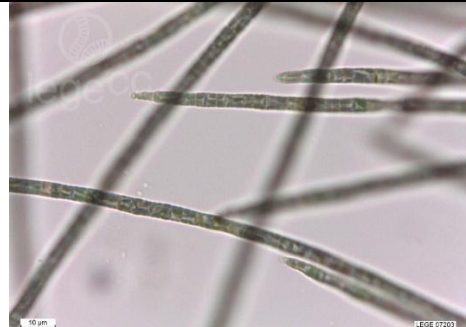
<i>Synechococcus</i> sp. LEGE 07172	<i>Synechococcus</i> sp.	Praia de Olhos d'Água, Albufeira (Portugal)	Aquatic – Marine Tide pool	
<i>Cyanobium</i> sp. LEGE 07183	<i>Cyanobium</i> sp.	Praia de Olhos d'Água, Albufeira (Portugal)	Aquatic – Marine Tide pool	
<i>Chroococcopsis</i> sp. LEGE 07187	<i>Chroococcopsis</i> sp.	Praia de Moledo, Caminha (Portugal)	Aquatic – Marine Intertidal zone	

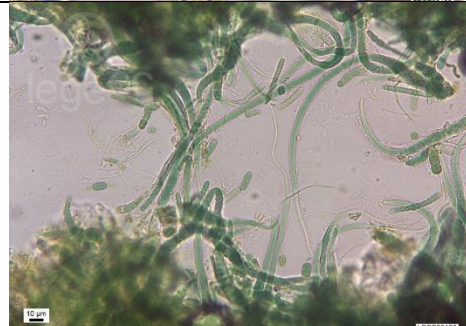
<i>Geminobacterium atlanticum</i> LEGE 07459	<i>Geminobacterium atlanticum</i>	Praia do Martinhal, Vila do Bispo (Portugal)	Aquatic – Marine Intertidal zone	
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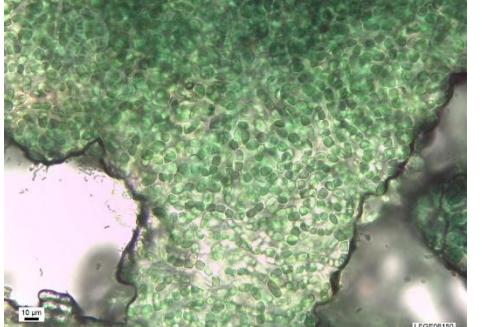
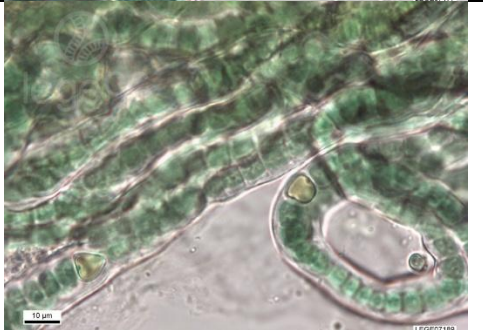
WP6				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
Unidentified unicellular <i>Synechococcales</i> LEGE 10387	Unidentified unicellular <i>Synechococcales</i>	Vila Nova de Mil Fontes (Portugal)	Aquatic – Marine Intertidal zone	

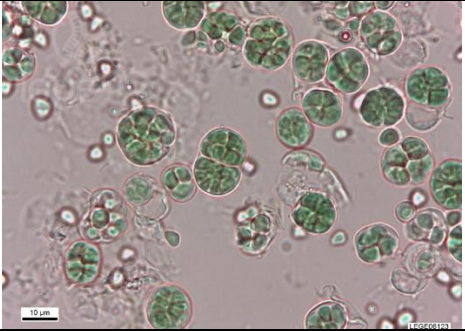
<i>Geitlerinema</i> sp. LEGE 11390	<i>Geitlerinema</i> sp.	Araruama lagoon, in the state of Rio de Janeiro (Brazil)	Aquatic - Hypersaline	
<i>Geitlerinema</i> sp. LEGE 11393	<i>Geitlerinema</i> sp.	Pernambuco lagoon, in the state of Rio de Janeiro (Brazil)	Aquatic - Hypersaline	
<i>Geitlerinema</i> sp. LEGE 11396	<i>Geitlerinema</i> sp.	Araruama lagoon, in the state of Rio de Janeiro (Brazil)	Aquatic - Hypersaline	

<i>Nostoc</i> sp. LEGE 12449	<i>Nostoc</i> sp.	Botanical Garden of the University of Coimbra (Portugal)	Subaerial – Terrestrial Cycad coralloid root	
<i>Nostoc</i> sp. LEGE 12450	<i>Nostoc</i> sp	ND	ND	
LEGE PCC7167	ND	ND	ND	

<i>Tychonema</i> sp. LEGE 07203	<i>Tychonema</i> sp.	Wastewater treatment plant at Febros river, Vila Nova de Gaia (Portugal)	Aquatic – Freshwater Biofilm	
<i>Nodosilinea</i> sp. LEGE 11429	<i>Nodosilinea</i> sp.	A Pedra', diving spot in front of the fort 'Castelo do Queijo' (Portugal)	Aquatic – Marine Subtidal-sample	
Unidentified <i>Oscillatorialles</i> LEGE 19967	Unidentified <i>Oscillatorialles</i>	Cais de Olhão (Portugal)	Aquatic – Marine Intertidal zone	

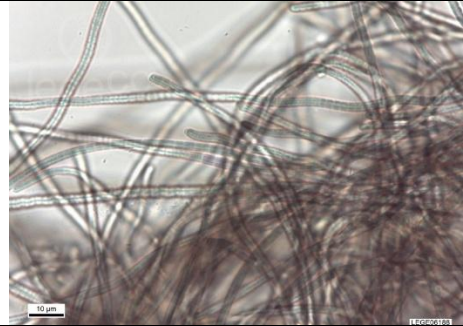
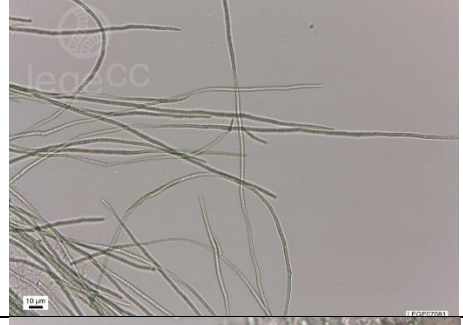
WP8				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
<i>Calothrix</i> sp. LEGE 06100	<i>Calothrix</i> sp.	Praia de Lavadores, Canidelo (Portugal)	Aquatic – Marine Tide pool	
<i>Plectonema</i> cf. <i>radius</i> LEGE 06105	<i>Plectonema</i> cf. <i>radius</i>	Praia da Luz, Lagos (Portugal)	Aquatic – Marine Intertidal zone	
<i>Calothrix</i> sp. LEGE 06122	<i>Calothrix</i> sp.	Praia de São Bartolomeu do Mar, Esposende (Portugal)	Aquatic – Marine Wave exposed tide pool	

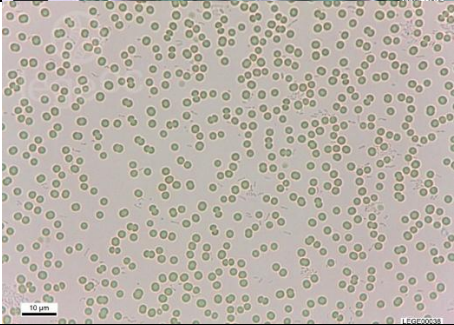
Unidentified <i>Nostocales</i> LEGE 06150	Unidentified <i>Nostocales</i>	Praia de São Bartolomeu do Mar, Esposende (Portugal)	Aquatic – Marine Tide pool	
<i>Rivularia</i> sp. LEGE 07177	<i>Rivularia</i> sp.	Praia do Martinhal, Vila do Bispo (Portugal)	Aquatic – Marine Intertidal zone	
<i>Scytonema</i> sp. LEGE 07189	<i>Scytonema</i> sp.	Praia de Moledo, Caminha (Portugal)	Aquatic – Marine Intertidal zone	

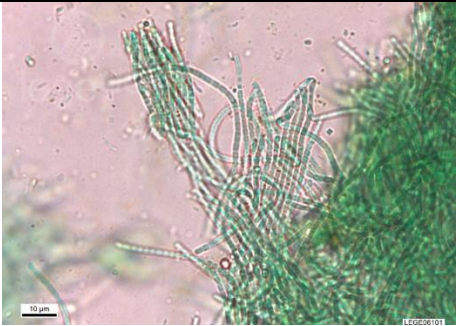
Unidentified <i>Nostocales</i> LEGE 11386	Unidentified <i>Nostocales</i>	Unknown	Aquatic – Marine Intertidal zone	
<i>Nostoc</i> sp. LEGE 13413	<i>Nostoc</i> sp.	Porto metropolitan area (Portugal)	Spirulina dietary supplements tablet form	
<i>Gloeocapsopsis</i> <i>crepidinum</i> LEGE 06123	<i>Gloeocapsopsis</i> <i>crepidinum</i>	Praia da Luz, Lagos (Portugal)	Aquatic – Marine Intertidal zone	

<i>Spirulina</i> sp. LEGE 11439	<i>Arthrospira</i> sp.	A Pedra', diving spot in front of the fort 'Castelo do Queijo' (Portugal)	Aquatic – Marine Subtidal-sample	
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WP9				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
<i>Phormidium laetevirens</i> LEGE 06103	<i>Phormidium laetevirens</i>	Praia da Luz, Lagos (Portugal)	Aquatic – Marine Tide pool	

<i>Calenema singularis</i> LEGE 06188	<i>Calenema singularis</i>	Praia de Lavadores, Canidelo (Portugal)	Aquatic – Marine Coastal	
<i>Coleofasciculus</i> sp. LEGE 07081	<i>Coleofasciculus</i> sp.	Minho estuary, Caminha (Portugal)	Aquatic – Brackish Mesotidal zone	
<i>Pseudanabaena</i> cf. <i>curta</i> LEGE 10371	<i>Pseudanabaena</i> cf. <i>curta</i>	Praia da Memória (Portugal)	Aquatic – Marine Intertidal zone	

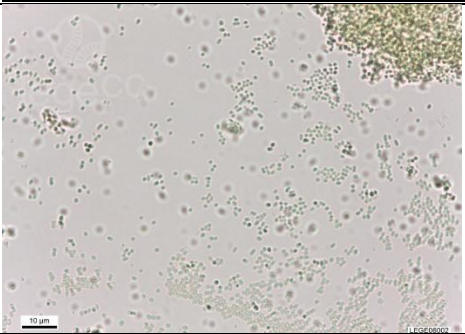
Unidentified <i>Oscillatoriales</i> LEGE 11467	Unidentified <i>Oscillatoriales</i>	A Pedra', diving spot in front of the fort 'Castelo do Queijo' (Portugal)	Aquatic – Marine Subtidal-sample	
Unidentified <i>Oscillatoriales</i> LEGE 11472	Unidentified <i>Oscillatoriales</i>	A Pedra', diving spot in front of the fort 'Castelo do Queijo' (Portugal)	Aquatic – Marine Subtidal-sample	
<i>Synechocystis salina</i> LEGE 00038	<i>Synechocystis salina</i>	Praia do Mindelo, Vila do Conde (Portugal)	Aquatic – Marine Intertidal zone	

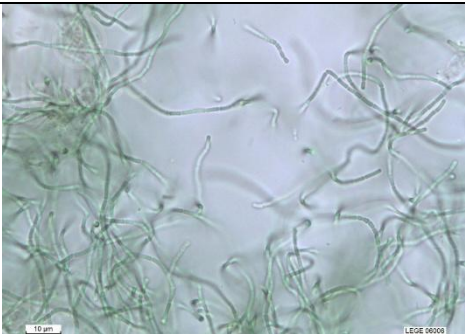
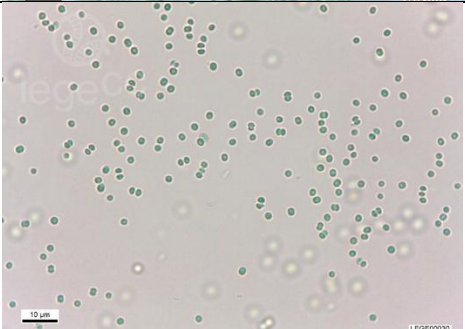
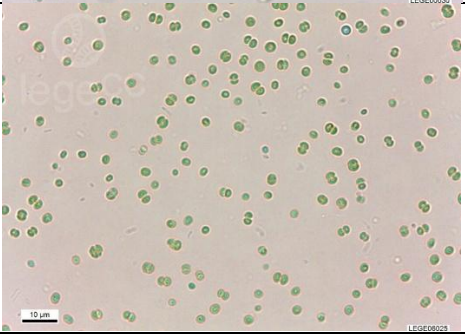
<i>Nodosilinea</i> sp. LEGE 06001	<i>Nodosilinea</i> sp.	Praia de Buarcos (Portugal)	Aquatic – Marine Coastal	
cf. <i>Romeria</i> sp. LEGE 06003	cf. <i>Romeria</i> sp.	Praia de Buarcos (Portugal)	Aquatic – Marine Intertidal zone	
<i>Nodosilinea nodulosa</i> LEGE 06101	<i>Nodosilinea nodulosa</i>	Praia da Luz, Lagos (Portugal)	Aquatic – Marine Intertidal zone	

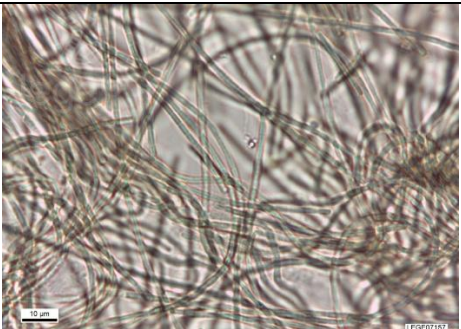
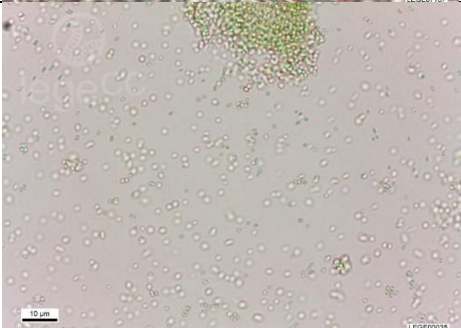
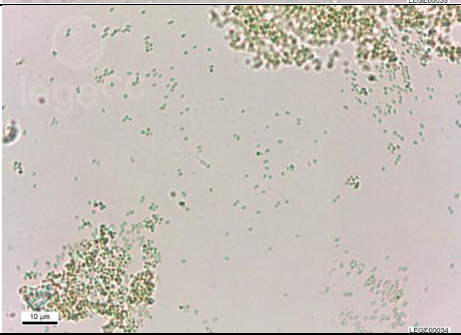
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Strain ID	Identification	Sampling place	Lifestyle	Microphotograph

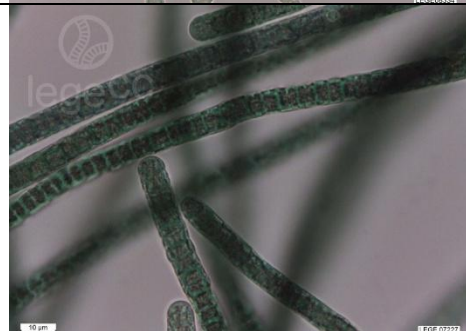
<i>Plectonema</i> cf. <i>radiosum</i> LEGE 06105	<i>Plectonema</i> cf. <i>radiosum</i>	Praia da Luz, Lagos (Portugal)	Aquatic – Marine Intertidal zone	
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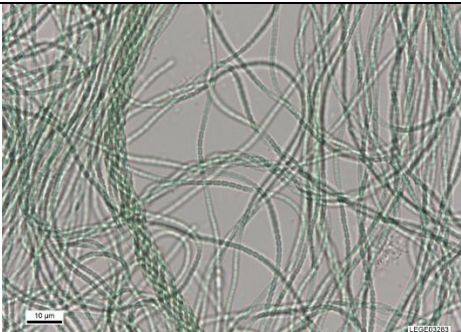
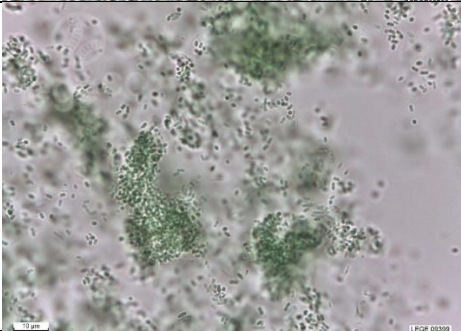
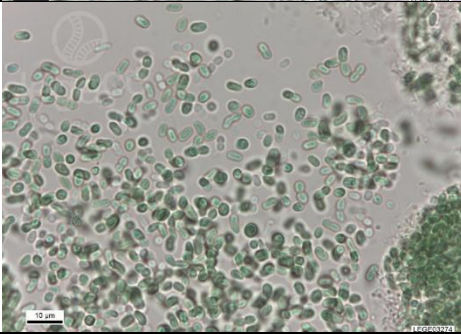
WP11				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
<i>Pseudanabaena</i> cf. <i>curta</i> LEGE 10371	<i>Pseudanabaena</i> cf. <i>curta</i>	Praia da Memória (Portugal)	Aquatic – Marine Intertidal zone	

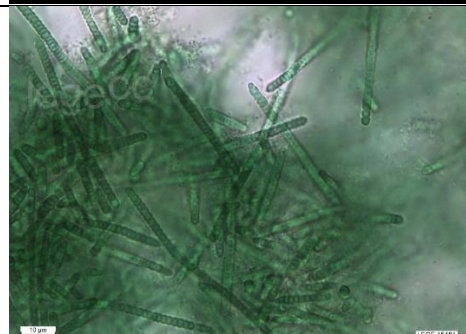
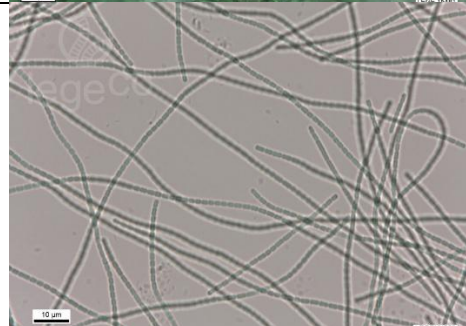
Unidentified <i>Synechococcales</i> LEGE 17966	Unidentified <i>Synechococcales</i>	Praia de Vale Figueiras, Aljezur (Portugal)	Aquatic – Marine Coastal	
<i>Cyanobium</i> sp. LEGE 06002	<i>Cyanobium</i> sp.	Praia de Buarcos (Portugal)	Aquatic – Marine Intertidal zone	
Unidentified <i>Synechococcales</i> LEGE 17965	Unidentified <i>Synechococcales</i>	Praia de Vale Figueiras, Aljezur (Portugal)	Aquatic – Marine Coastal	

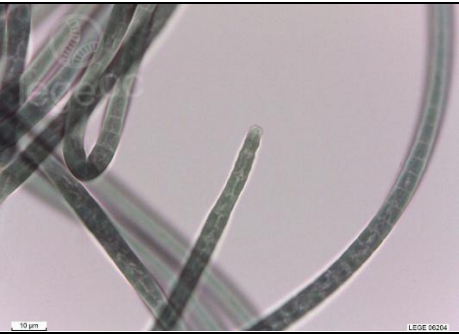
<i>Nodosilinea</i> sp. LEGE 06006	<i>Nodosilinea</i> sp.	São Pedro de Moel (Portugal)	Aquatic – Marine Coastal	
<i>Synechocystis salina</i> LEGE 00030	<i>Synechocystis salina</i>	Praia da Aguda, Arcozelo (Portugal)	Aquatic – Marine Intertidal zone	
<i>Synechocystis</i> sp. LEGE 06025	<i>Synechocystis</i> sp.	Praia da Empa, Ericeira (Portugal)	Aquatic – Marine Intertidal zone	

<i>Lusitaniella coriacea</i> LEGE 07157	<i>Lusitaniella coriacea</i>	Praia de Lavadores, Canidelo (Portugal)	Aquatic – Marine Tide pool	
<i>Cyanobium</i> sp. LEGE 00035	<i>Cyanobium</i> sp.	Praia de Olhos d'Água, Albufeira (Portugal)	Aquatic – Marine Intertidal zone	
<i>Cyanobium</i> sp. LEGE 00034	<i>Cyanobium</i> sp.	Praia da Aguda, Arcozelo (Portugal)	Aquatic – Marine Intertidal zone	

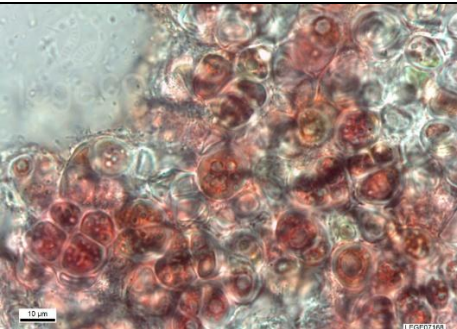
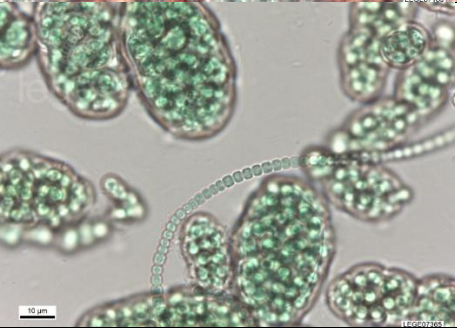
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
Unidentified filamentous cyanobacterium LEGE 00033	Unidentified filamentous cyanobacterium	Praia da Aguda, Arcozelo (Portugal)	Aquatic – Marine Intertidal zone	
<i>Sphaerospermopsis</i> sp. LEGE 08334	<i>Sphaerospermopsis</i> sp.	Lake Zumpango (Mexico)	Aquatic - Freshwater	
<i>aff. Planktothrix mougeotii</i> LEGE 07227	<i>aff. Planktothrix mougeotii</i>	Wastewater treatment plant at Febros river, Vila Nova de Gaia (Portugal)	Aquatic - Freshwater	

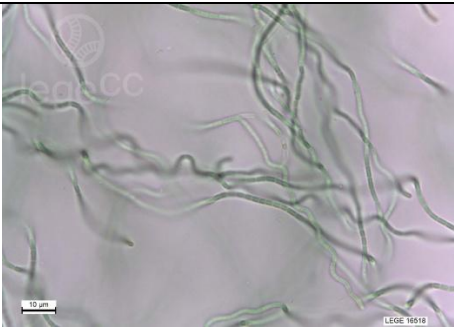
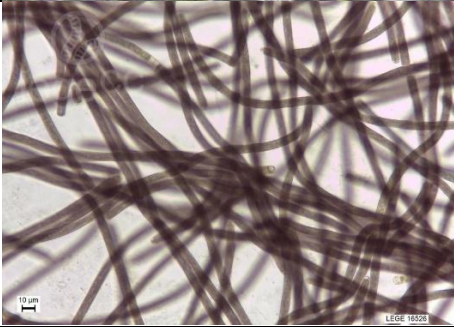
<i>Nodosilinea</i> sp. LEGE 03283	<i>Nodosilinea</i> sp.	Maranhão dam reservoir, campsite at Montargil (Portugal)	Aquatic - Freshwater	
<i>Cyanobium gracile</i> LEGE 09399	<i>Cyanobium gracile</i>	Lagoa da Vela, Mira (Portugal)	Aquatic - Freshwater	
<i>Cyanobacterium stanieri</i> LEGE 03274	<i>Cyanobacterium stanieri</i>	Lagoa dos Salgados, Albufeira (Portugal)	Aquatic - Freshwater	

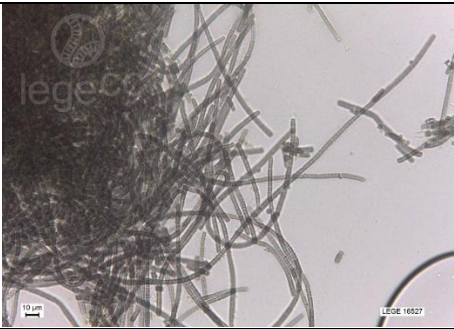
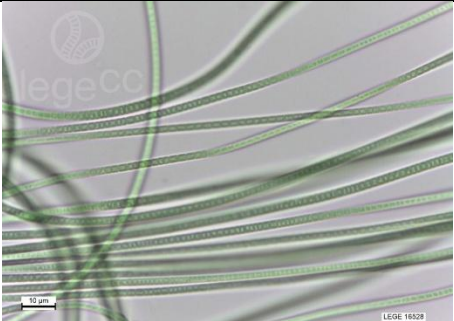
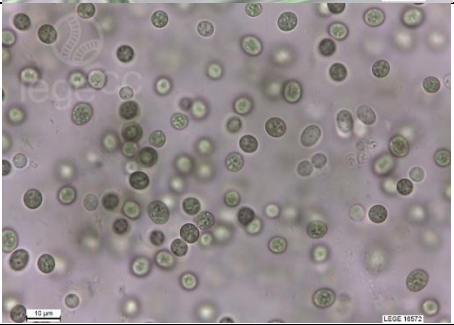
<i>Dolichospermum</i> sp LEGE 00248	<i>Dolichospermum</i> sp.	Maranhão dam reservoir, Benavila (Portugal)	Aquatic - Freshwater	
<i>Alkalinema</i> aff. <i>pantanalense</i> LEGE 15481	<i>Alkalinema</i> aff. <i>pantanalense</i>	Macapá, Amazon river (Brazil)	Aquatic - Freshwater	
<i>Planktothrix</i> sp. LEGE XX280	<i>Planktothrix</i> sp.	Guadiana river (Portugal)	Aquatic - Freshwater	

<i>Tychonema</i> sp. LEGE 06204	<i>Tychonema</i> sp.	Wastewater treatment plant at Febros river, Vila Nova de Gaia (Portugal)	Aquatic - Freshwater	
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WP13				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
<i>Leptolyngbya saxicola</i> LEGE 06131	<i>Leptolyngbya saxicola</i>	Praia de Burgau, Budens (Portugal)	Aquatic – Marine Intertidal zone	

<i>Chroococcopsis</i> sp. LEGE 07168	<i>Chroococcopsis</i> sp.	Praia de Lavadores, Canidelo (Portugal)	Aquatic – Marine Intertidal zone	
<i>Nostoc</i> sp. LEGE 07365	<i>Nostoc</i> sp.	Unknown	Aquatic - Brackish	
LEGE 11394	ND	ND	ND	

<i>Tildeniella</i> sp. LEGE 16518	<i>Tildeniella</i> sp.	Exploratório - Centro Ciência Viva de Coimbra, Coimbra (Portugal)	Aquatic - Freshwater	
<i>Microcoleus</i> sp. LEGE 16525	<i>Microcoleus</i> sp.	Rio Douro (Portugal)	Aquatic - Freshwater	
<i>Tychonema</i> sp. LEGE 16526	<i>Tychonema</i> sp.	Malhada Quente, Serra de Monchique (Portugal)	Aquatic - Freshwater	

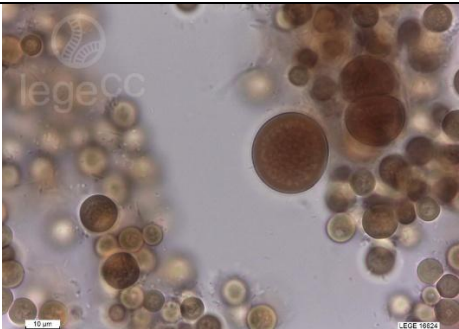
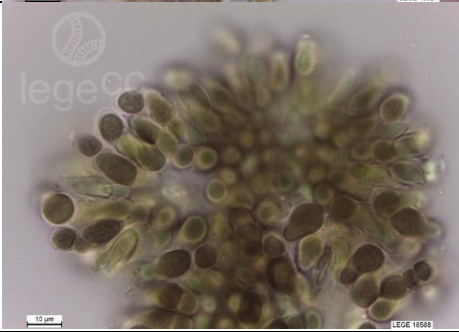
Unidentified <i>Synechococcales</i> LEGE 16527	Unidentified <i>Synechococcales</i>	Malhada Quente, Serra de Monchique (Portugal)	Aquatic - Freshwater	
<i>Pegethrix</i> sp. LEGE 16528	<i>Pegethrix</i> sp.	Arrábida (Portugal)	Aquatic - Freshwater	
Unidentified <i>Chroococcales</i> LEGE 16572	Unidentified <i>Chroococcales</i>	Malhada Quente, Serra de Monchique (Portugal)	Aquatic - Freshwater	

WP15				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph

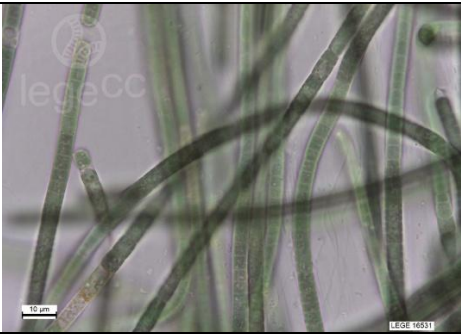
Unidentified filamentous <i>Synechococcales</i> LEGE 06144	Unidentified filamentous <i>Synechococcales</i>	Praia de Burgau, Budens (Portugal)	Aquatic – Marine Intertidal zone	
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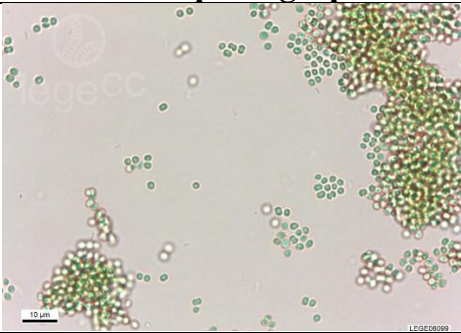
WP16				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
<i>Brasilonema</i> sp. LEGE 16513	<i>Brasilonema</i> sp.	Arrábida (Portugal)	Aquatic - Freshwater	

<i>Pseudanabaena</i> sp. LEGE 17503	<i>Pseudanabaena</i> sp.	Lousã (Portugal)	Aquatic - Freshwater	
Unidentified <i>Nostocales</i> LEGE 14679	Unidentified <i>Nostocales</i>	Av. Mendes Silva, Coimbra (Portugal)	Subaerial - Terrestrial	
Unidentified <i>Nostocales</i> LEGE 17626	Unidentified <i>Nostocales</i>	Rio Mondego, Coimbra (Portugal)	Aquatic - Freshwater	

Unidentified <i>Pleurocapsales</i> LEGE 16624	Unidentified <i>Pleurocapsales</i>	Rio Douro (Portugal)	Aquatic - Freshwater	
Unidentified <i>Synechococcales</i> LEGE 16588	Unidentified <i>Synechococcales</i>	Exploratório - Centro Ciência Viva de Coimbra, Coimbra (Portugal)	Aquatic - Freshwater	

WP17				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph

<i>Kamptonema</i> sp. LEGE 16531	<i>Kamptonema</i> sp.	Relva de Trás, Serra de Monchique (Portugal)	Aquatic - Freshwater	
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WP18				
Strain ID	Identification	Sampling place	Lifestyle	Microphotograph
<i>Synechocystis salina</i> LEGE 06099	<i>Synechocystis salina</i>	Praia de Moledo, Caminha (Portugal)	Aquatic – Marine Tide pool	

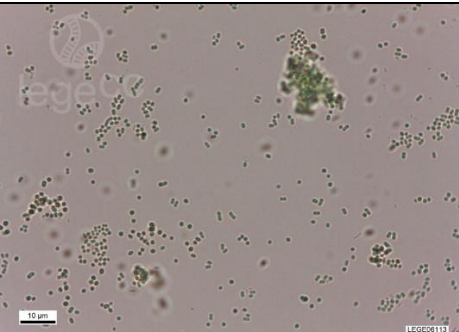
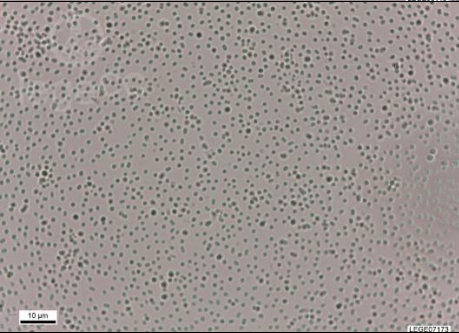
<i>Cyanobium</i> sp. LEGE 06113	<i>Cyanobium</i> sp.	Praia da Aguda, Arcozelo (Portugal)	Aquatic – Marine Intertidal zone	
<i>Cyanobium</i> sp. LEGE 07173	<i>Cyanobium</i> sp.	Praia de Olhos d'Água, Albufeira (Portugal)	Aquatic – Marine Tide pool	

Table III: Table showing the composition of the Z8 medium

SOLUTION A

REAGENT	NAME	g/L
NaNO ₃	Sodium nitrate	46.7
Ca(NO ₃) ₂ ·4H ₂ O	Calcium nitrate tetrahydrate	5.9
MgSO ₄ ·7H ₂ O	Magnesium sulfate heptahydrate	2.5

SOLUTION B

REAGENT	NAME	g/L
K ₂ HPO ₄	Potassium phosphate dibasic	3.1
Na ₂ CO ₃	Sodium carbonate	2.1

FE-EDTA SOLUTION

SOLUTION	mL/L
FeCl ₃ *	10
EDTA-Na **	9.5

*** FeCl₃ SOLUTION**

REAGENT	NAME	100 mL
FeCl ₃ ·6H ₂ O	Ferric chloride	2.8 g
HCl (0.1 N)	Hydrochloric acid	100 mL

**** EDTA-Na SOLUTION**

REAGENT	NAME	100 mL
EDTA	Ethylenediamine tetra acetic acid	3.9 g
NaOH (0.1 N)	Sodium hydroxide solution	100 mL

Micronutrients SOLUTION

REAGENT	NAME	g/L
[1] $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$	Sodium tungstate	0.33
[2] $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 2\text{H}_2\text{O}$	Ammonium heptamolybdate	0.88
[3] KBr	Potassium bromide	1.2
[4] KI	Potassium iodide	0.83
[5] $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	Zinc sulfate	2.87
[6] $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	Cadmium nitrate	1.55
[7] $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	Cobalt (II) nitrate	1.46
[8] $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	Cupric sulfate	1.25
[9] $\text{NiSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$	Ammonium nickel sulfate	1.98
[10] $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$	Chromium (III) nitrate	0.41
[11] V_2O_5	Vanadium (V) oxide	0.089
[12] $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$	Potassium aluminium sulfate	9.48
[13] H_3BO_3	Boric Acid	3.1
[14] $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	Manganese sulfate	2.23

SOLUTIONS	mL/L
1 to 12	10
13 to 14	100

Table III: Table presenting inflammation values >100% for the WP5, WP11, and WP12 plates, arranged in ascending order.

FRACTION	PLATE	INFLAMMATION
LEGE_08334_H	WP12	12,30
LEGE_08334_B	WP12	12,52
LEGE_08334_E	WP12	24,11
LEGE_08334_C	WP12	25,05
LEGE_00033_E	WP12	28,95
LEGE_08334_A	WP12	30,87
LEGE_08334_F	WP12	31,78
LEGE_06015_G	WP5	32,30
LEGE_00033_G	WP12	32,82
LEGE_07227_D	WP12	34,41
LEGE_06114_E	WP5	35,95
LEGE_09399_B	WP12	39,80
LEGE_09399_D	WP12	40,65
LEGE_03283_B	WP12	41,41
LEGE_03283_E	WP12	42,81
LEGE_07227_A	WP12	42,87
LEGE_09399_H	WP12	43,52
LEGE_07227_H	WP12	43,74
LEGE_07227_G	WP12	44,99
LEGE_09399_E	WP12	46,92
LEGE_00033_D	WP12	47,05
LEGE_03274_H	WP12	47,12
LEGE_09399_G	WP12	50,30
LEGE_07167_C	WP5	51,19
LEGE_00033_H	WP12	51,19
LEGE_03283_H	WP12	52,12
LEGE_03283_C	WP12	53,46
LEGE_09399_F	WP12	53,68
LEGE_03283_G	WP12	55,14
LEGE_03283_F	WP12	55,21
LEGE_00033_B	WP12	56,31
LEGE_07227_E	WP12	57,18
LEGE_07227_F	WP12	58,37
LEGE_06009_H	WP5	59,13
LEGE_03283_D	WP12	60,66
LEGE_11391_E	WP5	61,05
LEGE_06009_B	WP5	61,35
LEGE_03274_F	WP12	61,38
LEGE_07183_C	WP5	61,41
LEGE_07157_C	WP11	62,15
LEGE_07227_B	WP12	62,22

LEGE_10371_F	WP11	63,66
LEGE_06114_B	WP5	63,82
LEGE_03274_B	WP12	64,45
LEGE_07167_D	WP5	66,18
LEGE_07167_A	WP5	66,73
LEGE_03274_G	WP12	67,82
LEGE_06114_H	WP5	68,49
LEGE_15481_D	WP12	69,57
LEGE_06114_D	WP5	69,86
LEGE_09399_A	WP12	70,50
LEGE_00033_F	WP12	70,52
LEGE_03274_C	WP12	72,47
LEGE_06015_C	WP5	73,22
LEGE_06015_D	WP5	73,72
LEGE_08334_G	WP12	73,92
LEGE_03283_A	WP12	74,44
LEGE_07167_B	WP5	75,07
LEGE_10371_D	WP11	76,17
LEGE_06025_E	WP11	76,18
LEGE_06002_F	WP11	77,33
LEGE_07157_F	WP11	77,45
LEGE_06015_A	WP5	78,09
LEGE_06114_A	WP5	78,41
LEGE_10371_G	WP11	78,82
LEGE_06114_C	WP5	78,92
LEGE_11391_H	WP5	79,27
LEGE_06025_G	WP11	79,80
LEGE_07167_F	WP5	80,43
LEGE_00035_B	WP11	80,80
LEGE_06204_C	WP12	80,81
LEGE_17966_G	WP11	80,84
LEGE_07157_E	WP11	81,17
LEGE_06015_B	WP5	81,30
LEGE_07172_H	WP5	81,53
LEGE_06015_F	WP5	82,15
LEGE_06006_G	WP11	82,29
LEGE_06025_D	WP11	82,41
LEGE_07183_D	WP5	82,85
LEGE_10371_C	WP11	83,11
LEGE_06204_F	WP12	83,26
LEGE_10371_B	WP11	83,53
LEGE_00030_B	WP11	83,61
LEGE_XX280_F	WP12	83,71
LEGE_07167_H	WP5	83,73
LEGE_06009_A	WP5	83,79
LEGE_XX280_G	WP12	83,84

LEGE_XX280_E	WP12	84,05
LEGE_06002_D	WP11	84,06
LEGE_06025_B	WP11	84,44
LEGE_17966_A	WP11	84,77
LEGE_06025_H	WP11	84,78
LEGE_10371_H	WP11	84,90
LEGE_17965_G	WP11	84,93
LEGE_00248_A	WP12	85,23
LEGE_07187_E	WP5	85,36
LEGE_15481_E	WP12	85,43
LEGE_06204_E	WP12	86,04
LEGE_03274_E	WP12	86,72
LEGE_17965_H	WP11	86,83
LEGE_06204_H	WP12	86,92
LEGE_XX280_H	WP12	87,12
LEGE_06002_G	WP11	87,30
LEGE_07157_D	WP11	87,95
LEGE_07157_H	WP11	88,01
LEGE_06009_C	WP5	88,03
LEGE_17965_F	WP11	88,39
LEGE_00248_E	WP12	88,74
LEGE_06204_B	WP12	89,01
LEGE_00248_B	WP12	89,11
LEGE_00034_G	WP11	89,13
LEGE_07157_B	WP11	89,78
LEGE_06025_F	WP11	89,96
LEGE_06009_G	WP5	90,65
LEGE_06009_D	WP5	90,72
LEGE_09399_C	WP12	91,61
LEGE_06025_C	WP11	91,81
LEGE_11391_A	WP5	91,90
LEGE_07187_H	WP5	92,17
LEGE_00030_E	WP11	92,34
LEGE_06204_A	WP12	92,43
LEGE_XX280_C	WP12	92,64
LEGE_06114_F	WP5	92,70
LEGE_17966_B	WP11	92,85
LEGE_00034_F	WP11	93,18
LEGE_17966_E	WP11	93,29
LEGE_07157_G	WP11	93,75
LEGE_07183_F	WP5	93,83
LEGE_00035_E	WP11	94,30
LEGE_00248_F	WP12	94,56
LEGE_06002_H	WP11	94,58
LEGE_06009_F	WP5	95,28
LEGE_00035_G	WP11	95,29

LEGE_06009_E	WP5	95,29
LEGE_10371_A	WP11	95,44
LEGE_15481_A	WP12	95,83
LEGE_06006_H	WP11	95,85
LEGE_00034_B	WP11	95,88
LEGE_07183_G	WP5	96,00
LEGE_11391_C	WP5	96,81
LEGE_06006_C	WP11	96,99
LEGE_07183_A	WP5	97,18
LEGE_07183_H	WP5	97,22
LEGE_17966_F	WP11	97,27
LEGE_07187_B	WP5	97,48
LEGE_03274_A	WP12	97,49
LEGE_06015_H	WP5	97,87
LEGE_17966_D	WP11	97,97
LEGE_15481_F	WP12	98,40
LEGE_07187_G	WP5	99,08
LEGE_06002_C	WP11	99,17
LEGE_00035_C	WP11	99,32

Table IV: Table presenting the relative values of inflammation and cell viability corresponding to those fractions from the WP5 strains that meet the criteria of inflammation < 100% and cell viability > 100%. The table exclusively includes results obtained under the LPS-induced inflammation treatment (C/LPS). The "Score" column represents the difference between cell viability and inflammation values, providing a quantitative measure of the bioactive relevance of each fraction. The fractions have been ranked according to this value, with those showing the highest scores placed at the top and considered potentially more relevant from a bioactivity perspective.

FRACTION	INFLAMMATION	VIABILITY	SCORE
LEGE_07167_C	51,19	168,42	117,24
LEGE_07167_A	66,73	175,19	108,46
LEGE_07167_B	75,07	177,49	102,42
LEGE_06015_A	78,09	177,15	99,05
LEGE_11391_A	91,90	188,00	96,10
LEGE_06114_E	35,95	128,55	92,59
LEGE_06009_A	83,79	171,11	87,32
LEGE_06009_C	88,03	173,82	85,79
LEGE_06009_B	61,35	145,65	84,29
LEGE_06015_G	32,30	114,18	81,87
LEGE_06114_B	63,82	144,28	80,46
LEGE_06015_B	81,30	161,48	80,18
LEGE_11391_E	61,05	139,89	78,84
LEGE_06009_H	59,13	132,33	73,20
LEGE_07167_D	66,18	137,57	71,39
LEGE_06015_C	73,22	144,05	70,83
LEGE_11391_H	79,27	142,40	63,13
LEGE_06114_A	78,41	140,92	62,51
LEGE_07167_H	83,73	145,55	61,82

LEGE_07167_F	80,43	139,93	59,50
LEGE_11391_C	96,81	155,16	58,35
LEGE_06114_D	69,86	126,06	56,20
LEGE_06114_C	78,92	131,09	52,17
LEGE_06015_D	73,72	125,46	51,75
LEGE_06114_H	68,49	119,28	50,78
LEGE_07183_C	61,41	110,46	49,05
LEGE_06015_F	82,15	128,09	45,94
LEGE_06009_D	90,72	136,30	45,58
LEGE_07183_F	93,83	137,37	43,55
LEGE_06009_E	95,29	132,96	37,67
LEGE_06009_F	95,28	132,04	36,76
LEGE_07183_D	82,85	108,55	25,70
LEGE_07172_H	81,53	105,58	24,06
LEGE_06009_G	90,65	112,80	22,15
LEGE_07187_E	85,36	105,93	20,57
LEGE_06015_H	97,87	111,08	13,21
LEGE_07187_H	92,17	100,63	8,46
LEGE_07183_G	96,00	102,08	6,08

Table V: Table presenting the relative values of inflammation and cell viability corresponding to those fractions from the WP11 strains that meet the criteria of inflammation < 100% and cell viability > 100%. The table exclusively includes results obtained under the LPS-induced inflammation treatment (C/LPS). The "Score" column represents the difference between cell viability and inflammation values, providing a quantitative measure of the bioactive relevance of each fraction. The fractions have been ranked according to this value, with those showing the highest scores placed at the top and considered potentially more relevant from a bioactivity perspective.

FRACTION	INFLAMMATION	VIABILITY	SCORE
LEGE_07157_C	62,15	141,24	79,09
LEGE_06025_E	76,18	139,87	63,70
LEGE_06025_C	91,81	152,45	60,64
LEGE_06002_F	77,33	132,03	54,69
LEGE_10371_F	63,66	117,91	54,24
LEGE_07157_D	87,95	141,54	53,59
LEGE_06002_D	84,06	135,70	51,64
LEGE_10371_D	76,17	127,43	51,26
LEGE_06006_G	82,29	132,89	50,60
LEGE_10371_G	78,82	129,36	50,54
LEGE_07157_F	77,45	126,46	49,01
LEGE_00035_B	80,80	129,00	48,20
LEGE_07157_H	88,01	135,96	47,95
LEGE_06025_H	84,78	131,80	47,02
LEGE_06025_B	84,44	130,27	45,82
LEGE_10371_C	83,11	128,21	45,11
LEGE_00034_G	89,13	133,99	44,87
LEGE_00030_B	83,61	128,47	44,85
LEGE_17966_D	97,97	142,04	44,07

LEGE_07157_G	93,75	136,94	43,19
LEGE_00034_F	93,18	135,56	42,38
LEGE_07157_E	81,17	122,35	41,18
LEGE_00034_B	95,88	136,11	40,23
LEGE_00035_C	99,32	139,03	39,71
LEGE_06025_D	82,41	121,08	38,67
LEGE_00035_G	95,29	133,65	38,36
LEGE_17966_G	80,84	118,19	37,35
LEGE_06025_F	89,96	126,19	36,23
LEGE_00030_E	92,34	128,13	35,80
LEGE_06006_H	95,85	131,17	35,32
LEGE_17966_B	92,85	124,97	32,11
LEGE_17966_E	93,29	125,10	31,81
LEGE_06025_G	79,80	110,96	31,16
LEGE_17965_G	84,93	115,95	31,03
LEGE_06006_C	96,99	127,94	30,94
LEGE_06002_C	99,17	129,66	30,49
LEGE_17965_H	86,83	116,02	29,19
LEGE_00035_E	94,30	122,09	27,78
LEGE_07157_B	89,78	116,25	26,48
LEGE_17965_F	88,39	114,52	26,13
LEGE_17966_F	97,27	122,78	25,51
LEGE_06002_H	94,58	117,56	22,98
LEGE_06002_G	87,30	110,15	22,85
LEGE_10371_A	95,44	114,25	18,81
LEGE_17966_A	84,77	102,91	18,14
LEGE_10371_B	83,53	100,70	17,17

Table VI: Table presenting the relative values of inflammation and cell viability corresponding to those fractions from the WP12 strains that meet the criteria of inflammation < 100% and cell viability > 100%. The table exclusively includes results obtained under the LPS-induced inflammation treatment (C/LPS). The "Score" column represents the difference between cell viability and inflammation values, providing a quantitative measure of the bioactive relevance of each fraction. The fractions have been ranked according to this value, with those showing the highest scores placed at the top and considered potentially more relevant from a bioactivity perspective.

FRACTION	INFLAMMATION	VIABILITY	SCORE
LEGE_08334_A	30,87	215,56	184,68
LEGE_08334_C	25,05	206,26	181,22
LEGE_08334_B	12,52	190,73	178,21
LEGE_00033_G	32,82	206,19	173,37
LEGE_08334_H	12,30	182,23	169,93
LEGE_07227_D	34,41	199,31	164,91
LEGE_03274_C	72,47	230,89	158,42
LEGE_00033_H	51,19	205,63	154,44
LEGE_00033_E	28,95	179,98	151,03
LEGE_00033_D	47,05	196,57	149,53
LEGE_08334_E	24,11	162,85	138,74

LEGE_07227_A	42,87	181,42	138,55
LEGE_09399_A	70,50	207,72	137,21
LEGE_03274_B	64,45	195,22	130,77
hoLEGE_03283_C	53,46	179,11	125,66
LEGE_00033_B	56,31	181,41	125,10
LEGE_07227_E	57,18	178,70	121,52
LEGE_08334_F	31,78	150,87	119,09
LEGE_03274_F	61,38	178,86	117,49
LEGE_07227_G	44,99	162,43	117,44
LEGE_08334_G	73,92	190,50	116,58
LEGE_03283_E	42,81	159,28	116,47
LEGE_07227_H	43,74	157,89	114,14
LEGE_09399_D	40,65	153,86	113,22
LEGE_03283_B	41,41	153,90	112,49
LEGE_00033_F	70,52	182,09	111,58
LEGE_07227_B	62,22	170,64	108,41
LEGE_00248_B	89,11	196,67	107,56
LEGE_03283_A	74,44	177,09	102,65
LEGE_03283_F	55,21	156,89	101,67
LEGE_15481_E	85,43	185,89	100,46
LEGE_07227_F	58,37	157,20	98,83
LEGE_09399_B	39,80	136,04	96,24
LEGE_09399_H	43,52	139,58	96,06
LEGE_03283_H	52,12	146,94	94,83
LEGE_03283_D	60,66	153,91	93,25
LEGE_03274_H	47,12	138,17	91,04
LEGE_09399_F	53,68	141,24	87,56
LEGE_09399_E	46,92	132,46	85,54
LEGE_09399_G	50,30	125,89	75,59
LEGE_00248_F	94,56	167,33	72,78
LEGE_XX280_E	84,05	153,02	68,97
LEGE_00248_A	85,23	150,80	65,57
LEGE_15481_D	69,57	135,00	65,43
LEGE_XX280_F	83,71	149,02	65,31
LEGE_00248_E	88,74	153,42	64,68
LEGE_15481_A	95,83	160,26	64,43
LEGE_03283_G	55,14	119,02	63,88
LEGE_15481_F	98,40	159,65	61,25
LEGE_03274_G	67,82	125,75	57,93
LEGE_03274_A	97,49	154,83	57,35
LEGE_XX280_G	83,84	140,47	56,63
LEGE_03274_E	86,72	141,38	54,65
LEGE_06204_E	86,04	138,84	52,80
LEGE_09399_C	91,61	143,04	51,43
LEGE_XX280_C	92,64	142,31	49,68
LEGE_06204_A	92,43	141,26	48,83

LEGE_06204_C	80,81	128,32	47,50
LEGE_06204_F	83,26	130,71	47,44
LEGE_XX280_H	87,12	133,88	46,76
LEGE_06204_B	89,01	135,46	46,45
LEGE_06204_H	86,92	117,81	30,89

Table VII: Table presenting all evaluated fractions showing cell viability values > 100%. The fractions are ordered in descending order according to their cell viability values.

FRACTION	TREATMENT	PLATE	VIABILITY
LEGE_00033_H	S/LPS	WP12	234,47
LEGE_03283_B	S/LPS	WP12	233,31
LEGE_03274_C	C/LPS	WP12	230,89
LEGE_09399_B	S/LPS	WP12	228,94
LEGE_03283_A	S/LPS	WP12	218,08
LEGE_08334_C	S/LPS	WP12	215,78
LEGE_08334_A	C/LPS	WP12	215,56
LEGE_09399_C	S/LPS	WP12	215,29
LEGE_08334_D	C/LPS	WP12	215,02
LEGE_09399_D	S/LPS	WP12	214,17
LEGE_07227_C	S/LPS	WP12	213,44
LEGE_09399_E	S/LPS	WP12	212,78
LEGE_07227_G	S/LPS	WP12	212,50
LEGE_06204_E	S/LPS	WP12	208,02
LEGE_09399_A	C/LPS	WP12	207,72
LEGE_03274_D	C/LPS	WP12	206,77
LEGE_00033_F	S/LPS	WP12	206,44
LEGE_08334_C	C/LPS	WP12	206,26
LEGE_00033_G	C/LPS	WP12	206,19
LEGE_00033_H	C/LPS	WP12	205,63
LEGE_03283_E	S/LPS	WP12	205,60
LEGE_00033_C	C/LPS	WP12	204,39
LEGE_XX280_E	S/LPS	WP12	201,44
LEGE_06015_C	S/LPS	WP5	201,43
LEGE_00033_C	S/LPS	WP12	201,30
LEGE_09399_H	S/LPS	WP12	201,21
LEGE_03283_F	S/LPS	WP12	200,36
LEGE_08334_B	S/LPS	WP12	200,30
LEGE_06204_C	S/LPS	WP12	200,07
LEGE_03283_H	S/LPS	WP12	199,86
LEGE_07227_D	C/LPS	WP12	199,31
LEGE_XX280_G	S/LPS	WP12	197,21
LEGE_00248_C	C/LPS	WP12	197,08
LEGE_00033_G	S/LPS	WP12	196,91
LEGE_00248_B	C/LPS	WP12	196,67
LEGE_00033_D	C/LPS	WP12	196,57
LEGE_08334_F	S/LPS	WP12	196,06

LEGE_03274_B	C/LPS	WP12	195,22
LEGE_08334_G	S/LPS	WP12	195,08
LEGE_15481_E	S/LPS	WP12	194,19
LEGE_09399_A	S/LPS	WP12	194,02
LEGE_XX280_H	S/LPS	WP12	192,92
LEGE_XX280_F	S/LPS	WP12	192,17
LEGE_03283_C	S/LPS	WP12	191,37
LEGE_08334_B	C/LPS	WP12	190,73
LEGE_08334_G	C/LPS	WP12	190,50
LEGE_09399_G	S/LPS	WP12	189,71
LEGE_06204_H	S/LPS	WP12	189,64
LEGE_06204_F	S/LPS	WP12	188,68
LEGE_11391_A	C/LPS	WP5	188,00
LEGE_07227_H	S/LPS	WP12	187,59
LEGE_00033_A	C/LPS	WP12	187,32
LEGE_09399_F	S/LPS	WP12	186,60
LEGE_00033_B	S/LPS	WP12	186,09
LEGE_15481_E	C/LPS	WP12	185,89
LEGE_07227_C	C/LPS	WP12	185,86
LEGE_15481_F	S/LPS	WP12	185,47
LEGE_08334_A	S/LPS	WP12	185,43
LEGE_06015_D	S/LPS	WP5	184,56
LEGE_06204_B	S/LPS	WP12	183,54
LEGE_06204_G	S/LPS	WP12	183,02
LEGE_15481_G	S/LPS	WP12	182,84
LEGE_08334_H	C/LPS	WP12	182,23
LEGE_00033_F	C/LPS	WP12	182,09
LEGE_07227_A	C/LPS	WP12	181,42
LEGE_00033_B	C/LPS	WP12	181,41
LEGE_00033_E	C/LPS	WP12	179,98
LEGE_06204_A	S/LPS	WP12	179,21
LEGE_03283_C	C/LPS	WP12	179,11
LEGE_03274_F	C/LPS	WP12	178,86
LEGE_07227_E	C/LPS	WP12	178,70
LEGE_07167_B	C/LPS	WP5	177,49
LEGE_08334_H	S/LPS	WP12	177,47
LEGE_06114_D	S/LPS	WP5	177,37
LEGE_06015_A	C/LPS	WP5	177,15
LEGE_03283_A	C/LPS	WP12	177,09
LEGE_06114_A	S/LPS	WP5	175,96
LEGE_07167_A	C/LPS	WP5	175,19
LEGE_00248_G	S/LPS	WP12	174,19
LEGE_08334_E	S/LPS	WP12	173,90
LEGE_06009_C	C/LPS	WP5	173,82
LEGE_XX280_C	S/LPS	WP12	173,66
LEGE_00248_H	C/LPS	WP12	173,41

LEGE_15481_B	C/LPS	WP12	173,19
LEGE_00248_D	C/LPS	WP12	173,11
LEGE_07227_E	S/LPS	WP12	172,51
LEGE_06015_A	S/LPS	WP5	171,43
LEGE_15481_C	S/LPS	WP12	171,42
LEGE_06009_A	C/LPS	WP5	171,11
LEGE_00248_G	C/LPS	WP12	171,10
LEGE_07227_B	C/LPS	WP12	170,64
LEGE_06204_D	S/LPS	WP12	170,14
LEGE_07227_F	S/LPS	WP12	169,15
LEGE_15481_C	C/LPS	WP12	168,86
LEGE_07167_C	C/LPS	WP5	168,42
LEGE_00248_F	C/LPS	WP12	167,33
LEGE_00248_H	S/LPS	WP12	166,76
LEGE_00033_A	S/LPS	WP12	165,39
LEGE_11391_B	C/LPS	WP5	165,09
LEGE_XX280_D	C/LPS	WP12	164,04
LEGE_00033_E	S/LPS	WP12	163,78
LEGE_03274_F	S/LPS	WP12	163,50
LEGE_15481_G	C/LPS	WP12	163,45
LEGE_15481_H	S/LPS	WP12	163,13
LEGE_07227_D	S/LPS	WP12	163,10
LEGE_08334_E	C/LPS	WP12	162,85
LEGE_07227_G	C/LPS	WP12	162,43
LEGE_06114_B	S/LPS	WP5	161,73
LEGE_15481_A	S/LPS	WP12	161,57
LEGE_06015_B	C/LPS	WP5	161,48
LEGE_06015_B	S/LPS	WP5	161,40
LEGE_00034_B	S/LPS	WP11	161,32
LEGE_XX280_B	C/LPS	WP12	160,40
LEGE_15481_A	C/LPS	WP12	160,26
LEGE_06114_E	S/LPS	WP5	160,16
LEGE_06114_C	S/LPS	WP5	159,96
LEGE_15481_F	C/LPS	WP12	159,65
LEGE_03283_E	C/LPS	WP12	159,28
LEGE_03283_G	S/LPS	WP12	158,64
LEGE_XX280_B	S/LPS	WP12	158,62
LEGE_00248_C	S/LPS	WP12	158,58
LEGE_07227_H	C/LPS	WP12	157,89
LEGE_07227_F	C/LPS	WP12	157,20
LEGE_03274_B	S/LPS	WP12	157,05
LEGE_03283_F	C/LPS	WP12	156,89
LEGE_06015_E	S/LPS	WP5	156,53
LEGE_XX280_A	S/LPS	WP12	155,22
LEGE_11391_C	C/LPS	WP5	155,16
LEGE_07227_B	S/LPS	WP12	155,09

LEGE_03274_A	C/LPS	WP12	154,83
LEGE_03274_C	S/LPS	WP12	154,83
LEGE_08334_D	S/LPS	WP12	154,56
LEGE_03283_D	C/LPS	WP12	153,91
LEGE_00248_B	S/LPS	WP12	153,91
LEGE_03283_B	C/LPS	WP12	153,90
LEGE_09399_D	C/LPS	WP12	153,86
LEGE_00248_E	C/LPS	WP12	153,42
LEGE_XX280_E	C/LPS	WP12	153,02
LEGE_XX280_A	C/LPS	WP12	152,81
LEGE_06025_C	C/LPS	WP11	152,45
LEGE_06015_F	S/LPS	WP5	152,38
LEGE_15481_H	C/LPS	WP12	152,37
LEGE_00033_D	S/LPS	WP12	152,37
LEGE_00248_E	S/LPS	WP12	152,33
LEGE_XX280_D	S/LPS	WP12	151,96
LEGE_08334_F	C/LPS	WP12	150,87
LEGE_00248_A	C/LPS	WP12	150,80
LEGE_06114_G	S/LPS	WP5	150,34
LEGE_07187_F	S/LPS	WP5	150,30
LEGE_17965_D	C/LPS	WP11	150,21
LEGE_06114_H	S/LPS	WP5	150,16
LEGE_XX280_F	C/LPS	WP12	149,02
LEGE_00034_C	S/LPS	WP11	148,88
LEGE_06009_E	S/LPS	WP5	148,87
LEGE_06015_G	S/LPS	WP5	148,10
LEGE_00248_F	S/LPS	WP12	147,97
LEGE_07157_B	S/LPS	WP11	147,67
LEGE_17965_D	S/LPS	WP11	147,40
LEGE_07172_B	C/LPS	WP5	147,02
LEGE_03283_H	C/LPS	WP12	146,94
LEGE_06114_F	S/LPS	WP5	146,65
LEGE_07459_D	S/LPS	WP5	146,48
LEGE_17966_C	C/LPS	WP11	146,45
LEGE_07459_C	S/LPS	WP5	146,31
LEGE_06006_D	C/LPS	WP11	146,02
LEGE_07459_B	S/LPS	WP5	145,75
LEGE_06009_B	C/LPS	WP5	145,65
LEGE_07167_H	C/LPS	WP5	145,55
LEGE_03274_D	S/LPS	WP12	144,75
LEGE_06114_B	C/LPS	WP5	144,28
LEGE_06009_H	S/LPS	WP5	144,23
LEGE_11391_H	S/LPS	WP5	144,16
LEGE_06015_C	C/LPS	WP5	144,05
LEGE_00248_D	S/LPS	WP12	143,83
LEGE_00034_D	S/LPS	WP11	143,63

LEGE_07187_B	S/LPS	WP5	143,32
LEGE_06009_C	S/LPS	WP5	143,16
LEGE_09399_C	C/LPS	WP12	143,04
LEGE_07157_C	S/LPS	WP11	142,44
LEGE_11391_H	C/LPS	WP5	142,40
LEGE_XX280_C	C/LPS	WP12	142,31
LEGE_11391_C	S/LPS	WP5	142,21
LEGE_11391_E	S/LPS	WP5	142,18
LEGE_17966_D	C/LPS	WP11	142,04
LEGE_03283_D	S/LPS	WP12	141,88
LEGE_07157_D	C/LPS	WP11	141,54
LEGE_03274_E	C/LPS	WP12	141,38
LEGE_06204_A	C/LPS	WP12	141,26
LEGE_09399_F	C/LPS	WP12	141,24
LEGE_07157_C	C/LPS	WP11	141,24
LEGE_06114_A	C/LPS	WP5	140,92
LEGE_07167_A	S/LPS	WP5	140,87
LEGE_00034_C	C/LPS	WP11	140,80
LEGE_11391_D	C/LPS	WP5	140,62
LEGE_XX280_G	C/LPS	WP12	140,47
LEGE_06006_B	C/LPS	WP11	140,14
LEGE_07167_F	C/LPS	WP5	139,93
LEGE_11391_E	C/LPS	WP5	139,89
LEGE_06025_E	C/LPS	WP11	139,87
LEGE_06006_D	S/LPS	WP11	139,77
LEGE_06009_A	S/LPS	WP5	139,69
LEGE_09399_H	C/LPS	WP12	139,58
LEGE_17965_B	C/LPS	WP11	139,25
LEGE_06009_G	S/LPS	WP5	139,14
LEGE_00035_C	C/LPS	WP11	139,03
LEGE_06002_B	C/LPS	WP11	139,01
LEGE_15481_B	S/LPS	WP12	139,00
LEGE_17965_C	C/LPS	WP11	138,90
LEGE_06204_E	C/LPS	WP12	138,84
LEGE_06009_F	S/LPS	WP5	138,83
LEGE_07187_D	S/LPS	WP5	138,77
LEGE_03274_H	C/LPS	WP12	138,17
LEGE_00035_B	S/LPS	WP11	137,77
LEGE_07167_D	C/LPS	WP5	137,57
LEGE_06015_H	S/LPS	WP5	137,56
LEGE_07183_F	C/LPS	WP5	137,37
LEGE_00035_D	S/LPS	WP11	137,28
LEGE_17966_H	C/LPS	WP11	137,20
LEGE_11391_G	S/LPS	WP5	137,17
LEGE_07187_D	C/LPS	WP5	137,13
LEGE_07167_G	S/LPS	WP5	137,06

LEGE_07157_G	C/LPS	WP11	136,94
LEGE_06025_C	S/LPS	WP11	136,91
LEGE_07167_E	C/LPS	WP5	136,87
LEGE_06025_B	S/LPS	WP11	136,78
LEGE_00030_C	S/LPS	WP11	136,36
LEGE_06009_D	C/LPS	WP5	136,30
LEGE_00034_B	C/LPS	WP11	136,11
LEGE_09399_B	C/LPS	WP12	136,04
LEGE_07157_H	C/LPS	WP11	135,96
LEGE_06015_E	C/LPS	WP5	135,81
LEGE_06002_D	C/LPS	WP11	135,70
LEGE_00034_F	C/LPS	WP11	135,56
LEGE_07187_G	S/LPS	WP5	135,48
LEGE_06204_B	C/LPS	WP12	135,46
LEGE_06009_D	S/LPS	WP5	135,44
LEGE_00034_G	S/LPS	WP11	135,12
LEGE_00030_C	C/LPS	WP11	135,10
LEGE_07092_B	C/LPS	WP5	135,03
LEGE_15481_D	C/LPS	WP12	135,00
LEGE_11391_F	C/LPS	WP5	134,97
LEGE_17965_C	S/LPS	WP11	134,32
LEGE_07172_E	C/LPS	WP5	134,09
LEGE_17966_D	S/LPS	WP11	134,01
LEGE_00034_G	C/LPS	WP11	133,99
LEGE_00035_E	S/LPS	WP11	133,91
LEGE_XX280_H	C/LPS	WP12	133,88
LEGE_00035_G	C/LPS	WP11	133,65
LEGE_11391_F	S/LPS	WP5	133,53
LEGE_06009_E	C/LPS	WP5	132,96
LEGE_06025_D	S/LPS	WP11	132,90
LEGE_06006_G	C/LPS	WP11	132,89
LEGE_00034_A	S/LPS	WP11	132,82
LEGE_07157_A	S/LPS	WP11	132,55
LEGE_09399_E	C/LPS	WP12	132,46
LEGE_00030_D	C/LPS	WP11	132,45
LEGE_07167_G	C/LPS	WP5	132,41
LEGE_06204_D	C/LPS	WP12	132,40
LEGE_06009_H	C/LPS	WP5	132,33
LEGE_06009_F	C/LPS	WP5	132,04
LEGE_06002_F	C/LPS	WP11	132,03
LEGE_06025_H	C/LPS	WP11	131,80
LEGE_00034_H	C/LPS	WP11	131,43
LEGE_00030_B	S/LPS	WP11	131,39
LEGE_06006_H	C/LPS	WP11	131,17
LEGE_06114_C	C/LPS	WP5	131,09
LEGE_00035_D	C/LPS	WP11	130,95

LEGE_00030_H	C/LPS	WP11	130,77
LEGE_06204_F	C/LPS	WP12	130,71
LEGE_07157_D	S/LPS	WP11	130,46
LEGE_06025_B	C/LPS	WP11	130,27
LEGE_06002_C	C/LPS	WP11	129,66
LEGE_10371_G	C/LPS	WP11	129,36
LEGE_00035_C	S/LPS	WP11	129,32
LEGE_00035_B	C/LPS	WP11	129,00
LEGE_07187_C	S/LPS	WP5	128,85
LEGE_06009_B	S/LPS	WP5	128,82
LEGE_06114_E	C/LPS	WP5	128,55
LEGE_00030_B	C/LPS	WP11	128,47
LEGE_15481_D	S/LPS	WP12	128,42
LEGE_06204_C	C/LPS	WP12	128,32
LEGE_00030_G	C/LPS	WP11	128,28
LEGE_10371_C	C/LPS	WP11	128,21
LEGE_00030_E	C/LPS	WP11	128,13
LEGE_06015_F	C/LPS	WP5	128,09
LEGE_07187_H	S/LPS	WP5	128,03
LEGE_06006_F	C/LPS	WP11	128,02
LEGE_06204_G	C/LPS	WP12	127,95
LEGE_06006_C	C/LPS	WP11	127,94
LEGE_10371_D	C/LPS	WP11	127,43
LEGE_07459_H	S/LPS	WP5	127,27
LEGE_06002_D	S/LPS	WP11	127,19
LEGE_00030_D	S/LPS	WP11	127,12
LEGE_03274_E	S/LPS	WP12	126,96
LEGE_00030_F	C/LPS	WP11	126,81
LEGE_11391_G	C/LPS	WP5	126,71
LEGE_07459_G	S/LPS	WP5	126,69
LEGE_07157_F	C/LPS	WP11	126,46
LEGE_07183_E	C/LPS	WP5	126,45
LEGE_00035_G	S/LPS	WP11	126,28
LEGE_17965_H	S/LPS	WP11	126,26
LEGE_06025_F	C/LPS	WP11	126,19
LEGE_07157_G	S/LPS	WP11	126,13
LEGE_06114_D	C/LPS	WP5	126,06
LEGE_09399_G	C/LPS	WP12	125,89
LEGE_00248_A	S/LPS	WP12	125,89
LEGE_07459_E	S/LPS	WP5	125,82
LEGE_03274_G	C/LPS	WP12	125,75
LEGE_06015_D	C/LPS	WP5	125,46
LEGE_17966_E	C/LPS	WP11	125,10
LEGE_07167_E	S/LPS	WP5	125,07
LEGE_06006_H	S/LPS	WP11	124,99
LEGE_17966_B	C/LPS	WP11	124,97

LEGE_06002_C	S/LPS	WP11	124,95
LEGE_17966_C	S/LPS	WP11	124,92
LEGE_06006_A	C/LPS	WP11	124,87
LEGE_07167_F	S/LPS	WP5	124,61
LEGE_07092_F	S/LPS	WP5	123,61
LEGE_07172_C	C/LPS	WP5	123,52
LEGE_10371_E	C/LPS	WP11	123,48
LEGE_06002_A	C/LPS	WP11	123,36
LEGE_11391_D	S/LPS	WP5	123,02
LEGE_17966_F	C/LPS	WP11	122,78
LEGE_06025_G	S/LPS	WP11	122,51
LEGE_06006_G	S/LPS	WP11	122,42
LEGE_07157_E	C/LPS	WP11	122,35
LEGE_00035_E	C/LPS	WP11	122,09
LEGE_07172_D	S/LPS	WP5	121,77
LEGE_07092_E	C/LPS	WP5	121,76
LEGE_06025_A	C/LPS	WP11	121,74
LEGE_07167_H	S/LPS	WP5	121,73
LEGE_10371_C	S/LPS	WP11	121,62
LEGE_07459_A	S/LPS	WP5	121,56
LEGE_06006_E	C/LPS	WP11	121,44
LEGE_07459_B	C/LPS	WP5	121,41
LEGE_06002_E	C/LPS	WP11	121,32
LEGE_06002_B	S/LPS	WP11	121,13
LEGE_00035_H	S/LPS	WP11	121,12
LEGE_06025_D	C/LPS	WP11	121,08
LEGE_07167_C	S/LPS	WP5	120,56
LEGE_03274_H	S/LPS	WP12	120,52
LEGE_00034_D	C/LPS	WP11	120,40
LEGE_06006_C	S/LPS	WP11	120,31
LEGE_00034_E	C/LPS	WP11	119,96
LEGE_07183_B	C/LPS	WP5	119,44
LEGE_07187_E	S/LPS	WP5	119,35
LEGE_06114_H	C/LPS	WP5	119,28
LEGE_00035_F	S/LPS	WP11	119,22
LEGE_10371_D	S/LPS	WP11	119,21
LEGE_17966_G	S/LPS	WP11	119,18
LEGE_17966_H	S/LPS	WP11	119,08
LEGE_03283_G	C/LPS	WP12	119,02
LEGE_00035_H	C/LPS	WP11	118,83
LEGE_00035_F	C/LPS	WP11	118,75
LEGE_00034_E	S/LPS	WP11	118,51
LEGE_00035_A	S/LPS	WP11	118,37
LEGE_17966_G	C/LPS	WP11	118,19
LEGE_07187_C	C/LPS	WP5	117,93
LEGE_10371_F	C/LPS	WP11	117,91

LEGE_06204_H	C/LPS	WP12	117,81
LEGE_07227_A	S/LPS	WP12	117,76
LEGE_06002_H	C/LPS	WP11	117,56
LEGE_07157_H	S/LPS	WP11	117,28
LEGE_07092_B	S/LPS	WP5	117,27
LEGE_07172_F	S/LPS	WP5	117,26
LEGE_06025_E	S/LPS	WP11	117,06
LEGE_07092_C	S/LPS	WP5	116,97
LEGE_00034_A	C/LPS	WP11	116,69
LEGE_07092_D	S/LPS	WP5	116,39
LEGE_07157_B	C/LPS	WP11	116,25
LEGE_17965_H	C/LPS	WP11	116,02
LEGE_06025_A	S/LPS	WP11	116,00
LEGE_17965_G	C/LPS	WP11	115,95
LEGE_06002_H	S/LPS	WP11	115,75
LEGE_07172_B	S/LPS	WP5	115,71
LEGE_17966_A	S/LPS	WP11	115,68
LEGE_07172_G	S/LPS	WP5	115,62
LEGE_07157_F	S/LPS	WP11	115,54
LEGE_07172_D	C/LPS	WP5	115,42
LEGE_03274_G	S/LPS	WP12	115,40
LEGE_06002_G	S/LPS	WP11	115,23
LEGE_07167_B	S/LPS	WP5	115,00
LEGE_17965_F	C/LPS	WP11	114,52
LEGE_00030_E	S/LPS	WP11	114,47
LEGE_17965_B	S/LPS	WP11	114,39
LEGE_07172_E	S/LPS	WP5	114,25
LEGE_10371_A	C/LPS	WP11	114,25
LEGE_07092_F	C/LPS	WP5	114,25
LEGE_06002_E	S/LPS	WP11	114,23
LEGE_06006_B	S/LPS	WP11	114,23
LEGE_06015_G	C/LPS	WP5	114,18
LEGE_17965_E	S/LPS	WP11	113,82
LEGE_00034_H	S/LPS	WP11	113,73
LEGE_00030_G	S/LPS	WP11	113,47
LEGE_07157_E	S/LPS	WP11	113,02
LEGE_06009_G	C/LPS	WP5	112,80
LEGE_00035_A	C/LPS	WP11	112,37
LEGE_00030_A	C/LPS	WP11	112,34
LEGE_07092_C	C/LPS	WP5	112,21
LEGE_10371_E	S/LPS	WP11	112,08
LEGE_07157_A	C/LPS	WP11	111,82
LEGE_06002_A	S/LPS	WP11	111,53
LEGE_07183_B	S/LPS	WP5	111,16
LEGE_07092_E	S/LPS	WP5	111,13
LEGE_07172_C	S/LPS	WP5	111,13

LEGE_07183_E	S/LPS	WP5	111,12
LEGE_06015_H	C/LPS	WP5	111,08
LEGE_00034_F	S/LPS	WP11	110,98
LEGE_06025_G	C/LPS	WP11	110,96
LEGE_07187_A	S/LPS	WP5	110,91
LEGE_06025_F	S/LPS	WP11	110,72
LEGE_07092_G	S/LPS	WP5	110,70
LEGE_06114_G	C/LPS	WP5	110,67
LEGE_07183_C	C/LPS	WP5	110,46
LEGE_06025_H	S/LPS	WP11	110,41
LEGE_00030_H	S/LPS	WP11	110,25
LEGE_06002_G	C/LPS	WP11	110,15
LEGE_07183_G	S/LPS	WP5	110,02
LEGE_07172_G	C/LPS	WP5	109,73
LEGE_07183_C	S/LPS	WP5	109,58
LEGE_06006_F	S/LPS	WP11	109,48
LEGE_07459_F	S/LPS	WP5	109,46
LEGE_07183_F	S/LPS	WP5	109,19
LEGE_00030_A	S/LPS	WP11	109,03
LEGE_17966_B	S/LPS	WP11	108,90
LEGE_07183_H	S/LPS	WP5	108,86
LEGE_10371_G	S/LPS	WP11	108,78
LEGE_07183_D	C/LPS	WP5	108,55
LEGE_00030_F	S/LPS	WP11	108,32
LEGE_10371_B	S/LPS	WP11	108,13
LEGE_07092_G	C/LPS	WP5	108,06
LEGE_17965_G	S/LPS	WP11	108,01
LEGE_10371_A	S/LPS	WP11	107,38
LEGE_07459_G	C/LPS	WP5	106,66
LEGE_17965_F	S/LPS	WP11	106,21
LEGE_07187_E	C/LPS	WP5	105,93
LEGE_06002_F	S/LPS	WP11	105,66
LEGE_07172_H	C/LPS	WP5	105,58
LEGE_17966_E	S/LPS	WP11	104,97
LEGE_07459_C	C/LPS	WP5	104,13
LEGE_06006_A	S/LPS	WP11	103,95
LEGE_17965_A	C/LPS	WP11	103,44
LEGE_17965_A	S/LPS	WP11	103,26
LEGE_17966_F	S/LPS	WP11	103,26
LEGE_06006_E	S/LPS	WP11	103,12
LEGE_17966_A	C/LPS	WP11	102,91
LEGE_07183_G	C/LPS	WP5	102,08
LEGE_07092_A	S/LPS	WP5	101,41
LEGE_10371_B	C/LPS	WP11	100,70
LEGE_07187_H	C/LPS	WP5	100,63
LEGE_07172_A	S/LPS	WP5	100,23