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DETERMINATION OF PHYSICOCHEMICAL AND TOXICOLOGICAL PROPERTIES OF AZO DYES USING *IN SILICO* METHODS

İN SILICO YÖNTEMLERİ KULLANILARAK AZO BOYALARIN FİZİKOKİMYASAL VE TOKSİKOLOJİK ÖZELLİKLERİNİN BELİRLENMESİ

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ABSTRACT

In this study, Disperse Black 9 and Mordant Black 9 azo dyes were the objects of theoretical research. The characterization of the dyes was studied using the Density Functional Theory (DFT) method. The molecules were optimized, and their energy levels were calculated using the B3LYP theory level and the 6-311++G(d,p) basis set. Consequently, the frontier molecular orbital (FMO) was utilized to estimate the energy of the highest occupied molecular orbital (E_{HOMO}) and the energy of the lowest unoccupied molecular orbital (E_{LUMO}). Furthermore, global reactivity descriptor values such as global hardness, global softness, electronic chemical potential, electrophilic index, electronegativity, molecular electrostatic potentials, ionization energy, and electron affinity were calculated, including the values of the HOMO–LUMO energy gap. Concurrently, the toxicological and ADMET properties of the dyes were calculated. By calculating these values, the potential effects of azo dyes on human health and the environment were compared. Computer-based *in silico* programs such as ProTox 3.0, ToxTree, ECOSAR, and T.E.S.T. were used to collect the data. Molecular modeling methods have increased efficiency, saving time, reducing costs, and providing faster results. This study aims to inform researchers about the properties and toxicity of pollutants causing environmental and water pollution.

Keywords: Azo dyes, toxicology, DFT, molecular modeling.

ÖZET

Bu çalışmada, Dispers Black 9 ve Mordant Black 9 azo boya ları teorik araştırmanın konusu olmuştur. Boyaların karakterizasyonu, Yoğunluk Fonksiyonel Teorisi (DFT) yöntemi kullanılarak incelenmiştir. Moleküller optimize edilmiş ve enerji seviyeleri B3LYP teori seviyesi ve 6-311++G(d,p) temel seti kullanılarak hesaplanmıştır. Sonuç olarak, en yüksek dolu moleküler orbitalin (E_{HOMO}) enerjisini ve en düşük boş moleküler orbitalin (E_{LUMO}) enerjisini tahmin etmek için sınır moleküler orbital (FMO) kullanılmıştır. Ayrıca, HOMO–LUMO enerji aralığı değerleri de dahil olmak üzere, küresel sertlik, küresel yumuşaklık, elektronik kimyasal potansiyel, elektrofilik indeks, elektronegatiflik, moleküler elektrostatik potansiyeller, iyonlaşma enerjisi ve elektron afinitesi gibi küresel reaktivite tanımlayıcı değerleri hesaplanmıştır. Aynı zamanda, boya ların toksikolojik ve ADMET özellikleri hesaplandı. Bu değerler hesaplanarak, azo boya ların insan sağlığı ve çevre üzerindeki potansiyel etkileri karşılaştırıldı. Verilerin toplanması için ProTox 3.0, ToxTree, ECOSAR ve T.E.S.T. gibi bilgisayar tabanlı *in silico* programlar kullanılmıştır. Moleküler modelleme yöntemleri verimliliği artırmış, zamandan tasarruf sağlamış, maliyetleri düşürmüş ve daha hızlı sonuçlar elde edilmesini sağlamıştır. Bu çalışma, araştırmacılara çevre ve su kirliliğine neden olan kirleticilerin özellikleri ve toksisitesi hakkında bilgi vermeyi amaçlamaktadır.

Anahtar Kelimeler: Azo boyar maddeler, toksikoloji, DFT, moleküler modelleme.

INTRODUCTION

Environmental pollution is not a new phenomenon; it has been occurring since ancient civilizations. Among the primary causes of global environmental pollution are human activities such as urbanization, industrialization, mining, and mineral exploration (Ukaogo et al., 2020; Vesilind et al., 2013). Many raw materials and chemicals can be cited as causes of pollution. For instance, with the increasing demand for color, dyes are found in various industries. And these dyes are carelessly disposed of into environmental water sources after application. For instance, dyeing, dye manufacturers, and textile industries are responsible for the presence of dye waste in the environment (Bukola M Adesanmi et al., 2022; Katheresan et al., 2018). The textile industry is one of the main industries associated with water pollution due to the direct discharge of untreated wastewater and the release of toxic chemicals into the aquatic environment throughout the year (Al Prol, 2019; Gita et al., 2017).

Dyes are organic compounds that can be natural or synthetic and impart color through the absorption and reflection of light (Ölmez, 1999; Sneha et al., 2025). It is known that approximately 10-15% of the dyes used in the textile industry mix with wastewater (Karaçıray, 2019; Gita et al., 2017). Dyes found in wastewater are highly resistant to biological degradation and have a toxic effect on aquatic flora and fauna. Therefore, the proper and effective treatment of wastewater from the textile industry is of critical importance (Kocaer and Alkan, 2002; Gita et al., 2017; Al-Ghouti and Sweleh, 2019).

Approximately 70% of the dyes used in textiles are azo dyes (Bukola M Adesanmi et al., 2022). Azo dyes are synthetic colorants that can be found in nature and produce intense colors even in small quantities. In general, the chemical structure of an azo dye consists of several different types of groups, including azo functional groups (-N=N-), backbone auxochrome groups, and stabilizing groups (Zeyrekli et al., 2021; Omar et al., 2023). Azo dyes are primarily synthesized through diazotization and coupling reactions (Liao et al., 2024). Although azo dyes are easy and cost-effective to synthesize, only 10% of them are permanently transferred to the material, so careful sub-processes are required for their use and disposal (Ngo and Tischler, 2022; Bukola M Adesanmi et al., 2022). Azo dyes may be more toxic due to the production of dangerous aromatic amines under anaerobic conditions (Sardi et al., 2021). Studies conducted on azo dyes have clearly demonstrated that these substances have carcinogenic, mutagenic, and teratogenic effects on humans and animals (Song et al., 2024).

Although several studies have looked into azo dyes using computational methods, there is not much research that directly compares the structural and toxicological profiles of Disperse Black 9 and Mordant Black 9. This study uses high-level DFT calculations along with the latest toxicity prediction models to address this gap. Our goal is to provide a thorough theoretical evaluation of these dyes, clarifying the connection between their electronic properties, specifically HOMO-LUMO, and their potential effects on the environment and living organisms. While many *in silico* studies have examined the toxicity of azo dyes in general, the existing literature does not provide a thorough comparison focused specifically on Disperse Black 9 and Mordant Black 9. Previous studies tend to rely on single prediction models or concentrate only on ecological impacts. This research stands out by combining quantum chemical calculations (DFT) with a multi-model toxicological assessment using tools such as ProTox 3.0, ECOSAR, T.E.S.T., and ToxTree. This approach predicts toxicity profiles and explores the electronic structural features, like HOMO-LUMO gaps and global reactivity descriptors, that influence toxicological behaviors. By linking molecular reactivity to specific toxicity endpoints, such as organ toxicity and aquatic effects, this study helps enhance our understanding of the relationship between structure and toxicity for these particular industrial pollutants.

MATERIAL AND METHODS

Disperse Black 9 is used in thermosol dyeing and heat transfer printing processes and is generally suitable for acetate and polyester fibers. Figure 1 shows the chemical structure of the Disperse Black 9 dye.

Mordant dyes represent the largest portion of synthetic azo dyes, which are widely used in the dyeing of textile fibers such as wool, silk, polyester, cotton, and nylon (Zeyrekli et al., 2021). Mordant Black 9 is an acidic dye that can also be used in electro-chemical aluminum processes and leather dyeing. Figure 2 shows the chemical structure of the Mordant Black 9 dye.

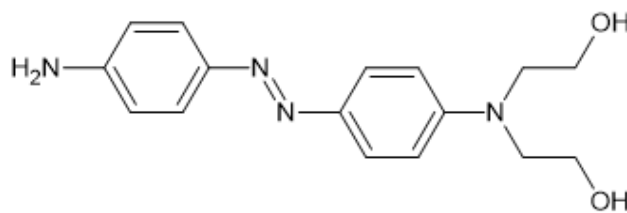


Figure 1. Chemical Structure of Disperse Black 9

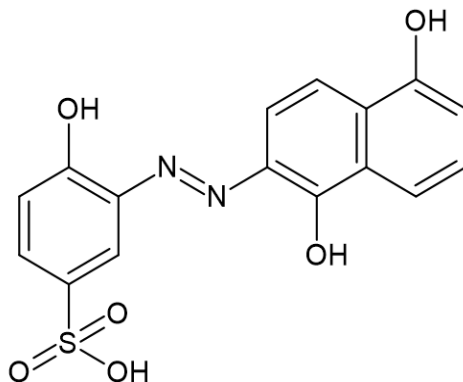


Figure 2. Chemical Structure of Mordant Black 9

Computational Details

Molecular modeling is a computer-assisted virtual laboratory technique that can be used to study the physical and chemical properties of molecules and atoms and observe their behavior. It can be particularly advantageous in terms of time and cost for complex structures. DFT is a powerful and cost-effective method for revealing fundamental information about a material, including its energy, geometric structure, electrical, and optical properties (Adekoya *vd.*, 2022; Frisch, 2009). It provides important theoretical predictions and assistance in material design (Bicheng *vd.*, 2019). Gaussian 09W is a program that includes methods such as molecular mechanics, semi-empirical, and AB-initio. Optimization can be performed with the Gaussian 09W program. The energies, vibration frequencies, and dipole moments of atoms and molecules can be calculated (Frisch, 2009). Gauss View 5.0.8 has been developed to prepare input files and visualize Gaussian outputs. It is used to make changes to molecules by visualizing them. It allows us to examine the calculated results graphically (Frisch, 2009).

In Silico Toxicology

The term *in silico* toxicology is defined as computational approaches that analyze, predict, and visualize the toxicity of substances. It offers advantages by reducing costs and animal testing (Myatt *et al.*, 2018; Valerio, 2014). *In silico* toxicology relies on the use of various computer-based data analysis programs designed to generate virtual yet realistic data related to the experimental toxicology of chemicals (Valerio, 2009). Data collected from the PubChem database provides basic information such as the chemical structure of substances. The next step is to obtain the sequence of azo dyes in SMILES format. The SMILES format is used to create sequential representations using basic elements such as carbon and oxygen (Worachartcheewan *et al.*, 2014). The SMILES format is useful when using programs that calculate the toxicity of molecules. ProTox 3.0 can be used to predict the potential target organs and toxicity of chemicals. It is an *in silico* methodology that provides information on safety scores, toxicity radar graphs (Nižnik *et al.*, 2024; Liu *et al.*, 2024). The program classifies chemicals according to their lethal dose (LD₅₀) value in mg/kg and assigns a safety score and toxicity class (Jurowski and Kobylarz, 2025). ToxTree is a free scientific software developed by the European Commission Joint Research Center in Ispra, Italy, and Ideacon Ltd. in Sofia, Bulgaria, which can be used for QSAR assessment. It is used to analyze the structure and properties of a chemical (Contrera, 2013). The Cramer decision tree classifies chemicals into three classes (I – low, II – medium, and III – high) and determines TTC levels in µg/day. The Cramer decision tree consists of 33 “yes” (Y) or “no” (N) questions or rules (Q). The answer to each question directs the user to another question until the final Cramer class for the chemical being studied is determined (Bhatia *et al.*, 2015; Frydrych and Jurowski, 2024). SARs in ECOSAR are used to predict the aquatic toxicity of chemicals by exploiting their structural similarity to chemicals whose aquatic toxicity

has already been measured. The focus is on the acute toxicity of a chemical to fish, water fleas (daphnia), and green algae. Using measured aquatic toxicity values and Kow values, regression equations can be developed for a chemical, and toxicity values can be calculated. ECOSAR is a pragmatic approach developed within the framework of the Toxic Substances Control Act (TSCA) regulations (Meyland and Howard, 1998; Sanderson et al., 2003). The Toxicity Estimation Software Tool (T.E.S.T.), developed by the United States Environmental Protection Agency (EPA), assesses chemical toxicity. T.E.S.T. is an in silico methodology that provides information on acute oral LD50 toxicity using three Quantitative Structure-Activity Relationship (QSAR) methodologies. It also predicts physical properties of chemicals such as melting point and water solubility (Jurowski and Kobylarz, 2025; Noga and Jurowski, 2025).

The choice of in silico tools came from the need for a complete toxicological assessment that includes human health, environmental impact, and mechanistic pathways. ProTox 3.0 was chosen for its powerful machine-learning algorithms, which can predict specific molecular targets and organ toxicity, such as hepatotoxicity. This capability is crucial for evaluating synthetic compounds like azo dyes. ECOSAR (Ecological Structure Activity Relationships) was selected because it is accepted by the U.S. EPA and can specifically predict aquatic toxicity in fish, daphnia, and algae. This directly addresses the potential ecological risks of dye waste. T.E.S.T. was used to provide consensus-based QSAR predictions that offer solid data on developmental toxicity and physical properties. Lastly, ToxTree was used to find structural alerts and classify the mode of action, such as mutagenicity identified by the Ames test, based on established decision tree methods. Together, this multi-tool approach reduces the uncertainty of single-model predictions and gives a complete safety profile for Disperse Black 9 and Mordant Black 9.

RESULTS AND DISCUSSION

Tables 1 and 2 present the structure's bond length and bond angles as determined using the 6-311++G(d,p) basis set and B3LYP method. With values, the C-N bond lengths for Disperse Black 9 and Mordant Black 9 are 1.38-1.45 Å and 1.40 Å, respectively. The C-S bond length for Mordant Black 9 is 1.87 Å. The range of all C-C-N angles for Disperse Black 9 is between 114° and 125°. And the range of all C-N-N angles for Mordant Black 9 is between 115° and 118°.

Table 1. Theoretically optimized geometric parameters of Disperse Black 9

Bond Lengths	B3LYP/ 6-311++G(d,p)	Bond Lengths	B3LYP/ 6-311++G(d,p)
C1-C2	1.40416	C30-N26	1.45705
C1-N11	1.38873	C30-H32	1.09175
C2-C3	1.38597	C30-C36	1.53075
C2-H7	1.08529	C36-O41	1.42363
C4-N14	1.40769	N11-H12	1.00866
C16-N15	1.40579	N14-N15	1.26037
C23-N26	1.38836	O41-H42	0.96103
Bond Angles	B3LYP/ 6-311++G(d,p)	Bond Angles	B3LYP/ 6-311++G(d,p)
C1-C2-H7	119.576	N11-C1-C2	120.970
C1-C2-C3	120.252	N14-N15-C16	115.458
C3-C4-N14	116.349	N26-C27-C33	114.275
C5-C4-N14	125.140	N26-C27-H29	110.566
C21-C23-N26	121.650	O39-C33-H35	111.623
C27-C33-H35	109.975	H12-N11-H13	112.908
C33-O39-H40	108.488	H37-C36-H38	107.962

Mulliken charge distribution is a technique used for charge analysis (Kaya, 2023). Tables 3 and 4 present the mulliken atomic charges as determined using the 6-311++G(d,p) basis set and B3LYP method. Some C atoms were found to be negatively charged, whereas other C atoms were positively charged. For Disperse Black 9, the N11 atom's mulliken atomic charge value was determined to be larger than that of different chemical structures. For Mordant Black 9, this atom is also O19. Figures 3 and 4 show the optimized geometry of the dyes using the B3LYP/6-311++G(d,p) method and basis set.

Table 2. Theoretically optimized geometric parameters of Mordant Black 9

Bond Lengths	B3LYP/ 6-311++G(d,p)	Bond Lengths	B3LYP/ 6-311++G(d,p)
C1-C2	1.42051	N9-N10	1.26073
C1-C8	1.38108	O19-H34	0.96295
C1-N9	1.40565	O23-H35	0.96766
C3-O19	1.36202	S20-O21	2.96494
C8-H28	1.08433	S20-O22	1.48407
C15-H31	1.85644	S20-O23	1.95322
C18-S20	1.87410		
Bond Angles	B3LYP/ 6-311++G(d,p)	Bond Angles	B3LYP/ 6-311++G(d,p)
C1-N9-N10	115.666	C18-C15-H31	149.999
C2-C1-N9	125.181	C18-C17-H33	117.811
C3-C4-C6	117.934	N9-N10-C11	118.890
C3-O19-H34	109.388	N10-C11-C14	129.452
C5-C13-C12	121.146	O21-S20-C18	53.173
C7-O24-H36	110.294	O22-S20-C18	96.087
C8-C6-C7	122.706	O22-S20-O23	90.401
C11-C14-O25	119.395	O23-S20-O21	120.339
C11-C15-H31	87.760	S20-C18-C15	117.177
C12-C7-O24	117.303	S20-C18-C17	125.203
C12-C13-H30	118.992	S20-O23-H35	103.386

Table 3. Mulliken atomic charges of Disperse Black 9

Mulliken Atomic Charges	B3LYP/ 6-311++G(d,p)	Mulliken Atomic Charges	B3LYP/ 6-311++G(d,p)
C1	0.017640	H22	0.087817
C2	-0.023674	C23	-0.036225
C3	-0.043081	H24	0.081792
C4	0.187874	H25	0.086833
C5	-0.039597	N26	-0.385104
C6	-0.054325	C27	-0.097876
H7	0.112294	H28	0.090944
H8	0.126061	H29	0.131606
H9	0.124401	C30	-0.077530
H10	0.099750	H31	0.132670
N11	-0.490919	H32	0.092450
H12	0.211368	C33	0.010444
H13	0.211422	H34	0.078958
N14	-0.304999	H35	0.102829
N15	-0.282383	C36	-0.011818
C16	0.272911	H37	0.082010
C17	-0.121764	H38	0.103491
C18	-0.121828	O39	-0.428505
C19	-0.033629	H40	0.234896
H20	0.089350	O41	-0.428578
C21	-0.022507	H42	0.234530

Molecular optimization was performed using the Gaussian 09W software with the DFT/B3LYP method and the 6-311++G(d,p) basis set. The molecular structures, optimized structures, and HOMO and LUMO orbitals of the basic dyes used to calculate quantum chemical parameters have been modeled. The frontier molecular orbital (FMO) has been used to estimate the E_{HOMO} (energy of the highest occupied molecular orbital) and E_{LUMO} (energy of the lowest unoccupied molecular orbital), followed by the values of the HOMO–LUMO energy gap, including global softness (η), global softness (S), electronic chemical potential (μ), electrophilic index (Ψ), electronegativity (χ), ionization

energy (IE), and electron affinity (EA) were approximately calculated using the following equations in terms of HOMO and LUMO orbital energies. Table 5 presents the calculated results of the global reactivity descriptors of the dyes in eV. The obtained Gaussian outputs have been converted from Hartree to eV. (1 a.u.= 27.2114 eV) The densities of the HOMO and LUMO orbital representations for Disperse Black 9 and Mordant Black 9 have been displayed in Figures 5 and 6. Determining the HOMO and LUMO levels between the electron donor and acceptor is crucial for determining whether efficient charge transfer will occur. The HOMO and LUMO energy band gap for Disperse Black 9 is 3.28 eV. This value is 1.91 eV for Mordant Black 9. Therefore, charge transfer interaction can easily occur in both molecules.

$$\mu = \frac{E_{HOMO} + E_{LUMO}}{2} \quad (1)$$

$$\eta = \frac{E_{HOMO} - E_{LUMO}}{2} \quad (2)$$

$$\Psi = \frac{\mu^2}{2\eta} \quad (3)$$

$$S = \frac{1}{2\eta} \quad (4)$$

$$\chi = -\frac{(E_{HOMO} + E_{LUMO})}{2} = -\mu \quad (5)$$

$$IE = -E_{HOMO} \quad (6)$$

$$EA = -E_{LUMO} \quad (7)$$

Table 4 Mulliken atomic charges of Mordant Black 9

Mulliken Atomic Charges	B3LYP/6-311++G(d,p)	Mulliken Atomic Charges	B3LYP/6-311++G(d,p)
C1	0.107067	S20	0.439402
C2	-0.071239	O21	0.022704
C3	0.190212	O22	0.035714
C4	-0.099489	O23	-0.244149
C5	0.005632	O24	-0.335958
C6	-0.097852	O25	-0.299681
C7	0.160126	H26	0.079312
C8	0.034223	H27	0.074463
N9	-0.318295	H28	0.030676
N10	-0.174097	H29	0.084595
C11	0.128301	H30	0.078921
C12	-0.057095	H31	-0.027621
C13	-0.064369	H32	0.074100
C14	0.220708	H33	0.001582
C15	-0.145514	H34	0.250061
C16	-0.033627	H35	0.251171
C17	-0.132929	H36	0.234567
C18	-0.259338	H37	0.211925
O19	-0.354210		

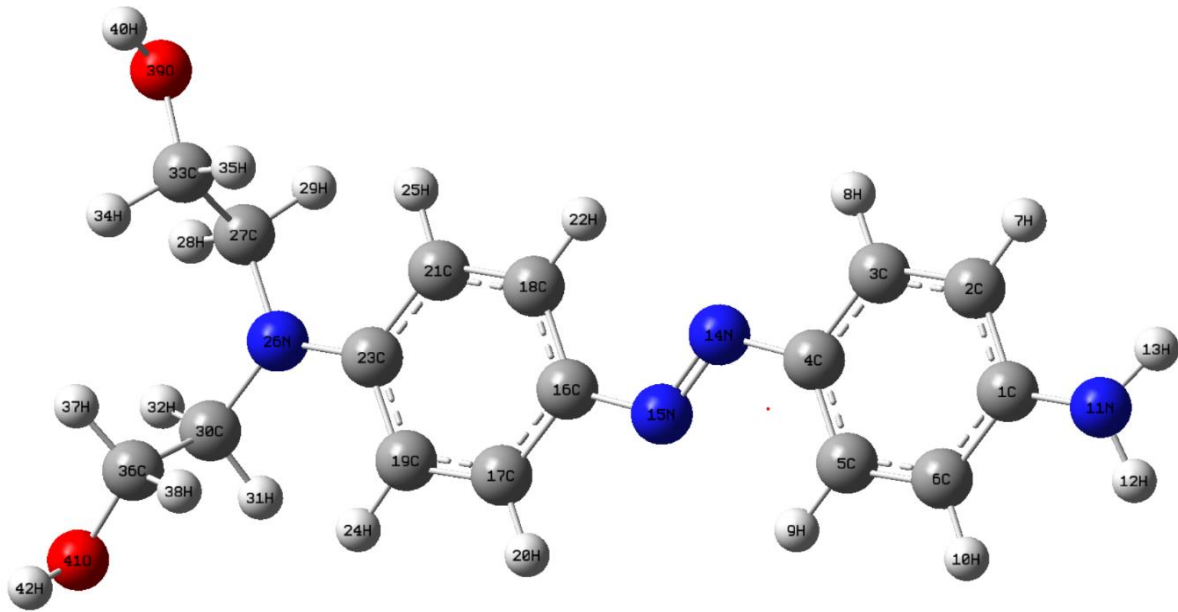


Figure 3. Optimized geometry display of Disperse Black 9
(red: O, grey: C, blue: N, white: H)

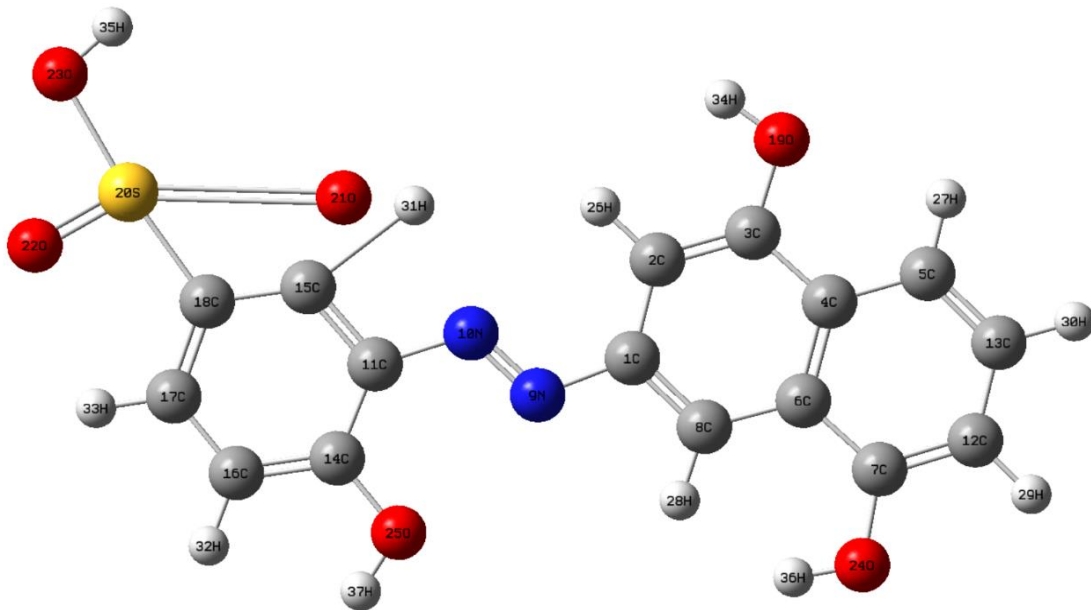


Figure 4. Optimized geometry display of Disperse Black 9
(red: O, grey: C, blue: N, yellow: S, white: H)

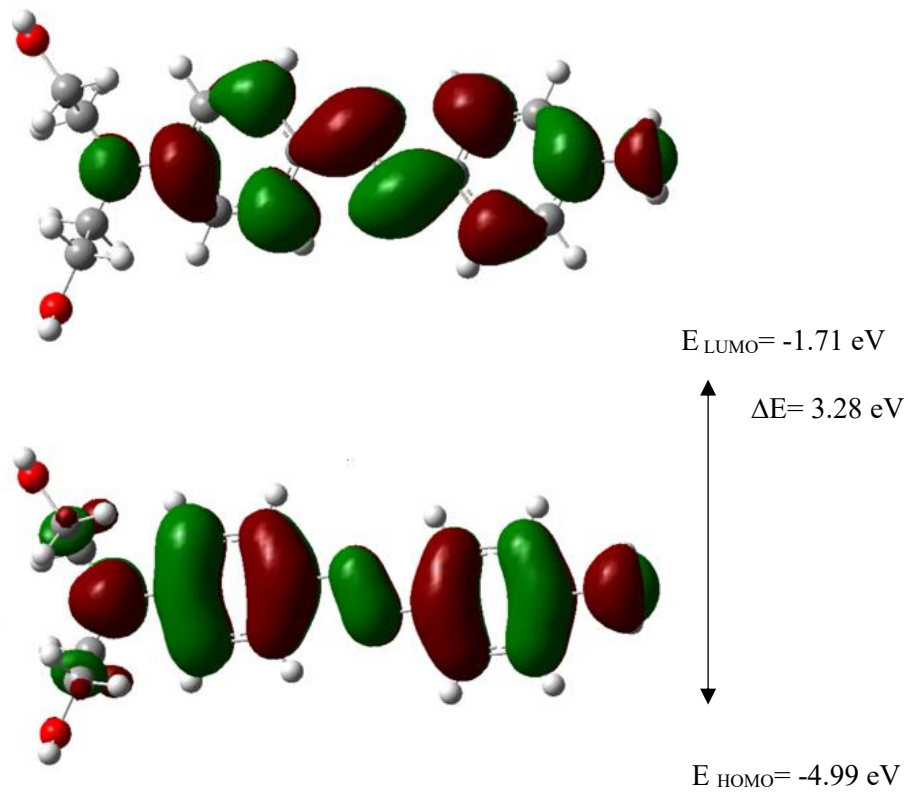


Figure 5. HOMO-LUMO energy gap of Disperse Black 9

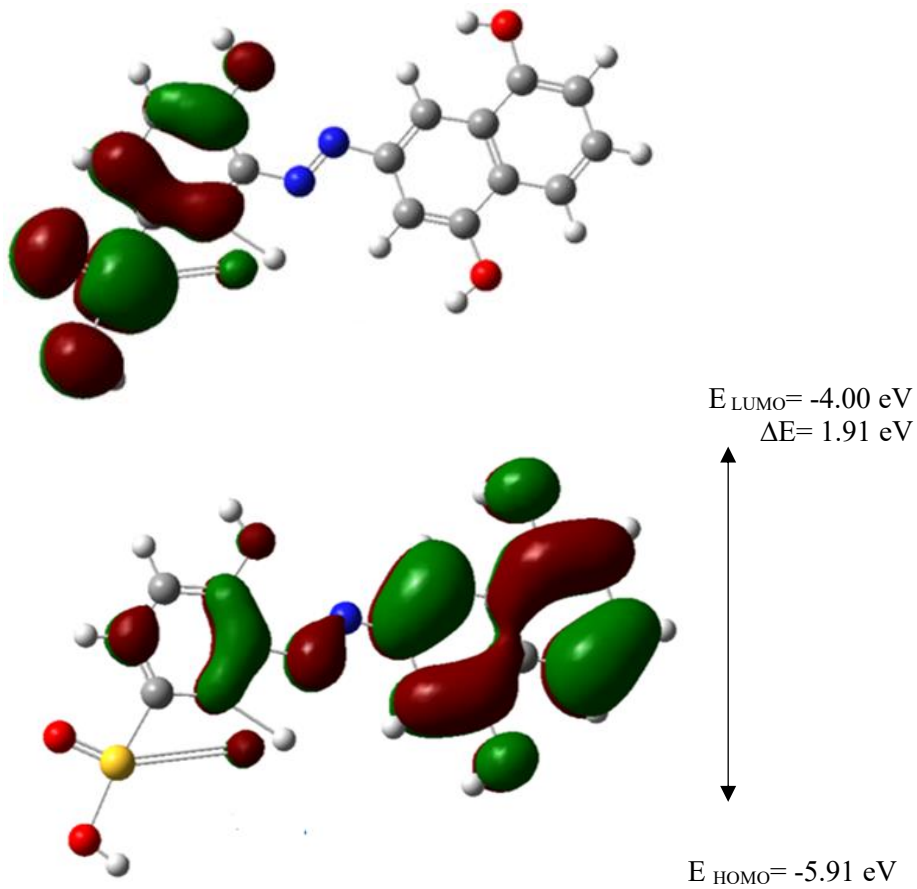
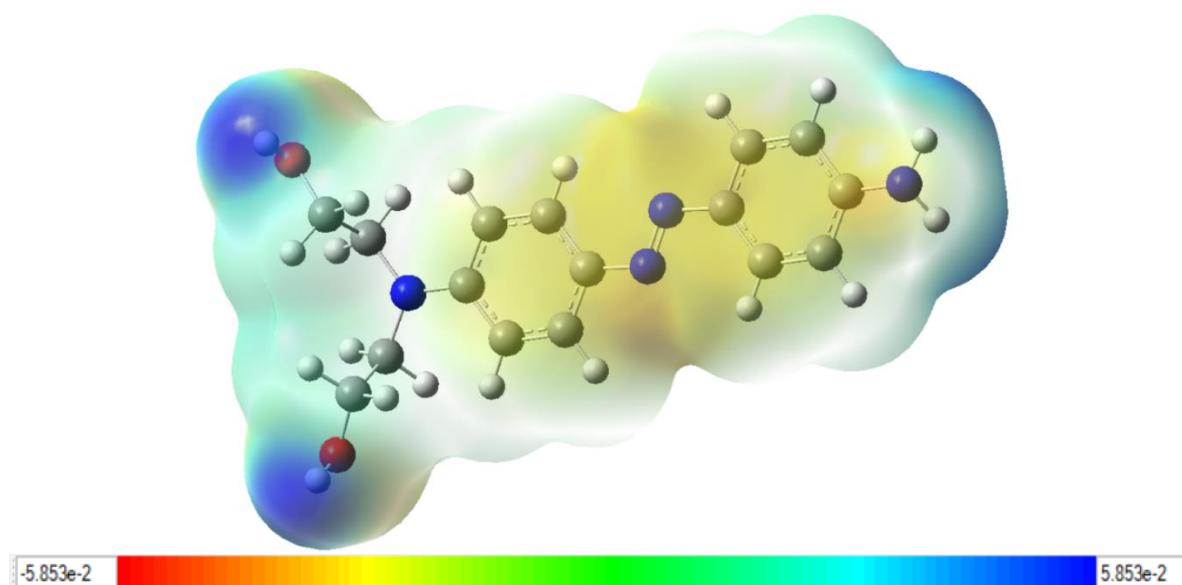
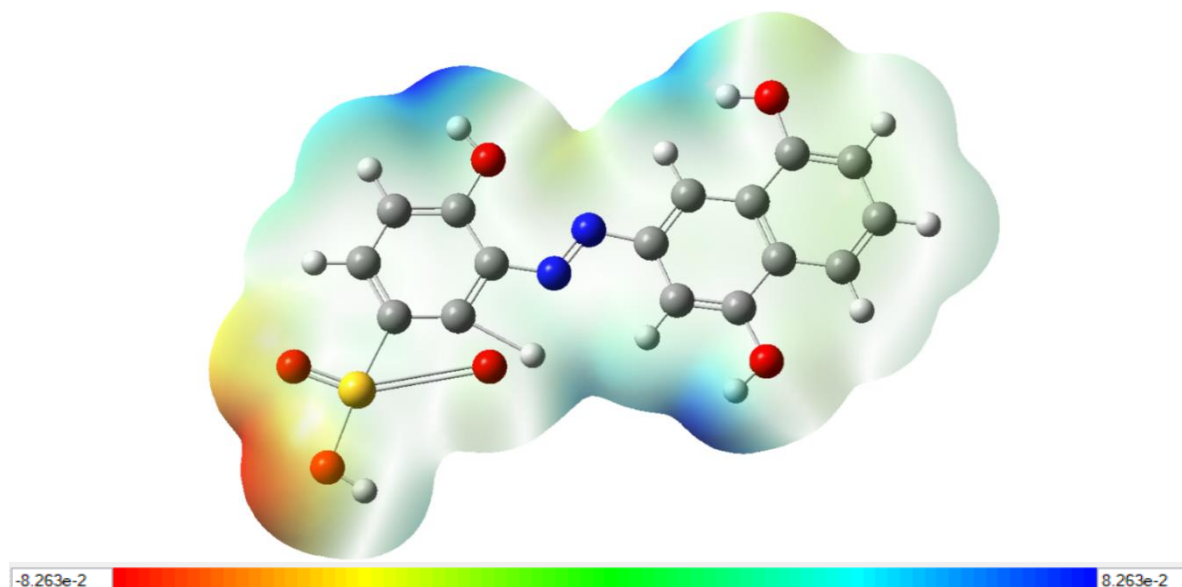


Figure 6. HOMO-LUMO energy gap of Mordant Black 9

Table 5. Global reactivity descriptors of Azo Dyes

Azo Dyes	Disperse Black 9	Mordant Black 9
ΔE	3.28	1.91
Chemical Potential (μ)	-3.35	-4.95
Global Hardness (η)	1.64	0.95
Global Softness (s)	0.30	0.52
Global Electrophilicity Index (Ψ)	3.41	12.85
Electronegativity (χ)	3.35	4.95
Ionization Energy (IE)	4.99	5.91
Electron Affinity (EA)	1.71	4.00

Figures 7 and 8 present the MEP maps of Dispers Black 9 and Mordant Black 9. Nucleophilic and electrophilic reactive sites indicate the binding sites of dyes. Red indicates electron richness, while blue indicates electron poverty.

**Figure 7.** Molecular Electrostatic Potential (MEP) surface of Disperse Black 9**Figure 8.** Molecular Electrostatic Potential (MEP) surface of Mordant Black 9

The sum of electronic values is shown in Table 6. The obtained Gaussian outputs have been converted from Hartree to kcal/mol. (1 a.u.= 627.51 kcal/mol) As a result of DFT calculations for Disperse Black 9, its total energy (ΔE) was

calculated as -621878.9995 kcal/mol, its enthalpy (ΔH) as -621878.4071 kcal/mol, and its Gibbs free energy (ΔG) as -621926.0828 kcal/mol. Besides, DFT calculations for Mordant Black 9, its total energy (ΔE) was calculated as -988842.2365 kcal/mol, its enthalpy (ΔH) as -988841.6442 kcal/mol, and its Gibbs free energy (ΔG) as -988890.0114 kcal/mol.

Table 6. Sum of electronic values of azo dyes

Azo Dyes	Thermal Energies	Thermal Enthalpies	Thermal Gibbs Free Energies
Disperse Black 9	-621878.9995	-621878.4071	-621926.0828
Mordant Black 9	-988842.2365	-988841.6442	-988890.0114

Results of ProTox 3.0 are shown in Tables 7 and 8. Results showed that Mordant Black 9 was considered non-toxic with predicted toxicity class 6 and lethal dose (LD₅₀) of 8000 mg/kg, showing prediction accuracy of 70.97%. Besides, Disperse Black 9 was considered toxic with predicted toxicity class 3 and lethal dose (LD₅₀) of 200 mg/kg, showing prediction accuracy of 68.07%. According to the toxicity target results, only Disperse Black 9 is active in terms of carcinogenicity and cytotoxicity. The other values were found to be inactive. Additionally, the results were further visualized using the ProTox 3.0 radar charts shown in Figures 9 and 10.

Table 7. Oral toxicity prediction results of Disperse Black 9 and Mordant Black 9 using ProTox 3.0 silico platform

Azo Dyes	Octanol/Water Partition Coefficient (logP)	LD ₅₀ (mg/kg)	Predicted Toxicity Class	Prediction Accuracy
Disperse Black 9	3.06	200	3	68.07%
Mordant Black 9	4.7	8000	6	70.97%

The connection between the calculated electronic properties and the camped toxicity profiles is an interesting argument that puts metabolic stability and bioavailability in the center of attention. The more toxic Disperse Black (Class 3) shows a significantly larger HOMO-LUMO energy gap ($\Delta E = 3.28$ eV) as compared to the non-toxic Mordant complex (Class 6), which has a much narrower gap ($\Delta E = 1.91$ eV). A smaller gap is generally associated with higher reactivity; however, the greater gap of Disperse Black points to a considerable chemical hardness ($\eta = 1.64$ eV) and thermodynamic stability.

In a toxicological sense, this increased stability indicates that the molecule is less susceptible to enzymatic degradation and metabolic detoxification (e.g., by cytochrome P450 enzymes). Unlike the very reactive Mordant complex ($\eta = 0.95$ eV, $\Psi = 12.85$ eV), which can be quickly and non-specifically degraded or hydrolyzed before it can interact with cellular targets, Disperse Black is stable enough to be retained in the systemic circulation and cause toxic effects.

Moreover, the difference in toxicity is greatly reinforced by the lipophilicity values. Disperse Black has a Log P value of 3.06, which is within the optimal range for membrane permeability, hence, it is very efficiently transported to the target organs. On the other hand, the Mordant complex has a significantly higher Log P of 4.7. Even though the compound is highly lipophilic, such high values usually result in accumulation in adipose tissues or poor solubility in aqueous biological fluids, thus, the effective free concentration of the compound available for acute toxicity interaction is limited. As a result, the higher toxicity of Disperse Black (Class 3) is due to the synergistic effect of its metabolic resistance (high ΔE) and optimal bioavailability, while the potential toxicity of the Mordant complex is lessened by its overly reactive nature and solubility drawbacks.

Table 8. Toxicity Target Results of Disperse Black 9 and Mordant Black 9 using ProTox 3.0 silico platform

Azo Dyes	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Disperse Black 9	Inactive	Active	Inactive	Inactive	Active
Mordant Black 9	Inactive	Inactive	Inactive	Inactive	Inactive

Results of ToxTree are shown in Table 9. For Disperse Black 9, a predicted toxic hazard suggests Cramer rules classification as high level (III), and biodegradation ability classification as Class 3. Disperse Black 9 exhibited binding alerts for DNA and protein bindings.

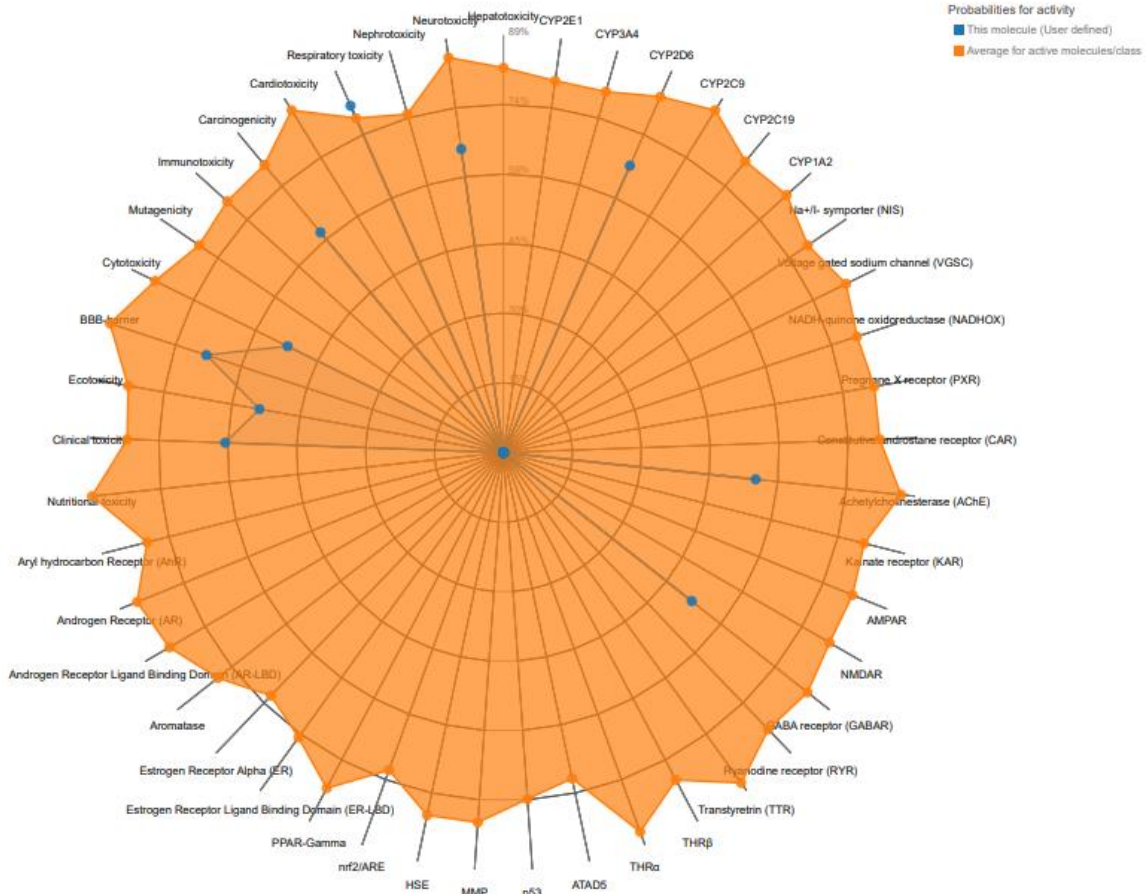


Figure 9. The ProTox 3.0 Radar Chart of Disperse Black 9

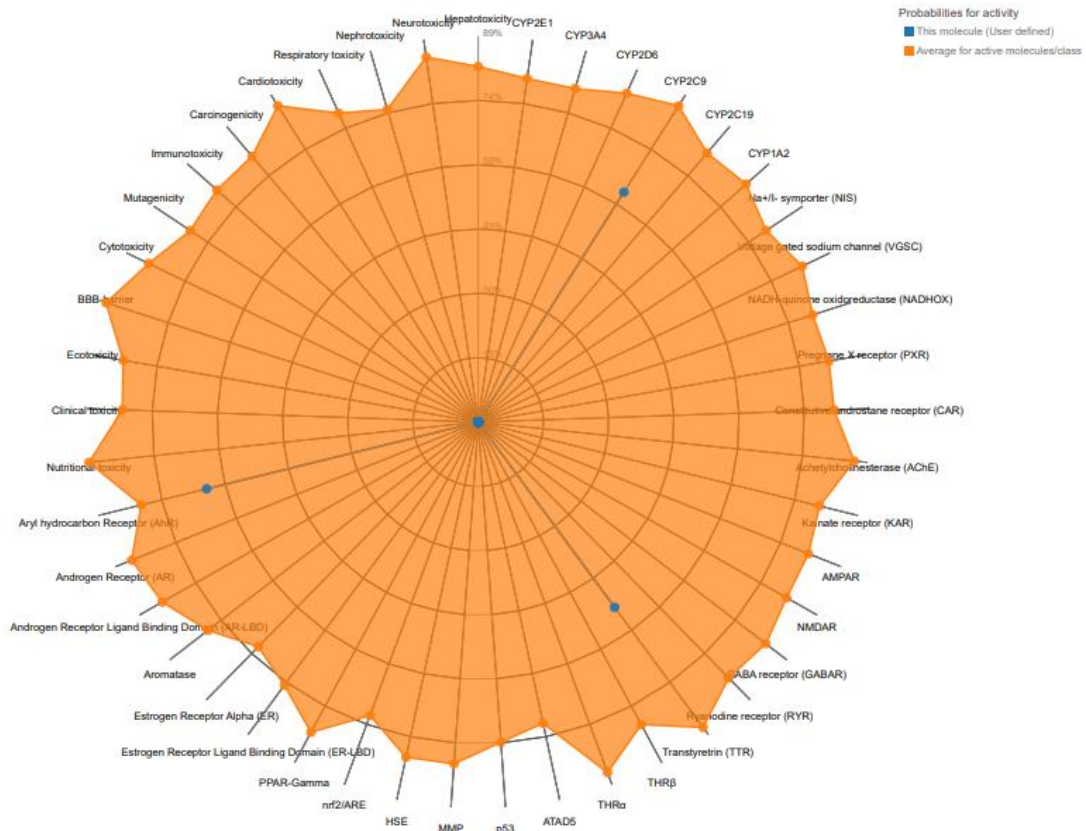


Figure 10. The ProTox 3.0 Radar Chart of Mordant Black 9

Table 9. The predicted toxicity of selected azo dyes using the ToxTree silico platform

Azo Dyes	Cramer Rules	Biodegradation Ability	Carcinogenicity (Genotoxic and Nongenotoxic)	Mutagenicity	DNA Binding Alerts	Protein Binding Alerts
Disperse Black 9	High Level (III)	Class 3 (unspecific reactivity)	N/A	N/A	Alert for SN1, Schiff base formation, Micheal Acceptor Identified	Alert for Schiff base formation and Micheal Acceptor Identified
Mordant Black 9	N/A	N/A	N/A	N/A	N/A	N/A

Results of ECOSAR are shown in Table 10. A considerably higher concentration (mg/L) in fish LC50 (96 hours) was noted for Mordant Black 9 to be 2.47E+3 while the lowest concentration was measured for Disperse Black 9 to be 41.8. Daphnid LC50 (48 hours) concentration (mg/L) for Disperse Black 9 was found to be the lowest as 3.44, whilst higher for Mordant Black 9 as 1.41E+3. Likewise, the lowest green algae EC50 (96 hours) concentration (mg/L) was observed for Disperse Black 9 as 9.25, whilst quite higher for Mordant Black 9 as 1.09E+3.

Table 10. The predicted aquatic toxicity of selected azo dyes using the ECOSAR silico platform

Azo Dyes	Fish (96 hours) LC50 (mg/L)	Daphnid (48 hours) LC50 (mg/L)	Green Algae (96 hours) EC50 (mg/L)	Log Kow
Disperse Black 9	41.8	3.44	9.25	1.8307
Mordant Black 9	2.47E+3	1.41E+3	1.09E+3	2.0885

Results of T.E.S.T. are shown in Tables 11 and 12. According to the T.E.S.T. software, both were identified as having developmental toxicant effects. The predicted toxicological endpoints serve as quantitative evidence for the observed class distinctions (Class 3 - Class 6). As indicated in the toxicity table, Disperse Black 9 has an Oral Rat LD50 value of 3331.80 mg/kg. On the other hand, the mordantization process dramatically reduces the acute toxicity, as the LD50 value for Mordant Black 9 is 6625.64 mg/kg, which is almost double the original value. This increase in the lethal dose by about two times is the confirmation that the structural modification, in particular the electronic softening and increased lipophilicity, has led to the compound's acute toxicity being reduced towards mammals, thus nicely correlating with the transition to a safer toxicity class (Class 6).

In terms of aquatic toxicity, Disperse Black 9 is a major source of environmental pollution, especially for invertebrates. The predicted LC50 for *Daphnia magna* is 2.85 mg/L, and the IGC50 for *Tetrahymena pyriformis* is 14.88 mg/L. These numbers put Disperse Black in the category of substances that are highly toxic to aquatic organisms, which is in agreement with the earlier conclusion that the high stability and optimal Log P make bioaccumulation and harm to the water column possible. On the other hand, the aquatic toxicity endpoints for Mordant Black 9 have been marked as 'N/A' (Not Available/Applicable). The absence of any aquatic toxicity is in line with its high lipophilicity (Log P ~4.7) and low aqueous solubility, both of which probably contribute to the limited bioavailability of pelagic organisms, thus reconfirming its profile as an environmentally safer alternative in terms of acute aquatic lethality.

Table 11. The predicted toxicological properties of selected azo dyes using the T.E.S.T. silico platform

Azo Dyes	Oral Rat LD ₅₀ (96 hours)		Fathead Minnow LC ₅₀ (96 hours)		Tetrahymena Pyriformis IGC ₅₀ (48 hours)		Daphnia Magna LC ₅₀ (48 hours)	
	Log ₁₀	LD ₅₀ (mg/kg)	Log ₁₀	LD ₅₀ (96 hr.) (mg/L)	Log ₁₀	IGC ₅₀ (mg/L)	Log ₁₀	LC ₅₀ (mg/L)
Disperse Black 9	1.96	3331.80	N/A	N/A	4.31	14.88	5.02	2.85
Mordant Black 9	1.74	6625.64	N/A	N/A	N/A	N/A	N/A	N/A

The identification of Disperse Black 9 as a toxic agent (Class 3; LD50 = 3331.80 mg/kg) is in line with the main mechanisms of azo dye toxicity on which the authors Chung and Cerniglia (1992) base their paper. They established that one of the main factors causing toxicity in such dyes is the enzymatic reductive cleavage of the azo bond (-N=N-) by azoreductases, leading to the production of potentially carcinogenic aromatic amines. In addition to that, the

research of Pinheiro et al. (2004) pointed out that such degradation products are, most of the time, far more toxic than the parent compounds, thus providing the reason for the high toxicity predicted for the free azo ligand in our study.

By means of DFT calculations, we found out that Disperse Black 9 has a very large energy gap between HOMO and LUMO levels ($\Delta E = 3.28$ eV) as well as high chemical hardness ($\eta = 1.64$ eV). The global reactivity descriptors concerning this case, as they were introduced for the first time by Parr and Pearson in 1983, speak of high thermodynamic stability. The Hard and Soft Acids and Bases (HSAB) principle established by Pearson (1963) explains that hard molecules are less reactive towards soft biological nucleophiles, but at the same time, they are more resistant to degradation. Apart from it being very stable, the Disperse Black 9 still remains an environmental hazard- having a Log P of 3.06, it, as Arnot and Gobas (2006) have addressed, can be regarded as a thermodynamically stable and moderately lipophilic organic pollutant which is prone to both environmental persistence and bioaccumulation in aquatic organisms what essentially explains the occurrence of aquatic toxicity ($LC50 = 2.85$ mg/L).

The drastic lowering of acute toxicity as a result of mordanzation (Class 6; $LD50 = 6625.64$ mg/kg) is in line with Sokolowska-Gajda et al. (1996) findings that have been confirmed by mordanzation-induced changes in the stability and physicochemical properties of azo dyes. Despite the fact that the Mordant complex has a high electronic reactivity ($\Psi = 12.85$ eV), biologically, it is less active due to its high lipophilicity (Log P = 4.7). This phenomenon is consistent with the 'solubility cut-off' effect in quantitative structure-activity relationships (QSAR) put forward by Hansch and Leo (1995). They considered that too much lipophilicity would limit bioavailability because it would lead to poor aqueous solubility or accumulation in adipose tissues; therefore, the limited amount of the free pool available to evoke acute toxic endpoints is present.

Table 12. The predicted developmental toxicity and bioconcentration factor of selected azo dyes using the T.E.S.T. silico platform

Azo Dyes	Predicted Value	Developmental Toxicity	Log ₁₀	Bioconcentration Factor
Disperse Black 9	0.51	Developmental Toxicant	0.60	3.94
Mordant Black 9	0.73	Developmental Toxicant	0.42	2.61

Although this study offers valuable insights into the physicochemical and toxicological profiles of the azo dyes examined, we must acknowledge some limitations tied to computational methods. First, when considering DFT functional sensitivity, the B3LYP functional works well for most organic molecules but may not fully capture long-range dispersion interactions or specific charge-transfer excitations as accurately as more advanced ab initio methods or range-separated functionals. Also, calculations done in the gas phase may not exactly represent the complex solvation environment of biological systems. Second, uncertainty in in silico predictions is a concern; the accuracy of tools like ProTox 3.0 and ECOSAR relies heavily on the applicability domain of their training sets. If a specific dye structure is significantly different from the chemical space of the training data, the reliability of predictions may drop. Therefore, these theoretical results should be seen as probabilistic risk assessments that help guide and prioritize future experimental validation.

CONCLUSION

This study utilized DFT-based computational methods to understand the electronic structure, physicochemical properties, and toxicity profile of Disperse Black 9 and Mordant Black 9. Comparative analysis shows that the presence of the (-N=N-) bond in azo dyes, along with different elements (C, O, S) and varying numbers of ring structures, causes significant changes in their electronic and toxicological properties. The calculations of the electronic structure showed that Disperse Black 9 has a wide HOMO-LUMO energy gap and high chemical hardness, which are features of stability and environmental persistence. The stability in question, combined with an ideal, leads to bioaccumulation and acute toxicity. On the other hand, Mordant Black 9 is much softer and drastically increases its electrophilicity. Nevertheless, the increased electronic reactivity is almost entirely offset by a big jump in lipophilicity, which causes a 'solubility cut-off,' thus limiting bioavailability and decreasing acute mammalian toxicity. *In silico* toxicology tools (ProTox 3.0, ToxTree, ECOSAR, T.E.S.T.) consistently predicted both dyes to be acutely toxic. Disperse Black 9 was generally predicted to be more toxic than Mordant Black 9 in both mammalian

and aquatic models. The high predicted aquatic toxicity and environmental persistence of these azo dyes highlight a significant potential environmental risk upon release into water systems. The utilization of *in silico* tools has led to a significant reduction in the necessity for animal and human testing. This method yields accurate results quickly and cost-effectively, resulting in savings on both fronts. This study demonstrates that computational methods are a valuable and cost-effective tool for the preliminary assessment of dye properties and toxicity, thereby guiding further experimental research. Proper treatment of industrial wastewater containing these dyes is crucial to mitigate their environmental impact. The study clarifies the structure-toxicity relationship of azo dyes and provides insight into numerous future research possibilities. The computational method utilized here should be applied to other dye classes that have industrial importance, like anthraquinone and phthalocyanine dyes. Comparing the studies of different chromophores will clarify whether the 'toxicity reduction via mordantization' is a general effect or just a case for azo ligands. It is highly advisable to use a hybrid method to confirm the precision of DFT and QSAR models, which were used in this study. Upcoming works should establish a connection between theoretical descriptors of reactivity (e.g., electrophilic index) and the results of biological experiments, such as the Ames test for mutagenicity or *in vitro* cytotoxicity on human cell lines. Though static DFT computations have shed light on electronic stability, Molecular Dynamics simulations can be employed in subsequent research to depict the dynamic mechanisms of these dye complexes interacting with the active sites of metabolic enzymes (e.g., Cytochrome P450) or DNA fragments. This will help understand the metabolic stability argument from an atomistic perspective more thoroughly. To summarize, apart from showing how electronic tuning plays an essential part in making dyes safer, this study also lays down a reliable computational guideline that can be used for toxicological assessment of novel textile auxiliaries.

Artificial Intelligence Contribution Statement

This manuscript was entirely written, edited, analyzed, and prepared without the assistance of any artificial intelligence tools. All content, including text, data analysis, and figures, was solely generated by the authors.

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