Degradation of anthraquinone dyes from effluents: a review focusing on enzymatic dye degradation with industrial potential

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Abstract

Up to 84,000 tons of dyes can be lost in water and 90 million tons of water are attributed annually to dye production and their application, mainly in the textile and leather industry, making dyestuff industry responsible for up to 20% of the industrial water pollution. The majority of dyes industrially used today are aromatic compounds with complex, reinforced structures, with anthraquinone dyes being the 2nd largest produced in terms of volume. Despite the progress on decolourisation and degradation of azo dyes, very little attention has been given to anthraquinone dyes. Anthraquinone dyes pose a serious environmental problem as their reinforced structure makes them difficult to be degraded naturally. Existing methods of decolorisation might be effective but are neither efficient nor practical due to extended time, space and cost requirements. Attention should be given to the emerging routes for dye decolorisation via the enzymatic action of oxidoreductases, which have already a strong presence in various other bioremediation applications. This review will discusses the presence of anthraquinone dyes in the effluents and ways for their remediation from dyehouse effluents, focusing on enzymatic processes.

1. INTRODUCTION – THE PROBLEM

A very important factor of our life is water, as it is not only vital for our physical existence but it is also necessary for numerous activities in domestic and industrial fields, varying from cleaning and agriculture to cooking and product formation[1]. Unsustainable exploitation and uncontrollable contamination are currently the "hot issues" regarding water management. The limited water resources need to be adequately distributed and carefully used to fulfil the constantly rising agricultural and industrial demand due to population growth[1]. The main strategies to address water scarcity are prevention, demand management and revalorization of water[2]. Following that perspective, industrial wastewater should be recycled and reused.

The dye sector and the sectors relevant to dye applications (textile, tannery, paper) are recognized among the most polluting industries, based on both the volume and the composition of effluents [3, 4]. Effluents released in the water bodies create aesthetic and environmental issues [5, 6] with a high societal unacceptance. Furthermore, they can cause pipe corrosion, blockages and bioaccumulation [7], and result in the production of hazardous sludge [7]. The presence of dyes in effluents makes their reuse difficult, as the presence of colour – and other substances – affects consecutive dyeing cycles[7].

Awareness of environmental protection has increased and minimization of water usage and wastewater production is required, in addition to the limitation on the amount of pollutants released to the environment. There are legislations regulating and monitoring the dyeing industry in Europe and the United States [8, 9], however these are not clearly defined and not comparable across countries in regards to the colour intensity of the discharged effluents[9]. These issues make the monitoring of coloured effluents released in the environment quite a challenge. The problem of the dye contaminated water is especially evident in Asia, which contributes to about 50 % of textile exports and more than 50 % of world's consumption of

53 dyes. However, many of the countries involved lack sufficient legislation about environmental
54 protection relevant to textile industries[10]. Having said that, there have been efforts for colour
55 restrictions to be included in legislation [7].

56 Although currently the relevant legislation might be vague and not properly applied [11-16], it is
57 clear that not only the volume of discharged effluents needs to be minimized, but the quality
58 of industrial effluents discharged in the environment needs to be fully monitored as well.

59 The dyeing and textile industry is responsible for dye discharge in the effluents, as well as for
60 a plethora of other hazardous and potentially hazardous substances. Such substances, mostly
61 surfactants and persistent organics, are used to accentuate dye stability/fastness or colour
62 intensity, to assist the process of dyeing and to give specific characteristics to textiles among
63 others [3, 17].

64 It is difficult to quantify the amount of dyes lost during production or during application on
65 textiles, as the available figures from the literature are based on estimations, or are
66 representative of very specific types of dyes or applications. Nevertheless, it is important to
67 discuss those data to understand the importance of the problem. Dye production may vary
68 between 10,000[8] and 770,000[18] tons per year and the losses are estimated around 2 % during
69 production and around 10 % during application[7], with wastewaters being discarded directly
70 into the environment in developing countries [19]. Based on the data from 2013, the annual
71 production of textiles was around 30 million tons, increasing every year [18]. Each ton of textile
72 requires around 30 tons of water for the dyeing process[10], while each ton of dye production
73 requires an average of 200 tons of water [5, 20]. That means a total of 80 million tons and 90
74 million tons of water respectively is attributed to dye production and textile dyeing process of
75 per year. Taking into account the amount of contaminated water (2 % and 10 % respectively
76 during their production and application), a staggering sum of about 11 million tons of water is

polluted per year, making the dyestuff industry responsible for about 20 % of the total industrial water pollution [21] . It is thus evident that water pollution from dyes is an existing and growing problem that demands attention.

The majority of dyes industrially used today are aromatic compounds with complex, reinforced structures, leading to difficult degradation [18]. Of the industrially important dye categories (Figure 1), the most common "azo" dyes are making up of almost 60 % of the synthetic dyes used industrially, followed by "anthraquinones" (15 %), and indigoids in respect of the chromophore group present[22].

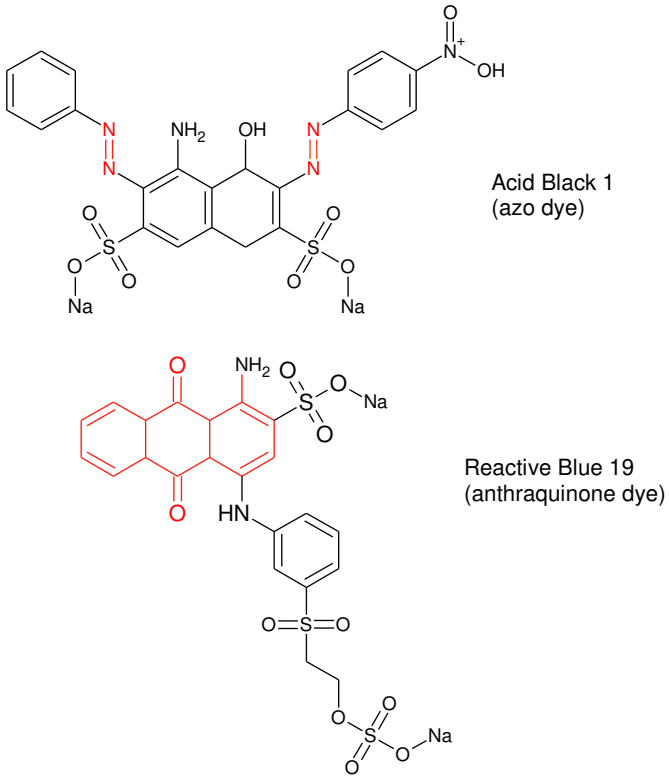

Figure 1: Representation of the two most important chromophore groups, with examples shown being Acid Black 1 (azo dye) and Reactive Blue 4 (anthraquinone dye).

While for azo dyes, relevant data is easily available, it is difficult to find current or accurate data for the annual production of AQ dyes. Nevertheless, data found from previous years can be used to roughly estimate a production volume. For the US, within a period of about 15 years (1986-2002), the annual production of anthraquinone (a precursor for dyes and other

chemicals) had a staggering 5,000 % increase (from 500 to 25,000 tons)[23]. Given the increase in production volume of dyes, it is safe to assume that the production of AQ dyes increased as well; a rough estimation of about 100,000 tons of AQ dyes per year can be made.

The specific chemistry of the anthraquinone group is based on the anthracene and consists of three fused benzene rings (basic anthracene structure) with two carbonyl groups on the central ring (highlighted in Figure 1). This structure is naturally colourless, but substitution of the aromatic rings gives colour and controls its intensity[24]. Colour gets deeper with increased basicity of the substituents, for an aniline-based substituent (NHC_6H_5) is used, the maximum absorption length rises to from 327nm (case of H) to 508nm[24].

The difference from azo dyes is that in the anthraquinone structure, the carbonyl group acts as an electron acceptor, thus requiring an electron donor to react and break their structure [5]. This combined with resonance effects among the anthracene structure leads to higher difficulty in AQ dyes degradation compared to azo dyes [19, 25] and makes the choice of an appropriate degradation/decolorisation method challenging [26]. The majority of the industrially important AQ dyes are derived from anthraquinonesulfonic acids, using sulfonation or nitation [27], and research has shown that presence of sulfone groups in dye structure can reduce their degradability [28]. Due to their highly stable structure, AQ dyes are known for their great fastness, stability and brightness[24].

2. AVAILABLE METHODS FOR DECOLORISATION

2.1. Industrially available methods

The most known and extensively applied methods in the industry are adsorption, coagulation, membrane filtration, as well as various oxidative processes [10, 29]. Regarding biological methods, aerobic and anaerobic processes are currently widely applied for general water treatment, offering distinct advantages compared to physicochemical methods (e.g. products of added

value, environmentally friendlier), but also facing challenges regarding their efficiency (e.g. sensitivity, long contact time) [29]. There are many examples of papers reviewing the current and future industrial methods for general dye decolorisation [3, 8, 30-34]. Every method has advantages and disadvantages related to the following criteria: efficiency under various conditions, practicality, requirements of pre- and post-treatment and environmental impact; ultimately relating to the cost. Given this complexity, a single method can rarely satisfy these demands simultaneously [5], hence, typically, a combination of available and under-development methods is preferred, maximizing their strengths and compromising their disadvantages [17, 29].

Despite the significant amount of research about the decolorisation and degradation methods applied for azo dyes [6, 35-39], not much research is available on AQ dyes, with only two reviews available, both discussing AQ dye decolorisation mainly by biological methods [40, 41] and comparably fewer research papers compared to those available on azo dyes[a]. What is worth mentioning, is that there is a review paper focusing on the degradation of a specific AQ dye, reactive Blue 19, covering various methods and research examples dated up to 2011 [42].

2.2. Physical, chemical and biological methods applied for AQ dye removal

The most common physical methods for treatment of dye house effluents are adsorption, and filtration (using membranes and reverse osmosis). As there are numerous research studies on dye removal assisted by adsorption, we have summarised the best performing literature findings on AQ dye removal and degradation in Table 1, with associated comments, while below we discuss selected examples. Best performance was arbitrarily evaluated based on the amount of dye removed per litre, per hour, assuming continuous use of the system described at the optimal state identified by the researchers. This arbitrary metric allows for a comparison

[a] *about 500 papers on anthraquinone dye degradation compared to about 8,700 for azo dyes, according to Web of Knowledge search engine over the period of 1975 to 2019.*

between results found in literature, as given the lack of a consistent approached followed, superficial comparison of results does not produce valuable conclusions.

2.2.1.Physical methods

Amongst many adsorbents explored such as activated carbon, peat, silica-based adsorbents, zeolites or other naturally derived substances, activated carbon is widely studied for dye adsorption. It is also the dominant adsorbent in industry, based on its great adsorption ability, high surface area, stability and homogeneity [43], which compromise the high cost of production and regeneration and the possibility of decreased efficiency due to material loss during regeneration [8, 44, 45]. A few examples using activated carbon for AQ dye adsorption showed that uptake was higher for acidic solutions [46-48], and that pore structure of the materials could facilitate [46, 48] or hinder [47] adsorption. Another frequently discussed option in the area of adsorption are the abundant in nature zeolites, with substantially lower adsorption capacity and again facing high regeneration costs [49, 50]. Silicon based materials have been studied extensively for pollutants adsorption as well [51, 52]. Their interesting properties such as ability for a wide range of pore size and surface areas, durability, ease of functionalisation and relatively cheaper regeneration compared to activated carbon, have made them excellent candidates for water treatment with many examples on dye adsorption [53-56]. However, issues such as manufacturing and regeneration cost as well as diffusional limitations arising from high throughput in industrial scale applications, have prevented them from being widely applied in water treatment yet, although research is showing positive signs on their industrial implementation [57]. Newer trends in adsorption, with application for AQ dyes, include the use of agricultural waste [58, 59]. As per filtration, the usually encountered textile effluent treatments include nanofiltration (pore diameter up to 10 nm) and reverse osmosis [60], but there was no example of their application on AQ dyes found in literature.

Major issues about the application of physical methods for dye removal are the relatively high required contact time, hence large spaces required, as well as the need for adsorbent (or membrane) regeneration, issues that are not usually addressed in literature, but are of great importance for industrial implementation.

2.2.2.Chemical methods

2.2.2.1. Coagulation – Flocculation

The most common chemical treatment methods applied to textile effluents are chemical coagulation and oxidation processes, while electrochemical methods are gaining attention as well (Table 1). Chemical coagulation or flocculation is being replaced by newer methods or used in combination with other methods, in order to reduce the effect of some major drawbacks such as potentially toxic sludge production and need for further treatment of the effluent [61]. The principle of coagulation and flocculation methods is the opposite charge between the soluble pollutant (e.g. dye) and the usually aluminum, iron, or most recently polymeric coagulant, that makes the pollutant become insoluble [61]. The factors of importance during coagulation are the type and dose of coagulant needed and the size and "sturdiness" of the floccs (coagulated pollutants), which dominates their ease of removal [62]. Table 1 summarises some distinct examples of AQ dye treatment using chemical methods, while we elaborate on relevant research below. When degradation of Reactive Blue 19 and 49, individually and in a mixture, was attempted using active chlorine, it was shown that degradation was much faster for individual dyes [63]. Contradicting these findings, decolorisation of Disperse Blue 3 via coagulation with magnesium chloride or ferrous sulphate, as individual dye or in mixture with azo dyes, showed that there is a synergistic effect. Dye removal increased from 68 % (individual dye) to up to 90 % (mixture with azo dyes) in presence of ferrous sulphate, whereas for magnesium chloride the decolorisation percentage was maintained very high, at 93 %, regardless the presence of other dyes [64].

2.2.2.2. Advanced Oxidation Processes (AOP)

The available oxidative methods include Fenton's process with or without external energy supply, or ozonation, and they operate via the production of active OH^- radicals that non-selectively oxidise dyes [65]. Their application in water treatment has been illustrated recently [28, 34, 65, 66] gaining much attention over the last few years. Selected stellar examples of AOP applied for AQ dye degradation are shown in Table 1 and some of them are discussed below. Studies on Reactive Blue 19 conducted by different groups, using the same starting concentration (100 mg/mL) but different combination of AOP, showed highly different results. It was shown that using a combination of methods such as Fenton reaction coupled with adsorption on pyrite ash [67], or ozonation coupled with UV radiation[68] can be much more efficient compared to ozonation only [69], based on the . Using the same dye on a much higher starting concentration (about 2,000 mg/L) and examining its decolorisation by Fenton's reaction, photocatalysis and UV radiation, as single methods or combined, Radovic resulted in generally very high dye removal (above 90 % for a combination of Fenton reagent coupled with photocatalysis) [70]. This shows that combination of AOP methods does work synergistically, and usually better than single methods. What is worth highlighting about AOP when applied in AQ dye degradation, is the very short reaction times required, usually few minutes, their very good efficiency and mineralisation of dye, but also their high cost, which poses difficulties on their consideration for scale-up [71, 72].

2.2.2.3. Combination of methods

Emerging combinations of the once very popular chemical coagulation with newer dye removal methods are implemented, in order to reduce the effect of some major drawbacks such as sludge production and need for further treatment of the effluent [61]. Electrochemical coagulation producing in-situ coagulants based on aluminium or iron, showed great dye removal potential (Reactive Blue 19 was used as a representative AQ dye, but other dyes were studied as well) and associated time [73]. That work also presented an economic evaluation of some

decolorisation processes, which suggested that electrochemical and oxidative processes are advantageous to adsorption, however, biological/enzymatic methods were not included. Furthermore, what was only acknowledged but not commented further is sludge production and the need to deal with it, but, it was shown qualitatively that use of different conditions can have an effect on the amount and type of produced sludge. In the area of coupling photocatalysis with nanoparticles, a study optimised the degradation of Acid Green 25 using immobilised TiO_2 nanoparticles coupled with UV light photocatalysis, resulting at an optimised system operating at a relatively low dye concentrations (18 mg/mL) [74]. The same group examined the importance of the chemical structure of various dyes in degradation through the same method [28]. Their critical review analysis showed that degradation of AQ dyes was more difficult compared to azo dyes –without further elaboration on the differences between the dye structures– and also that presence of sulfone groups reduces the efficiency of dye degradation. The application of other nanomaterials for AQ dye degradation from effluents is gaining more and more attention, although not yet thoroughly developed, but their potential advantages lead to an increased interest for their industrial application [75]. A study coupling use of zinc and titanium oxides with photocatalysis by irradiation, showed that nanoparticles can be quite effective in degradation of a model AQ dye, but their efficiency depends on the type [25]. A more recent study examined the degradation of Reactive Blue 4 based on the coupled use of copper nanoparticles and showed that initially the dye gets adsorbed onto the nanoparticles and then is oxidised based on the production of hydroxyl radicals from added mediators and the action of monovalent copper [76].

2.2.3.Biological methods

The available biological methods can be divided on whether they are performed inside a cell (of bacteria, fungi, yeasts, algae), or using isolated enzymes. The challenge is to create methods

241 based on bioremediation that can bypass the disadvantages of conventional methods, yet be
242 efficient, cost-effective and environmentally benign [77, 78].

243 Degradation of AQ dyes using aerobic and anaerobic cultures has been reviewed recently [40, 41],
244 so in Table S1 we report some newer (or unreported) examples on biological degradation of
245 AQ dyes. Some of the clear differences between research examples on biological and non-
246 biological methods include the usually low starting concentration of dyes examined and the
247 longer time needed for decolorisation in biological methods. Although usually individual
248 cultures are examined in research papers, a recent study [79] showed that when the
249 microorganisms were acting in a consortium, the decolorisation of Reactive Blue 4 and 19 was
250 dramatically improved. Another study [80] examined *Escherichia coli* cultures for degradation of
251 AQ dyes at higher concentration and found that dye decolorisation occurred primarily due to
252 microbial induced precipitation, followed by adsorption on cells and cell metabolism. A very
253 interesting observation was that the dye structure affected the decolorisation mechanisms and
254 the kinetics, indicating that this method might not be applicable in real effluents where a
255 mixture of dyes is present. When the degradation of a mixture of dyes was examined, (including
256 Acid Blue 350 of AQ structure) using a specific strain of *Trametes Versicolor*, over 90 % %
257 degradation could be achieved for after 48 h of treatment, that being slightly lower to the almost
258 complete decolorisation achieved for the individual dyes [81].

259 Upon comparison of the –arbitrary- throughput value calculated for the examples shown for
260 physical/chemical and biological methods, it is evident that biological methods cannot compare
261 in terms of efficiency with the chemical and physical methods examined. This leads to the
262 conclusion that biological methods might not be as effective as physical and chemical methods
263 However, biological methods are generally recognised as more benign, environmentally
264 friendly and economically viable, with the ability for in-situ degradation of pollutants,

compared to physical removal of the dye or transformation to other substances requiring further treatment [82, 83].

2.3. Challenges with current methods

Based on the data discussed and presented in Table 1, it is clear that AQ dye removal, is highly specific to the dye and the method used. It has been shown that different degrees of decolorisation are observed for a single dye when using different treatment methods [84, 85]. It can be quite difficult to choose an appropriate method among the available conventional methods to decolorise AQ dyes, due to their fused ring structure that enhances their stability [86]. Although anthraquinone on its own is not toxic [23], research has shown that some AQ dyes as well as their degradation intermediates (often unidentified), can be potentially toxic, few of them are mutagenic and/or potentially carcinogenic [26, 87-90]. Also the lack of data on the intermediate degradation products [91] makes it difficult to speculate any residual toxicity, as well as to find appropriate degradation pathways [26]. There are research examples where a degradation pathway for a specific AQ dye has been proposed, based on collected data and in few cases the pathway has been fully determined, usually using analytical techniques to identify the by-products. This indicates that methods which can result in complete destruction of the dye structure rather than isolation or coupling with other chemicals, are in need. In these examples, anthraquinone dyes were degraded using ozonation [68, 69, 92, 93], electrochemical methods and their combinations [94] or via enzymatic degradation [95-97]. A discussion on the degradation pathways on a model AQ dye by various techniques is available in Section 4.

We note that most studies examined a lower dye concentration spectrum, sometimes coupled with quite a low concentration of adsorbent. These scenarios may be ideal for scanning a method or optimizing the process conditions, but they do not give information on realistic conditions of industrial applications and may cause barriers during commercialisation.

When using physical and chemical methods for dye removal, there is sludge generation, which can be difficult to handle, as well as the materials used cannot be regenerated easily, if at all. In addition some of these methods are not very efficient due to the large cost, time and space requirements [10].

Existing literature on decolorisation of AQ dyes from water effluents, acknowledges the problem and explores potential solutions, however, the lack of consistency on the way the issue is approached highlights the need for an evaluation of proposed solutions on a consistent basis, on the merit of the potential of industrial implementation and socially acceptable practices.

299 Table 1: Exceptional performance of decolorisation of anthraquinone dyes by various physical and chemical methods. Please refer to Table S1 for biological methods.

Decolorisation method	**Dye name/ initial dye concentration (mg/L)**	**% max decolorisation * /timescale (h)**	**Throughput (mg/L/h)****	**Comments**	**Ref.**
Adsorption (activated carbon from *T. dealbata*)	Reactive Brilliant Blue X-BR / 150	About 81 % / 0.5	**121.5**	Pseudo second order kinetic model used, higher capacity than commercial carbon products)	46
Adsorption (on silica)	Reactive Blue 19 / 800	99 % / 4	**198**	Amount of silane affects decolorisation (the more the better) and elution of dye	55
Adsorption (on clay)	Acid Blue 25 /100	About 100 % / 1	**100**	Full material characterization and adsorption kinetics analysis, chemisorption dominates, lower pH facilitates adsorption	98
AOP (wet air, wet peroxide, photocatalytic, Fenton)	Reactive Blue 4 / 100	100 %, 100 %, 99 % / 1 and 100 % / 0.75	**100**	Very high degrees (>75 %) of mineralization, wet peroxide oxidation worked best, examination of degradation pathway	93
AOP (Photodegradation) + nanoparticles	Reactive Blue 19 / 30-70	Over 95 % / 0.5	**126**	Degradation based on ZnO and TiO2 nanoparticles assisted by photocatalysis, multifactorial design analysis and optimization, ZnO performs better (both cost of energy + dye degradation effect), TOC analysis shows low residual toxicity, activity of ZnO regulated only by pH	25
AOP (Photodegradation + TiO_2)	Reactive Blue 19 / 800	About 75 % / 3	**200**	Examination of various factors affecting dye degradation such as dye, catalyst and peroxide concentration, identification of intermediate products through UPLC-MS, fragments show reduced cytotoxicity	99
AOP (ozonation)	Reactive Blue 19 / 200	Almost 100 % / 0.3	**200**	Ozone feed rate and presence of electrolytes affect decolorisation, identification of oxidation products through IC	100
AOP (Fenton reaction) coupled with Pyrite Ash	Reactive Blue 4 / 100	100 % /0.5	**200**	Coupled process enhances degradation due to enhancement of Fenton process reaction, high concentration of dye is prohibitive, metal removal is necessary	67
Ozonation and UV radiation	Reactive Blue 19 / 111	100 % / 0.1	**1665**	Ozonation is better for decolorisation, combination with UV radiation is better for mineralization, proposed degradation pathway, toxicity studies	68
AOP (Fenton/ photo-Fenton reaction), UV radiation	Reactive Blue 19/ 2500	81 %-98 %-42 %/ 0.3	**1050-2450**	Decolorisation examined in pure dye (higher) and simulated effluent (lower), AOPs are more effective than UV radiation, dye structure affects efficiency of each process, optimization study for each process	70
Electrochemical coagulation	Reactive Blue 19/ 2000	95 % /0.1	**19000**	Use of Fe (better action) and Al (can have reversed effect depending on pH) as coagulants, higher voltage increases removal percentage, overall quite fast method, no mention of proposed sludge treatment	73

300 * values shown are for the optimised methods as presented by researchers and refer to removal of colour unless stated otherwise.

301 ** arbitrary value calculated to show the maximum removal capacity of any given method within an hour, based on the best results presented in each reference. In cases where
302 the timescale of the decolorisation is within a few minutes, the assumption of decolorisation ability over continuous use for 1 h is made.

3. FOCUS ON ENZYMATIC DECOLORISATION

Isolated enzymes are very effective as they are highly specific catalysts that produce by-products of lower toxicity and volume. The overall process is considered environmentally friendly and less intrusive. The enzymes responsible for dye degradation belong mainly to the family of oxidoreductases, including peroxidases, reductases and laccases [17, 101]. These enzymes have the ability to act on dyes by either creating precipitants that can be easily removed or chemically transforming the dyes into compounds easily dealt with [102].

The use of both isolated enzymes and the whole cell/micro-organism has considerable advantages and disadvantages. Use of isolated enzymes does not depend on culture/microorganism's "well-being" or growth rate [101, 103, 104]. Also, diffusional limitations of substrate and/or product in-between the cell compartments can be avoided as well as any other actions besides enzymatic [105-107]. Isolated enzymes are relatively easier to use under harsher conditions, offer higher specificity and easier regulation of catalytic activity, as well as easier handling/storage compared to whole cells [8, 77, 108]. It is also easier to implement the use of isolated enzymes in an industrial context since their development as biocatalysts can be faster than whole cells. Further, recombinant enzymes and/or their immobilisation is possible to improve the performance [108-111]. On the other hand, some enzymes may require co-factors or mediators [17] and may be too specific/selective to degrade multiple dyes simultaneously [108, 109].

3.1. Oxidoreductases – Peroxidases

The enzymes responsible for dye decolorisation belong to the family of oxidoreductases (EC: 1), which catalyse oxidation and reduction reactions, finding application in various domains varying from diagnostics to wastewater treatment and production of chemicals or potentially biofuels [112-118]. They have been studied extensively for dye decolorisation and bioremediation,

with much research focusing on the oxidative action of laccases and peroxidases as well as the reductive action of azoreductases (azo dye specific enzymes), with many review papers available targeting dye degradation in general [104, 110, 119-121] or focusing on azo dyes [6, 38, 39], but none focusing specifically on anthraquinone dyes.

Peroxidases catalyse the reduction of peroxides simultaneously with the oxidation of various organic and inorganic substrates. This "dual action" mechanism has been named ping-pong bi bi mechanism due to the fact that the electrons liberated by the enzyme from the reduction of peroxides are recovered through the oxidation of the main substrate, with the aid of the intermediate enzymatic compounds [122].

Recently peroxidases from white-rot fungi (WRF) have attracted interest in the general area of bioremediation [84, 104], as actions such as lignin degradation and dye degradation are dominated by similar mechanisms, around structurally similar substrates [123, 124]. The advantage of non-specific binding of WRF peroxidases allows them to act on a wide range of substrates [125, 126]. Enzymes secreted from WRF include various known peroxidases, like manganese, lignin and versatile peroxidase and a less known category of enzymes, dye decolorising peroxidases (DyPs). DyPs were first reported almost 20 years ago, showing a great activity over the decolorisation of anthraquinone dyes [97, 127], followed by lignin-like compounds [128]. Although their action mechanism resembles that of other peroxidases, anthraquinone dye degradation is not yet fully mapped [129-134]. The characterization of DyPs can be found in recent reviews [135-138]. Anthraquinone dyes used as a model system to examine decolorisation using DyPs include Reactive Blue 19 (RB19 or RBBR) [129, 139-143] and Reactive Blue 5 (RB5) [97, 130, 144-151].

3.2. Oxidoreductases in enzyme-based bioremediation

There are quite a few examples of isolated oxidoreductases applied in dye degradation and decolorisation, some of them focusing on anthraquinone dyes (Table 2). Focusing on DyPs,

there are many research examples qualitatively examining their activity on anthraquinone dyes and a few researchers have tried to consider an industrial implementation. For example, recombinant DyP was used to treat Reactive Blue 19 in a single batch system and also in a step fed batch reactor [107] to assess a cyclic operation, leading to a very high decolorisation performance, regardless the soluble nature of the enzyme. However, a major concern for industrial application is the continuous ingress of effluents that can make batch treatment tricky. Another study tested free horseradish peroxidase on a single anthraquinone dye and a real effluent. The results showed that although single dye degradation was very fast and effective (90 % within 2 mins), only 52 % decolorisation was achieved for the real effluent (undefined period of time) [152]. Research conducted using again horseradish peroxidase for the decolorisation of 2 anthraquinone dyes, showed that the structure of the dye affected its decolorisation, despite the similar optimised operational conditions and the high decolorisation degrees achieved [122]. When laccase was examined for dye decolorising potential on several types of dyes, it was shown that there was better affinity towards the anthraquinone Reactive Blue 19, resulting in almost 90% decolorisation over 30 mins[153]. The preference of laccase towards anthraquinone dyes compared to other types was also confirmed by a different study, where complete decolorisation of the same dye concentration was achieved in the much higher time point of 72 h [154]. However, whereas in the aforementioned cases laccase did not need a mediator, this has not been always the case. Soares [103]used laccase for the decolorisation of Reactive Blue 19 and reported that in absence of mediator almost no decolorisation was observed, but upon use of mediators decolorisation was able to reach 100 % success. The different results obtained by the same combination of enzyme and dye allows us to understand the complexity of decolorisation and the difficulty to generalise results and expectations. When degradation of a mixture of 3 azo and 1 anthraquinone dye was examined using isolated enzyme extracts from *Funalia Frogii*, it was shown that degradation of dyes in the mixture followed a

pattern based on the ease of structure breakdown. Once the Reactive Blue 69 anthraquinone dye (easiest to degrade) was almost fully degraded, degradation of the other dyes would occur, leading to a time dependant decolorisation and resulting to 84 % colour removal after 48 h [155].

Although enzyme use in dye degradation specifically can be quite effective under laboratory conditions, their application to an industrial scale has many limitations, mainly due to the production cost (culture, isolation, equipment) and operational cost (use/reuse, downstream processing) of the enzymes [29, 112, 156-158]. Also, some of the by-products can inhibit the enzymatic action [146, 159, 160], thus limiting potential reusability of the enzymes. Furthermore, as the pH for enzymatic action is important, difficulties can occur when treating real effluents as it was shown in literature [152, 161, 162].

There are mainly three ways to address the aforementioned limitations from isolated enzyme use, which can be applied alone or together. The "invasive" way is altering the properties of the enzyme via genetic engineering. The "excluding" way is screening for new, better enzymes. The "external improvement" way refers to the improvements and optimization of the process and enzyme [109, 163], e.g. immobilisation, optimization of reactor configurations and design of effective downstream processing. In order for isolated enzymes to become applicable for water treatment of industrial potential, they have to be immobilised. Even if enzymatic action has been improved via genetic engineering and screening, the production cost of "optimised enzyme" can be inhibitory for application at industrial scale, without the option of reuse. Hence, even after overcoming limitations through the invasive or the exclusive way, external improvement –usually via immobilisation– needs to be applied. In the next section we shed light on the applicability of immobilisation, focusing on immobilised oxidoreductases and their application for dye removal.

401 Table 2: Decolorisation of anthraquinone dyes by isolated enzymes

Decolorisation method	**Dye name/ initial dye concentration (mg/L)**	**% max decolorisation* /timescale (h)**	**Throughput (mg/L/h)****	**Comments**	**Ref.**
Horseradish peroxidase	Reactive Blue 19 / 120	96 % / 0.1	**115.2**	Better results shown for anthraquinone dyes compared to other types, reduction of toxicity after degradation	106
Horseradish peroxidase	Remazol Blue / 1000	35 % / 9	38.8	Inactivation of enzyme due to dye concentration, precipitation can occur depending on dye	164
Horseradish peroxidase	Lanaset Blue 2R/ 10-100	90 % / 0.033	**270-2700**	Very good decolorisation of single anthraquinone dye, examination of enzyme performance on real effluents (see discussion), examination of 2 bioindicators on toxicity of effluents before and after treatment	152
Horseradish peroxidase	Acid Blue 225, Acid Violet 109/ 30	83 %, 70% / 0.5, 0.25 (for AB225 and AV109 respectively)	53, **113**	Different anthraquinone dye structures lead to different decolorisation degrees under the same conditions, decolorisation of AB225 is affected more by temperature and concentration of enzyme, but dye concentration affects AV109 decolorisation more	122
Laccase	Reactive Blue 19/ 100	89 %/ 0.5	**178**	Laccase showed better decolorisation performance on anthraquinone dyes, compared to azo, triphenylmethane or indigo dyes, no mediators were necessary	153
Laccase	Reactive Blue 19/ 300	100 %/ 72	**100**	Laccase showed better decolorisation performance on anthraquinone dyes compared to other types of dyes. Furthermore, activity of purified enzyme was higher than use of mother culture.	
Laccase followed by biosorption	Reactive Blue 4 / 1000	90 (61+29) % / 12	50.8+24.1	Reduced phytotoxicity, individually, biosorption works better, enzymatic degradation results in brown-ish products, dye fragments are more polar compared to original dye	165
Dye-decolorising peroxidase	Reactive Blue 19/ 150	95 % / 0.167	**855**	Decolorisation examined in batch reactor and fed batch reactor, examination of stepwise and continuous feed of H_2O_2, batch and continuous fed reactor, residual activity of DyP is 80 % after 80 mins, increased dye concentration prolongs the decolorisation time, increased addition of H_2O_2 deactivated the enzyme, through batch fed system one dose of enzyme managed to decolorise 3,650 mg/L RB19	107

402 * values shown are for the optimised methods as presented by researchers and refer to removal of colour unless stated otherwise

403 ** arbitrary value calculated to show the maximum removal capacity of any given method within an hour, based on the best results presented in each reference. In cases where
404 the timescale of the decolorisation is within a few minutes, the assumption of decolorisation ability over continuous use for 1 h is made.

405

3.3. Immobilisation

3.3.1.General information

The stability of enzymes under "un-natural" conditions, enzyme production cost and the need for downstream separation are crucial for their industrial potential [166]. Immobilisation is an established technique with the aim to facilitate separation and reuse of enzymes as well as maintain the most active conformation [167, 168] by "securely attaching" the enzyme onto usually solid supports that offer molecular rigidity [169]. Major advantages of immobilisation include the ability for enzyme reuse and the simplified downstream processing, as well as the enhancement of operational stability of enzymes and the option for cascade reactions; ultimately offering cost effective solutions [166, 170]. However, distinct disadvantages include the rigorous design of the system, which has to be tailored to the enzyme and application in mind, minimising mass transfer limitations between the enzyme and the substrates and the possibilities for enzyme deactivation [166, 170]. It should be noted that immobilisation does not necessarily aim to make the enzyme perform better when it is applied in its optimal operational conditions, but to maintain or ideally increase its performance when the conditions are not optimal [171].

3.3.2.Methods and supports for immobilization

There are many extensive reviews on methods and supports used for enzyme immobilisation[172, 173] focusing on a specific support (e.g.[174]), immobilisation method (e.g.[175, 176]) or enzyme (e.g.[177]). Among many available ways of immobilisation as shown in Figure 2, the most widely preferred ones are adsorption, entrapment or encapsulation and covalent bonding [174]. The typical enzyme content in the final product is usually less than 10 % by weight, remaining being the support [176].

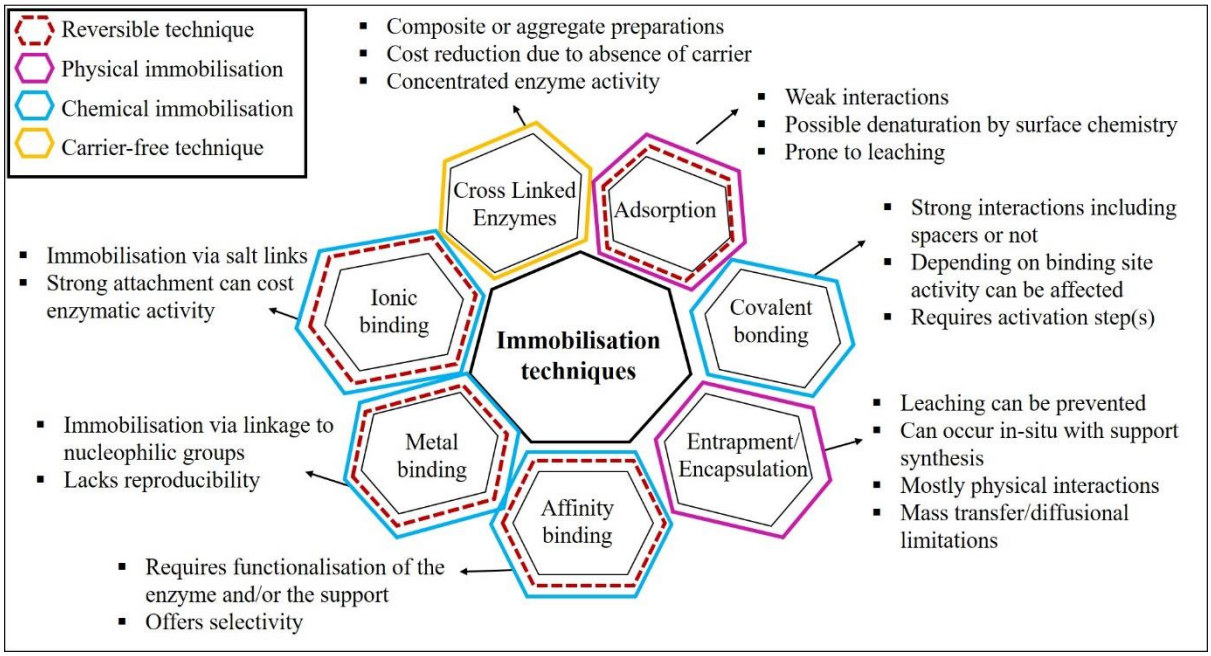

Figure 2: Brief description of immobilisation methods, with the most common methods underlined (adapted from [167, 176, 178]).

There is not a set combination of technique, support and enzyme, as immobilisation highly depends on the targeted reaction/process, the given conditions and the possible interactions. Thus for achieving a golden mean for the factors affecting its efficiency, approaches ranging from trial and error to predictive designing of the targeted system are used [179]. The selected combination should satisfy both catalytic (productivity, stability and specificity) and non-catalytic requirements (control of the process, separation, robustness and need for further processing)[180]. A suitable support for enzyme immobilisation should fulfil requirements in regards to mechanical properties, ease of synthesis and functionalization, environmental friendliness, leaching prevention, toxicity, loading capacity, low steric hindrance effects but high availability of reactive groups [167, 172, 181]. Also, it should fulfil requirements about the microenvironment and mass transfer during enzymatic actions [169, 182]. What is aimed is to create a stable and active biocatalyst that can be applied on an industrial level [172, 183], at an acceptable total cost [184, 185]. Immobilised enzymes can be cost-effective if the cost of immobilisation (total cost of every step of the process) is lower than the cost of separation of soluble enzymes from

446 the product (and of further product purification if needed), in addition to the cost of using fresh
447 enzyme in every "catalytic round" [186].

448 3.3.3. Anthraquinone dye decolorisation by immobilised
449 oxidoreductases
450

451 There are some reviews available on water decontamination by immobilised enzymes, focusing
452 either on specific pollutants or on specific enzymatic sub-categories of oxidoreductases [119, 120,
453 125, 177, 187, 188]. Generally, the operational stability of enzymes is enhanced by immobilisation
454 but the activity of the enzyme is reduced, mainly due to disruption of the active conformation,
455 difficulty of the substrate to reach the enzyme, or deactivation of the enzyme due to
456 accumulation of toxic substances. The decontamination efficiency highly depends on the
457 combination of enzyme and support used, as well as on the system investigated (dye structure
458 and concentration, presence of other substances). Hence, enzymatic performance is typically
459 investigated by varying some of those parameters, as well as operational parameters like
460 temperature and pH (Table 3). From Table 3 it is clear that immobilisation enhances the
461 stability and it facilitates enzyme reusability. Some examples include increased performance
462 of the immobilised enzyme compared to free (7 % higher performance was noted when
463 polyphenol oxidase was immobilised) [189]. It is also worth mentioning that both the available
464 examples of immobilised DyP showed great performance of the composite, with high
465 reusability and stability [190, 191]and very high throughput, comparable to the values observed for
466 AOPs..

467

468

469

470 Table 3: Decolorisation of anthraquinone dyes by immobilised oxidoreductases.

Enzyme	**Method/Support**	**Substrate/ Dye concentration (mg/L)**	**Throughput (mg/L/h)***	**Comments**	**Ref.**
Horseradish peroxidase	Covalent binding/ methacrylated polysulfones	Reactive Blue 19/ 40	34	85 % decolorisation within 1h, decent reuse potential (7 times, 20 % activity left by 7th), increased storage stability. Increased T stability	192
Dye-decolorising peroxidase	Adsorption/ immobilized FSM-16 and AlSBA-15	Reactive Blue 19 /150	**1800**	100% removal within 5mins, immobilisation support affects enzymatic activity hence decolorisation, pH affects decolorisation and enzyme leaching from support	190
Dye-decolorising peroxidase	Adsorption/ Meso Cellular Foam	Reactive Blue 19 /150	**3600**	High adsorption yield but low residual activity, pH affects decolorisation and enzyme leaching from support, very good reuse potential (20 cycles) in pH4	191
Polyphenol oxidase	Adsorption/ Celite 545	Reactive Blue 4 /50-100	43.5-87	Immobilised enzyme shows better results than free, pH affects decolorisation, immobilized enzyme treatment leads to reduced TOC post-treatment compared to free enzyme	189
Horseradish Peroxidase	Cross linked Enzyme Aggregates	Acid Violet 109 /30	36-46	High decolorisation degree (70-90 %), decolorisation experiments in batch/packed bed reactors (packed bed performs better), enhanced pH stability and higher dye and peroxide concentration tolerance, reduced toxicity after enzymatic treatment of dye solution	193
Horseradish Peroxidase	Adsorption/activated kaolin	Acid Violet 109 /40	52.2	Adsorption conditions examined, good decolorisation (87% after 40mins), improved pH stability during decolorisation, better tolerance of high dye concentration, considerably lower substrate affinity but not very lower initial rate, high (7) reuse cycles (35 % activity left)	19
Hematin (not enzyme, but of structure resembling peroxidases) and Horseradish Peroxidase	Covalent adsorption/ chitosan and APTS	Alizarin red/ 200	97.4 for Hematin 40 for Horseradish Peroxidase	Decolorisation is based on action of hematin as peroxidase active site, comparison with immobilised horseradish peroxidase is taking place, about 50 % efficiency on 1st cycle, after 6 cycles efficiency drops to 34%, identification of possible reasons for decreased activity, comparison between 2 dyes (anthraquinone and azo)	194
Laccase	Adsorption/ magnetic carbon capsules	Reactive Blue 19/ 100-300 Acid Green 25/ up to 2000	18-54 for Reactive Blue 19, 18-**360** for Acid Green 25	Decolorisation experiments for the 2 dyes were under different conditions, very high loading achieved (1g enzyme/g support), almost 80 % decolorisation within 1st hour, 90 % within 5 hours, excellent reusability (activity almost intact after 6 cycles), acknowledgment of dye adsorption on support, good storage stability (10 % activity loss after 2 months)	195
Horseradish Peroxidase	Adsorption/ chitosan	Reactive Blue 19 / 100	17.5	Use of glutaraldehyde for added functionalisation, about 70% decolorisation regardless of the dye concentration (25 mg/L and 100 mg/L	196

				examined), biocatalysts were reused for up to 7 cycles with more than 60% residual activity, main body of the work was done for an azo dye	
Laccase	Adsorption/ silanised silica beads	Disperse Blue 3, Reactive Blue 19 / ~20	0.9 for Disperse Blue 3 and 3.2 for Reactive Blue 19	Examination of various dye structures, decolorisation of 80-90 % of AQ dyes within 5 h for Reactive Blue 19 and 17 h for Disperse Blue 3, reduction of toxicity further to free enzyme, free laccase leads to a throughput of about 34 for both AQ dyes,	[197]

471 * arbitrary value calculated to show the maximum removal capacity of any given method within an hour, based on the best results presented in each reference. In cases where

472 the timescale of the decolorisation is within a few minutes, the assumption of decolorisation ability over continuous use for 1 h is made.

Immobilisation of laccase on silanised alumina pellets has also shown to reduce the inhibitory effects of components usually present in industrial dye-baths such as wetting, soaping or sequestering agents [198]. When decolorisation of two structurally similar anthraquinone dyes (Reactive Blue 19 and Acid Blue 25) was examined using immobilized laccase in epoxy activated Sepabeads, researchers got greatly different results (almost 0 % for Reactive Blue 19 and about 40 % for Acid Blue 25), indicating that structure of dye –even if of the same general type– has an important role [199]. Presence of a mediator in the examined systems increased the decolorisation of Reactive Blue 19 from 0 % to about 30 %, whereas the effect on decolorisation of Acid Blue 25 was negligible. Following the same argument, researchers [195] studying the decolorisation of two anthraquinone dyes (Reactive Blue 19 and Acid green 25) using again laccase but immobilised on magnetic carbon nanoparticles, achieved highly positive results (more than 80 % decolorisation efficiency, good reusability potential, stability) for both dyes. This shows that the immobilisation support also has a great effect on decolorisation efficiency, since using the same enzyme (laccase) acting on the same dye (Reactive Blue 19) yield different results when different supports were examined. In terms of methods and matrixes used for immobilisation, looking at Table 3 one can see that adsorption on inorganic matrices – usually silicates – is highly favoured over other methods and matrices combinations. This is possibly due to the extensive research available on those materials [174, 200] and their wide industrial presence [201], thus allowing easier industrial implementation of the immobilized biocatalyst. An issue usually faced with immobilisation supports is adsorption of dye on the actual support instead of decolorisation due to enzymatic action, which might lead to false results if it is not accounted for. Indeed, another study showed an initial step of dye adsorption onto the carrier (silica beads), followed by decolorisation by the enzyme (laccase), allowing for fresh substrate to be used [202]. Also, another issue is the adsorption of degradation products [192, 202], which might lead to enzyme deactivation.

It should be noted that researchers have developed artificial enzyme mimics – namely nanozymes – that mimic specific enzymatic actions. Their action is based on a chemically synthesized active site that is very similar to the one of the targeted natural enzyme, e.g. enzymes that contain metals or metal oxides [203]. So far, there have been examples mimicking the action of peroxidases [204-208] but only two reports of peroxidase mimetic nanozymes for dye decolorisation exist (for an azo, a xanthene [209] and a thiazine dye [206]), and none on anthraquinone dyes. . It has been reported that nanozymes are easy to use over natural enzymes due to lower cost, easier scale-up production, higher durability and stability. However, nanozymes applications are very limited due to lack of selectivity and substrate recognition, as well as lower activity compared to natural enzymes [203, 210, 211].

4. ON THE DEGRADATION PATHWAY OF ANTHRAQUINONE DYES

As it has been already mentioned, it is quite difficult to confirm the degradation pathway of a dye, mainly due to the possibility of spontaneous oxidations and our inability to quickly isolate fragments. Nevertheless, there are few examples where based on the initial dye structure, some identified fragments and the identified as final products, researchers have been able to propose a degradation pathway for model anthraquinone dyes. Although Li et al.[41] discuss the degradation pathway of AQ dyes by biological methods and Siddique et al.[42] have collected examples of Reactive Blue 19 degradation by different methods without however touching on the degradation pathway, a comprehensive discussion around the degradation pathway of AQ dyes by various methods is missing. In Table 4 one can see a list of research examples studying the degradation of a model AQ dye (Reactive Blue 19, structure shown in Figure 1) using 4 different methods and proposing degradation pathways based on the identified fragments. The chemical structure of fragments is shown in Table S2. By comparing the identified fragments, it is evident that each method can lead to different results. Degradation of Reactive Blue 19

using thermal-pressure hydrolysis [212] was the only case where the identified fragments were not of aromatic structure (small carboxylic acids were detected). In every other research example studied the identified fragments were considerably larger, especially for the examination of degradation via enzymatic action. In this case, the proposed degradation pathway did not progress mush further than the deamination of the anthraquinone structure and the ring's rupture, as well as the deamination and desulfonation of the main auxiliary structure [95]. The AOPs, chemical and biological methods used for Reactive Blue 19 degradation (Table 4) resulted in phthalic acid and its derivatives (as identified products), with expectations for further degradation to lower by products, which however were not confirmed by the analytical methods used. What can be assumed based on the degradation pathways proposed is that the anthraquinone structure is eventually broken down to simpler aromatic derivatives, allowing for easier manipulation afterwards. However, this is not the case for the enzymatic methods, where degradation seems to be a more lengthy procedure [95, 97]. Based on that absence of further degradation might be attributed to loss of enzymatic activity over prolonged time of use and/or exposure to the reaction mixture.

A first comparison between the identified as final fragments across different methods used, show that electrochemical methods lead to generally smaller fragments, with higher mineralization potential compared to biological methods. As it can be seen in Table S3, of the most common fragments identified were phenol, phthalic acid, their derivatives and low molecular hydrocarbons. These fragments were mainly produced by the cleavage and subsequent degradation of the anthraquinone ring through various steps. In some cases it was noted that different dyes (Reactive Blue 19 [213] and Reactive Blue 4 [214]) treated with the same method led to the same degradation products (as derived by the anthraquinone ring). This observation could be an indication of some control over the end products if a specific method is applied. However, looking more in depth into a specific method, ozonation, for the

548 degradation of the same dye, Reactive Blue 19, results from different researchers showed
549 slightly different fragments. Identified fragments ranged from a mixture of phenol, acetic acid
550 and propandioic acid [68], to a mixture of phenol, acetic acid and oxalic acid [92] and to a mixture
551 of phthalic acid and unspecified carboxylic acids [69]. Upon examination of the conditions used,
552 Fanchiang et al. [69] used slightly higher ozone feed rate compared to the other studies, which
553 might have been responsible for the higher mineralisation potential.

554 Based on the few research examples showing a degradation pathway and fragments of AQ dyes
555 treated using biological methods as shown in the lower end of Table S3, we can see that the
556 identified fragments are not different to those shown for chemical and AOP methods. However,
557 the identified fragments upon degradation using enzymes were substantially larger compared
558 to those identified by other methods, as shown in Table S3. This observation shows that
559 although isolated enzymes can potentially be very efficient in decolorisation, when it comes to
560 dye breakdown and mineralisation, the requirements for degradation are higher than what
561 currently available from enzymes. That being said, combination of enzymes, as it is the case in
562 biological systems, might be a potential avenue to explore.

563 It should be noted that in all the research examples examined, the suggested degradation
564 pathways based on some identified fragments and the discussion around the specific
565 breakdown mechanisms, show that the initial fragments could not be identified by the
566 analytical methods used, but were speculated retrospectively. This shows the lack of control
567 over the dye degradation reactions and the existence of spontaneous reactions that can lead to
568 the same lower fragments via multiple paths [69, 74]. With regards to the auxiliary groups present
569 on the dye structures (such as amino-groups, sulfone-groups, halogen-groups), mapping down
570 their degradation pathway was more difficult. Research examples either did not identify further
571 degradation past the original rupture from the dye structure [93, 95, 214], or identified big fragments
572 and even polymerized by-products [97].

573 *Table 4: Final identified fragments during degradation of Reactive Blue 19 via various methods. Please refer to Table S2 for the chemical structures of the identified fragments.*

Method	**Final identified fragments**	**Identification methods* and relevant comments**	**Ref.**
Thermal-pressure hydrolysis	Acetic acid, oxalic acid	Use of GC-MS, comprehensive table with identified fragments and their time occurrence during degradation	212
Ozonation	Phthalic acid, carbon dioxide, water	Use of UV-Vis, FTIR, LC-MS and GC-MS, comprehensive discussion around proposed degradation pathway	69
Photodegradation on nano-TiO_2 in presence of H_2O_2	3,6-dihydroxyphthalic acid, ethyl-sulfate-phenyl-sulfone	Use of UPLC-MS	99
Electrochemical degradation in presence of chloride	1,3 indanone, phthalide, phthalic anhydride, phthalimide, benzoic acid	Use of GC-MS, potential for further breakdown upon increased contact time, no chlorinated by-products detected	213
Biological (bacterial flora *DDMY2*)	benzenesulfonic acid, hexan-1-amine, 3,6-dihydroxyphthalic acid,	Use of UV-Vis, FTIR and LC-TOF-MS, comparative discussion on degradation products with literature	215
Enzymatic (immobilised laccase)	5-sodium-benzenesulfonyl-ethanone, opened anthraquinone ring fragment	Use of LC-MS, 2 intermediates and 2 final products identified, no observations for backward reactions	95

574 *H/UPL/C: high/ultra pressure liquid chromatography, GC: gas chromatography, MS: mass spectrometry, TLC: thin layer chromatography, UV-

575 Vis: Ultra Violet-Visible Spectrophotometry, FTIR: Fourier Transformation Infra-Red Spectroscopy, TOF: Time of flight

5. CONCLUSIONS AND FUTURE CHALLENGES

Degradation of anthraquinone dyes poses an environmental problem that has been ignored due to their smaller volume compared to azo dyes. Existing research shows that anthraquinone dyes can be sturdier when it comes to decolorisation due to their structure. Furthermore, their removal is highly specific on the dye and method used, hence it can be quite difficult to choose a single solution among the available conventional methods. Comparing the existing physical/chemical methods with the biological ones, it is clear that while every method has their distinctive advantages and disadvantages, Advanced Oxidation Processes and isolated enzymes stand out it terms of fastness of degradation. In this review we focused mainly on enzymatic decolorisation of anthraquinone dyes, and showed that it has gone a long way but still needs extensive research before industrial implementation. Immobilisation can help create powerful biocatalysts that can be both environmentally friendly and industrially applicable. Currently, immobilized oxidoreductases can show activity comparable to free enzyme when it comes to smaller substrates, but they sometimes suffer when it comes to dyes of larger sizes causing inaccessibility to the enzyme inside a porous support.

A main challenge we identified during literature review, was the lack of consistency in approaches used in various research examples. This makes the comparison of the ability of suggested methods to treat anthraquinone dyes (or pollutants in general) quite tricky, as we showed that different conditions using the same method and the same dye, of using a method under the same conditions for different dyes can lead to different results. A way to circumnavigate that would be to set a benchmark set of parameters per available method, making comparison across methods easier. For example, setting a standard dye concentration for experiments, a fixed ratio of dye to enzyme, nanomaterial, oxidant or energy used. It could also mean setting an arbitrary unit that allows to compare results on a common basis, such as

productivity. These approaches would make comparison across methods easier, showing the strong and weak points of each method in a more comparable manner.

Another challenge that became obvious across examined literature is the unrealistic approach usually followed, pinpointed by the fully controlled systems used. Although some level of control is needed to define and characterise a dye degradation system, a more realistic approach towards system development should be adopted, incorporating research on real effluents or mixtures of dyes. Although it is mainly the mixtures of dyes that are encountered in the actual effluents from textile industry, there are very limited reports examining such mixtures of dyes [216-218] and even fewer that include an anthraquinone dye within the dye mixture [155, 219, 220]. The absence of extended research on dye mixtures (only one review paper on dye mixture decolorisation was found [221]) highlights the need to investigate the ability of existing technologies to treat a more realistic form of effluent, that being dye mixtures, or solutions containing other auxiliaries used in the textile industry. Focusing on decolorisation of anthraquinone dyes by immobilized oxidoreductases, the lack of information of the performance of such systems on real (or realistic) effluents was also noted. This can be attributed to the high sensitivity of biocatalysts towards operational conditions, but if such methods are aimed to be applied industrially, then a more realistic approach is ought to be explored.

Another identified challenge with respect to dye degradation is the relatively limited available information on the degradation pathways. All the research examples proposing a degradation pathway, reached their conclusions based on a few identified fragments via analytical techniques, assuming previous and further reaction steps. The existence of free radicals and the recalcitrant structure of anthraquinone dyes make oxidation, hence degradation, difficult to predict. In a few examples in literature, there were more than one pathways proposed for the degradation of an anthraquinone dye, indicating the need for better control over the process of

degradation. This could be achieved through more careful control of the reaction and the development of techniques to pause the reaction before progressing further, or techniques to slow it down enough so that samples during the initial stages can be withdrawn.

Finally, with regards to immobilised oxidoreductases, future studies need to tackle the "value for money" equation when it comes to the synthesis of a biocatalyst for such a specific, yet absolutely crucial application. So far the main aim of the studied examples seems to be the performance of the biocatalyst, without trying to minimise synthesis cost. Majority of examples examined are using lengthy synthesis procedures with many subsequent steps for the materials synthesis and the immobilisation of the enzyme, and further hazardous chemicals and unsustainable practices are usually employed [189, 192, 196, 197, 199, 222-224]. These lead to final products which might tick the performance brief, but by no means tick the industrial implementation brief, rendering them basically unusable. Research should focus on the identification of a golden means between synthesising a material-enzyme complex able to perform as a powerful dye degradation agent, but also have the potential for easy and economical scale up in order to be industrially relevant. Alternatively, research could also focus on the combination of methods in order to address their limitations and enhance their advantages. For example, initially treating anthraquinone dyes – or effluents generally – with immobilised oxidoreductases and following up with AOPs, could help minimise the energy cost associated with AOPs and prevent biocatalysts from prolonged use, hence increase their industrial potential.

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