Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

Anthraquinone

EC Number: 201-549-0
CAS Number: 84-65-1

CLH-O-0000001412-86-86/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

4 December 2015

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

Substance Name: Anthraquinone

EC Number: **201-549-0**

CAS Number: **84-65-1**

Index Number: **-**

Contact details for dossier submitter:

BAuA
Federal Institute for Occupational Safety and Health
Federal Office for Chemicals
Friedrich-Henkel-Weg 1-25
D-44149 Dortmund, Germany

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Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	anthraquinone
EC number:	201-549-0
CAS number:	84-65-1
Annex VI Index number:	-
Degree of purity:	≥ 98.5%
Impurities:	See confidential Annex

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	-
Current proposal for consideration by RAC	Carc. 1B, H350
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Carc. 1B, H350

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives				Not assessed in this dossier.
2.2.	Flammable gases				Not assessed in this dossier.
2.3.	Flammable aerosols				Not assessed in this dossier.
2.4.	Oxidising gases				Not assessed in this dossier.
2.5.	Gases under pressure				Not assessed in this dossier.
2.6.	Flammable liquids				Not assessed in this dossier.
2.7.	Flammable solids				Not assessed in this dossier.
2.8.	Self-reactive substances and mixtures				Not assessed in this dossier.
2.9.	Pyrophoric liquids				Not assessed in this dossier.
2.10.	Pyrophoric solids				Not assessed in this dossier.
2.11.	Self-heating substances and mixtures				Not assessed in this dossier.
2.12.	Substances and mixtures which in contact with water emit flammable gases				Not assessed in this dossier.
2.13.	Oxidising liquids				Not assessed in this dossier.
2.14.	Oxidising solids				Not assessed in this dossier.
2.15.	Organic peroxides				Not assessed in this dossier.
2.16.	Substance and mixtures corrosive to metals				Not assessed in this dossier.
3.1.	Acute toxicity - oral				Not assessed in this dossier.
	Acute toxicity - dermal				Not assessed in this dossier.
	Acute toxicity - inhalation				Not assessed in this dossier.
3.2.	Skin corrosion / irritation				Not assessed in this dossier.
3.3.	Serious eye damage / eye irritation				Not assessed in this dossier.
3.4.	Respiratory sensitisation	None		None	Not assessed in this dossier.
3.4.	Skin sensitisation				Not assessed in this dossier.
3.5.	Germ cell mutagenicity	None		None	Conclusive but not sufficient for classification.
3.6.	Carcinogenicity	Carc. 1B		None	

3.7.	Reproductive toxicity	None		None	Not assessed in this dossier.
3.8.	Specific target organ toxicity –single exposure				Not assessed in this dossier.
3.9.	Specific target organ toxicity – repeated exposure				Not assessed in this dossier.
3.10.	Aspiration hazard				Not assessed in this dossier.
4.1.	Hazardous to the aquatic environment				Not assessed in this dossier.
5.1.	Hazardous to the ozone layer				Not assessed in this dossier.

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Pictogram:

GHS08

Signal word: Danger

Hazard statements: H350 (May cause cancer.)

Proposed notes assigned to an entry:

2 BACKGROUND TO THE CLH PROPOSAL

This CLH proposal aims to classify and label anthraquinone (AQ) for carcinogenicity. For the purpose of this dossier the available REACH registration data has been taken into account (January 2015).

AQ can be applied in the production of cellulose fibres. In case of use of such fibres for manufacturing of paper and board for food contact any traces of the substance that remain in the cellulose can transfer to foodstuffs.

So far, AQ was not listed in any priority list of Existing Substance Regulation (Regulation 793/93/EC).

AQ was evaluated in the framework of Directive 91/414/EEC (concerning the placing of plant protection products on the market). As result of that review AQ has not been included in the Annex I to Directive 91/414/EEC (list of active substances that are authorised for the use in plant protection products). An EFSA opinion (2012) stating, that a carcinogenic potential for AQ cannot be excluded.

There is also a decision not to include AQ in the Annex I, IA and IB to Directive 98/8/EC concerning the placing of biocidal products on the market (Commission Directive 2007/565/EC).

2.1 History of the previous classification and labelling

AQ is not listed in Annex VI to Regulation (EC) 1272/2008 on Classification, Labelling and Packing of Dangerous Substances.

Based on the results of two carcinogenicity studies (oral administration of AQ to mice and rats; NTP 2005) it was concluded by IARC in 2012 that there is sufficient evidence in the experimental animals for carcinogenicity. Therefore, AQ was evaluated as possible carcinogenic to human (group 2B). Since this evaluation no further animal data has become available. The same experimental studies are re-evaluated for justification of classification of AQ as carcinogenic according to CLP Regulation.

2.2 Short summary of the scientific justification for the CLH proposal

The available carcinogenicity studies on AQ as tested by the NTP (2005) showed that there was clear evidence of carcinogenic potential of AQ in female F344/N rats as well as clear evidence of carcinogenic activity in male and female $B6C3F_1$ mice. The tumour findings are consistently observed in target organs showing toxic effects and precursor lesions in repeated dose studies and in organs with highest tissue concentrations (see toxicokinetic data). It is not possible to determine to what extent, if any, the impurity 9-nitroanthracene (9-NA) has influenced the outcomes of the NTP carcinogenicity study on AQ. The low exposure level, the bioavailability, and the weak mutagenicity (positive effects in bacterial gene mutation tests but equivocal effect (L5178Y cells) or marginal positive effect (h1A1v2 cells) in gene mutation tests) make it unlikely and implausible that 9-NA was solely and totally responsible for the carcinogenic response. Due to biotransformation processes mutagenic metabolites of AQ appear, which are at least five times more potent and present at systemically higher concentration than 9-NA. Therefore, the results are attributable to AQ (and its active metabolites) and AQ is considered to be carcinogenic. AQ was not clearly demonstrated as mutagenic in the available tests; however, a contribution of mutagenicity (by mutagenic metabolites) cannot be excluded.

Overall, the NTP studies are valid and there is sufficient evidence for carcinogenicity. A classification of AQ as carcinogenic Category 1B with the hazard phrase 'H350: May cause cancer.' is proposed according to CLP Regulation.

2.3 Current harmonised classification and labelling

AQ is not classified according Annex VI to Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging of Dangerous Substances.

2.4 Current self-classification and labelling

Table 4: Notified classification and labelling according to CLP criteria (December 2014)

Classification		Labelling			Specific Concentration limits, M-Factors	Notes	Number of Notifiers
Hazard Class and Category Code(s)	Hazard Statement Code(s)	Hazard Statement Code(s)	Supplementary Hazard Statement Code(s)	Pictograms, Signal Word Code(s)			
Skin Sens. 1	H317	H317		GHS07 Wng			96
Not Classified							33
Acute Tox. 4 Skin Sens. 1 Acute Tox. 4	H312 H317 H332	H312 H317 H332		GHS07 Wng			24
Acute Tox. 4 Acute Tox. 4 Aquatic Acute 1	H312 H332 H400	H312 H332 H400		GHS07 GHS09 Wng			4
Skin Sens. 1B	H317	H317		GHS07 Wng			3
		H351 H317		GHS07 GHS08 Wng			2
Acute Tox. 4 Acute Tox. 4 Acute Tox. 4	H302 H312 H332	H302 H312 H332		GHS07 Wng			1
Acute Tox. 4	H332	H332		GHS07 Wng			1

Number of Aggregated Notifications: 8

RAC general comment
Anthraquinones are derived from an anthracene ring (tricyclic aromatic) with two keto groups, one each on carbon atoms nine and ten (figure below). The substituents present in the anthracene ring of anthraquinone derivatives can have a major impact on the mutagenic and carcinogenic properties of this family of substances.

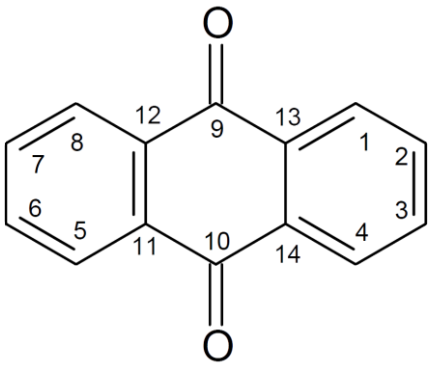


Figure: Anthraquinone: Basic structure and carbon numbering

Anthraquinone (AQ), also known as 9,10-dioxoanthracene, is a widely used raw material for the manufacture of many synthetic dyes and naturally occurring pigments such as alizarin (a 1,2-dihydroxyl derivative). AQ is also used as a catalyst in chemical alkaline pulp processes in the paper and pulp industry for the production of cellulose. AQ is reported as a seed dressing in its intended function as a bird repellent but it is not registered for use as a plant protection product in Europe. Other uses include the production of chemically similar derivatives used as medicines (laxative, antimalarial and antineoplastic drugs). Anthraquinone-derived substances are found naturally from diverse sources, in aloe latex, senna, rhubarb, cascara buckthorn, fungi, lichens, and are typically cathartic when ingested.

Anthraquinone has no existing entry in Annex VI to the CLP regulation. The hazard classes considered in the CLH proposal by the dossier submitter (DS) Germany are mutagenicity and carcinogenicity only. Repeated dose toxicity data (and some other information) were provided by the DS in the CLH report as supportive information for assessment of the carcinogenicity potential of anthraquinone. A specific section on repeated dose toxicity is not included in this opinion but the relevant information is incorporated into the carcinogenicity section.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Harmonised classification and labelling of AQ as Carc. 1B, H350 (May cause cancer) is proposed, which is considered a community wide action according to Article 36 regulation (EC) No 1272/2008. It is recommended that the classification proposal is considered for inclusion on Annex VI to Regulation (EC) No 1272/2008, table 3.1, with regard to Article 42.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

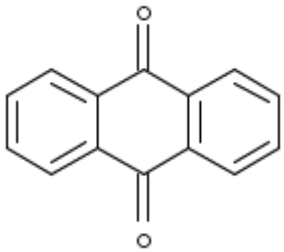
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 5: Substance identity

EC number:	201-549-0
EC name:	anthraquinone
CAS number (EC inventory):	84-65-1
CAS number:	84-65-1
CAS name:	9,10-Anthracenedione
IUPAC name:	9,10-anthraquinone
CLP Annex VI Index number:	-
Molecular formula:	$C_{14}H_8O_2$
Molecular weight range:	208.2 g/mol

Structural formula:



1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks

Current Annex VI entry:

Table 7: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks

Current Annex VI entry:

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks

Current Annex VI entry:

1.2.1 Composition of test material

1.3 Physico-chemical properties

Table 9: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Light yellow to almost colorless crystals	O´Neil 2001	Handbook data (Merck Index)
	Yellow odourless powder	DEZA a.s. 2010	EPA OPPTS 830.6302 (Colour) EPA OPPTS 830.6303 (Physical State) EPA OPPTS 830.6304 (Odour)
Melting/freezing point	ca. 284.6 °C, ca. 100 kPa	DEZA a.s. 2008	OECD Guideline 102 (capillary method)
Boiling point	377 °C, 760 mm Hg	O´Neil 2001	Handbook data (Merck Index)
Relative density	1.42 — 1.44, 20 °C	O´Neil 2001	Handbook data (Merck Index)
	1.261, 20 °C	DEZA a.s. 2008	OECD Guideline 109 (buoyancy method)
Vapour pressure	0.000000116 mm Hg 25 °C	Shimizu et al. 1987	Experimental result
Surface tension			water solubility is below 1 mg/l at 20 °C
Water solubility	74.6 µg/L at 20 ºC, pH 7	Cook 2010	EPA Pesticide Assessment Guidelines Subdivision N, Section 161-1 (column elution method)
	190 µg/L at 20 ºC, pH: ≥5.5 - ≤ 6.5	DEZA a.s. 2009	OECD Guideline 105, (column elution method)
	0.17 mg/L at 20 ºC, pH 5.6	Lewandowska 2012	OECD Guideline 105, EU Method A.6 (flask method) Value might not be valid as for soluble amount of 0.17mg/L the wrong method was used, as the column elution method should be used for water solubility's < 10 mg/L
Partition coefficient n-octanol/water	Log Pow = 3.4 at pH=7, 30 °C	Cook 2010	ASTM E 1147-92 (HPLC Method)
Flash point			
Flammability			
Explosive properties			
Self-ignition temperature			
Oxidising properties			
Granulometry	Mass median diameter: 70.98 — 71.62 µm D10: 15.52 µm D50: 53.74 µm D90: 141.97 µm	Al Amin 2012	volumetric distribution, OECD Guideline 110 (Coulter LS 230)
	Mass median diameter:	VUOS a.s. 2010	counted distribution, OECD

	15.49 µm D10: 8.05 µm D90: 32.46 µm		Guideline 110 (LA-950 Laser Scattering Particle Size Distribution Analyzer - WET was used)
Stability in organic solvents and identity of relevant degradation products	-		
Dissociation constant	-		
Viscosity	-		

2 MANUFACTURE AND USES

AQ can be applied in the production of cellulose fibres.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not evaluated in this report.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

An overview on toxicokinetics of AQ is given for information.

4.1.1 Non-human information

Table 10: Summary table of toxicokinetics studies

Method	Results	Remarks	Reference
Fischer 344 rat/ $B6C3F_1$ mice 1. Imbedded toxicokinetic study (in NTP carcinogenicity assay): 18M rats dosed 469, 938, 1,875 or 3,750 ppm and 36M mice dosed 833, 2,500, or 7,500 ppm for 8 d. Also 10M/10F rats dosed 469, 938, 1,875 or 3,750 ppm and 10M/10F mice dosed 833, 2,500, or 7,500 ppm. Blood collection and analysis after 8 d, 3, 6, 12, and 18 months (rats) / 8 d, 12 months (mice). 2. Single dose toxicokinetics in 18 months old animals: 100 mg/kg in rats (14F/14M) and 200 mg/kg in mice (13F/14M) per gavage Blood collection and analysis after 2, 6, 12, 24, and 36 h (rats) / 1, 2, 4, 8, and 12 h (mice).	AQ absorbed from gastrointestinal tract and distributed to tissues. No bioaccumulation. Majority of AQ eliminated in the faeces and urine within 24 h. 96 h after dosing, less than 5% of the dose remained in the tissues. Metabolites identified: yes Details on metabolites: 1-hydroxyanthraquinone (1-OH-AQ) 2-hydroxyanthraquinone (2-OH-AQ)	Test material (EC name): 9,10-^{14}C-anthraquinone	NTP 2005
Chester-Beatty rat (4 rats) metabolism study diet containing 5% (w/w) for 4 d Urine collection and analysis.	Sulfate and glucuronide conjugates of 2-hydroxyanthraquinone, 9,10-dihydroxyanthracene and 2,9,10-trihydroxyanthracene were found in pooled urine samples.	Test material (EC name): 9,10-anthraquinone	Sims 1964

rat metabolism study 100 mg in diet Urine collection and analysis.	Detection of 1-hydroxy and 2-hydroxy-anthraquinone in the urine	Test material (EC name): 9,10-anthraquinone	Sato et al. 1956
Male Mura SPRA (SPF 68 Han) rat (M/F) Dose levels: 0.1, 1.0 and 3.0 mg/kg bw Route of exposure: intravenous and oral Measurement of toxicokinetic parameters. Deviation from official protocol: Too limited description.; no high dose; no repeated doses; no observation of toxicity. GLP: no	Almost complete absorption and distribution throughout the body (highest relative concentrations in liver and kidney). 90-95 % AQ excreted via urine and faeces within 48 h. Metabolites identified: no	Test material (EC name): 9,10-^{14}C-anthraquinone	Bayer AG 1983
Sprague-Dawley rats (5M) Single dose: 5.0 mg/kg bw Urine and faeces were collected until 48 h post administration. Metabolites were studied and identified in naïve urine and in methanolic extracts of faeces. Deviation from official protocol: Too limited description. No proposed biotransformation pathway. GLP: no	Metabolites identified: yes Details on metabolites: 1-OH-AQ 2-OH-AQ	Test material (EC name): 9,10-^{14}C-anthraquinone	Bayer AG 1985

Absorption

The National Toxicology Program (NTP 2005) found in their toxicokinetic experiments that doses of 0.35 to 350 mg/kg bw [^{14}C] AQ were well absorbed (> 99% of the administered dose from the gastrointestinal tract in male F334 rats). Studies conducted by Bayer AG (1983 and 1985) also suggested that AQ is almost completely absorbed after oral administration and that a short lag period of approximately 2-3 minutes occurs before absorption commences).

Distribution

After absorption AQ was distributed to various tissues, with the highest concentration observed in the adipose tissue, but no bioaccumulation was shown in any particular tissue (NTP 2005). The plasma concentration of AQ in female rats was approximately twice the concentration in male rats (NTP 2005). In addition to the NTP studies, Bayer AG (1983) indentified that the liver and the kidneys are the organs with the highest relative concentration. The concentrations of rat organs at 48 h following a single oral administration of 1 mg/kg bw were up to 0.47 % and 0.55 % in the liver or kidney, respectively, in female rats and up to 0.44 % and 0.29 % in the liver or kidney, respectively, in male rats. The maximum plasma level (C_{max}) was reached after 2.5 h after 0.1 mg/kg bw, or after 5 h or 12 h for male or female rats treated with 1 mg/kg bw, respectively (Draft Assessment Report – Anthraquinone 2006). Also, results of repeated oral administration studies showed distribution of AQ throughout the whole body (incidences of non-neoplastic lesions of the kidney, liver, spleen, and bone marrow in male and female rats, the liver, urinary bladder, and spleen in male and female mice, and the thyroid gland and kidney in male mice; NTP 2005).

Metabolism

The major metabolite detected by the NTP (2005) in the urine samples of rats after oral or intravenous exposure was 2-hydroxyanthraquinone (2-OH-AQ). This is in accordance with earlier studies, where 1-hydroxyanthraquinone (1-OH-AQ) and 2-OH-AQ were identified in the urine of rats after daily oral administration of AQ (Sato et al. 1956). Bayer AG (1985) found that approximately 25% of the administered dose was metabolised and 2-OH-AQ was the main metabolite (20% of the total recovered radioactivity in urine, 4% in faeces) followed by 1-OH-AQ. In this study 41% of the recovered radioactivity (urine + faeces + body) consisted of not metabolised AQ, with only 1% unchanged AQ detected in urine. Furthermore, sulphate and glucuronide conjugates (of 2-OH-AQ, 8,10-dihydroxyanthracene and 2,9,10-trihydroxyanthracene) were found in the pooled urine samples of 4 male Chester-Beatty rats, which had received a diet containing 5% AQ for 4 days (Sims 1964). Last, anthrone was reported as metabolite by the NTP (2005). However, no further robust evidence for its occurrence as metabolite was given.

Excretion

In the NTP studies the majority of the radiolabeled substance was eliminated in the faeces and the urine by 48 h after administration. Within 96 h more than 95% of the administered dose was metabolised and eliminated in the bile, faeces and urine at all dose levels (NTP 2005). Bayer AG (1983) reported an excretion of 90-95% of the administered dose (oral and intravenous) within 48 h, whereby 55-59% were represented in the faeces and 33-37% in the urine. A single dose intra-duodenal administration demonstrated that the biliary route is an important pathway for faecal excretion of AQ (Bayer AG 1983). Bayer AG (1985) found 40% excretion of the radioactivity via urine and 60% via faeces within 48 h after oral administration of a single oral dose of 5 mg/kg bw AQ.

4.1.2 Human information

No information is available.

4.1.3 Summary and discussion on toxicokinetics

AQ is readily, rapidly and completely absorbed in the gastrointestinal tract of rats and distributed throughout the whole body. The highest concentrations after distribution with no indication of bioaccumulation have been found in adipose tissue as well as in liver and kidney. Identified metabolites comprise 1-OH-AQ and 2-OH-AQ as major ones as well as probably their sulphate and glucuronide conjugates. Sulphate and glucuronide conjugates of 2-OH-AQ, 8,10-dihydroxyanthracene and 2,9,10-trihydroxyanthracene were identified. Besides urine bile fluid represented an important route of excretion. The majority (95%) of the radiolabeled substance is eliminated within 48-96 h via faeces and urine, whereby over 50% of the eliminated radioactivity was found in the faeces.

4.2 Acute toxicity

Not evaluated for this dossier.

4.3 Specific target organ toxicity – single exposure (STOT SE)

Not evaluated for this dossier.

4.4 Irritation

Not evaluated for this dossier.

4.5 Corrosivity

Not evaluated for this dossier.

4.6 Sensitisation

Skin sensitisation as well as respiratory sensitisation was not evaluated for this dossier.

4.7 Repeated dose toxicity

The studies on repeated dose toxicity are reported for purpose of background information only. The findings are of relevance to identify toxic effects and possible precursor lesions with regard to hazard class toxicity.

4.7.1 Non-human information

Table 11: Summary table of repeated dose toxicity studies

Method	Results	Remarks	Reference
rat (Wistar) (5M/5F/per dose group) subacute (oral: gavage) 0, 2, 10, 20, 50 and 250 mg/kg (actually ingested) Exposure: 28 days GLP: no Not compliant with 67/548/EEC Annex V methods or OECD 407 (no guidelines existed at that time)*	NOAEL: 2 mg/kg bw/day LOAEL: 10 mg/kg bw/day M/F ≥ 10 mg/kg bw/day: - Rel. liver weight ↑ (8%/10% M/F) associated with minimal hepatocyte enlargement centrilobular/ midzonal/periportal - Rel. kidney weight↑ (10% F) - Rel. spleen weight↑ (13%/22% M/F) associated with minimal spleen congestion	experimental result Test material (EC name): Anthraquinone	Bayer AG 1976
rat (Wistar) (20M/20F per dose group) subchronic (oral: feed) 0, 15, 150, 1,500 ppm (0, 1.36, 12.58, 126.25 mg/kg bw/d for M, 0, 1.79, 16.79 and 175.21 mg/kg bw/d for F) (nominal in diet) Exposure: 3 months (daily) GLP: no Not compliant with 67/548/EEC Annex V methods or OECD 408 (no guidelines existed at that time)**	NOAEL: 15 ppm (1.36/1.79 mg/kg bw M/F) Not taking into account the slightly reduced body weight (5% in M/F), increased reticulocyte count (108%/92% M/F week 6, 11%/54% M/F week 13) and minimal decreases in RBC counts (6%/5% M/F week 6, 2%/3% M/F week 13) LOAEL: 150 ppm (12.58/16.79 mg/kg bw M/F) M/F ≥ 150 ppm: reduced body weight (↓6%/↓7% M/F) and food consumption (↓10 % M/F) Increases in reticulocyte counts ↑149%/↑92% M/F week 6, ↑99%/↑136% M/F week 13 Increased RBC ↑6%/↑6% M/F week 6, ↑5%/↑6% M/F week 13 Cholesterol ↑ F Albumin ↑ M/F Abs. liver weight (↑22%) Same effects at M/F≥ 1,500 pmm with increasing effect strength and at this concentration also changes in abs. thyroid weight (↑42%) appeared.	experimental result Test material (EC name): Anthraquinone	Bayer AG 1979

F344/N rats (10M/10F) subchronic (oral: diet) 0, 1,875, 3,750, 7,500, 15,000, and 30,000 ppm (0, 135, 275, 555, 1,130 and 2,350 mg/kg bw) Exposure: 14 weeks (daily)	LOAEL: 1,875 ppm M/F ≥1,875 ppm: Slight anaemia (Hct, Hb and RBC↓, levels ≤10%), MCV↑, MCHC↓, reticulocytes↑, platelet counts ↑ ALP↓, creatinine ↑, total protein↑, albumin ↑ M≥7,500 ppm urea nitrogen↑ M≥1,875 ppm, F ≥15000 ppm: Normalized urine AST↑, NAD↑, urine protein↑ M/F ≥1,875 ppm: Abs/rel. liver weight ↑ Abs/rel. kidney weight↑ Abs. testis weight ↑ (rel from 7500 ppm) F ≥15,000 ppm: Estrous cycle length↑ *Liver*: M/F ≥1,875 ppm: Centrolobular liver cell hypertrophy: minimal to moderate *Kidney*: M/F ≥1,875 ppm: Renal tubular hyaline droplet accumulation: M mild-moderate (PAS negative), F Minimal-mild (PAS positive) M ≥1,875 ppm; F ≥ 15,000 ppm: Nephropathy in all males including controls, lesion more severe in all exposed groups (positive immune resposnse to alpha2 u-globulin) of M and F ≥ 15,000 ppm *Spleen*: M/F ≥1,875 ppm extramedullar haematopoiesis ↑,(iron-positive) pigmentation↑ *Bone marrow:* M ≥3,750 ppm,F ≥1,875 ppm Haematopoiesis↑ minimal-slight *Thyroid* : M/F ≥3,750 ppm Follicular cell hypertrophy, minimal Urinary bladder: F 30,000 ppm Inflammation & transitional epithelial hyperplasia	experimental result Test material (EC name): Anthraquinone	NTP 2005

$B6C3F_1$ mice (10M/10F) subchronic (oral: diet) 0, 1,875, 3,750, 7,500, 15,000, or 30,000 ppm (250, 500, 1,050, 2,150, or 4,300 mg/kg in males and 300, 640, 1,260, 2,600, or 5,300 mg/kg in females) Exposure: 14 weeks (daily)	LOAEL: 1875 ppm. M ≥7,500 ppm F ≥1,875 ppm Slight anaemia reticulocytes↑, (Hct↓ (high dose M 12%, high dose F 8%), Hb ↓ (6%, 5%) and RBC↓ (13%, 12%)), M/F ≥15,000 ppm MCV↑, MCH↑, MCHC↑, M ≥15,000 ppm F ≥1875 ppm platelet counts ↑ (no data on clinical biochemistry and urine) <u>M/F≥1,875 ppm</u> Abs/rel liver weight ↑ <u>M/F 30,000 ppm</u> Abs/rel kidney weight ↑ <u>*Liver*</u> M/F≥3,750 ppm Centrilobular hypertrophy, mild-moderate Urinary bladder cytoplasmic alteration (intracellular eosinophilic granules) minimal - severe <u>*Spleen*</u> M/F≥1,875 ppm Haematopoiesis (minimal/mild), pigmentation (minmal-mild)	experimental result Test material (EC name): Anthraquinone	NTP 2005
rat chronic (inhalation: dust) (whole body) 12.2 ± 0.7 mg/m³ (analytical conc.) 5.2 ±0.8 mg/m³ (analytical conc.) Vehicle: air Exposure: 5-6 h (daily, 4 months) Due to the lack of information, no statement of compliance with OECD Guideline 413 is possible***	NOAEC: 5.2 mg/m³ air (analytical) LOAEC: 12.2 mg/m³ air (analytical) ↓body weight Slight anaemia (↓Hb, ↓RBC, reticulopenia) <u>*Lungs*</u>: emphysema, atelectasis, cellular proliferation, in particular perivascular hyperaemia of the capillaries and exsudation in the alveolar lumen without any information on affected species, number of animals, sexes and treatment.	Test material (EC name): Anthraquinone <u>Clear limitations</u>: Number and species of exposed animals is unclear, only two dose groups were tested, it is not specified which organs were examined (in histological investigations).	Volodchenko et al. 1970

* Deviation from to date standard protocol: No weight of epididymes and thymus, no histology of nerve, thymus and trachea, no reactivity test, only one observation a day, no measurement of electrolytes and total proteins in blood and no measurement of food consumption (Draft Assessment Report – Anthraquinone 2006).
** Deviation from to date standard protocol: No reactivity test and weekly checking out of cage, no platelets count, no weighing of the brain, epididymes and uterus, no histology of a nerve and the uterus, blood tests on 5 rats/sex/dose only, no measurement of electrolytes and no ophthalmological tests (Draft Assessment Report – Anthraquinone 2006).
*** Deviation from to date standard protocol: number and species of exposed animals is not clear, only two dose groups were tested, no statement concerning clinical and cage-side observation, food consumption not reported, it is not sound enough specified which parameters were measured (clinical chemistry haematology) and which organs were examined (in the histological investigations); the same is true for a in-depth analysis of the respiratory tract.

Repeated dose toxicity: oral

1. Subacute study (Bayer AG 1976)

The study is summarised in the Draft Assessment Report – Anthraquinone (2006), which was the base for the following paragraph.

In a 28 day subchronic oral study male and female Wistar rats were administered 0, 2.0, 10, 20, 50 and 250 mg/kg bw AQ per gavage. Clinical observations reported that the animals showed lowered general conditions (effects not specified) at doses of 10 mg/kg bw/d. Body weight was decreased at 50 mg/kg bw/d onwards during week 1 up to week 4 in males and at 20 mg/kg bw/d onwards during week 2 up to week 4 in females. In haematology and clinical chemistry no adverse effects or abnormalities were reported. Sometimes absolute organ weights were altered, but no dose relationship was observed. Therefore, these effects were considered of no toxicological relevance. Relative liver weights as well as relative spleen weights were increased beginning at 10 mg/kg bw/d. Relative kidney weights were increased at 50 mg/kg bw/d and at higher doses. Histological investigations showed enlarged hepatocytes (which frequently involve periportal as well as centrilobular and midzonal hepatocytes) beginning at doses of 10 mg/kg bw/d and becoming more pronounced at 250 mg/kg bw/d. Males were more severely affected than females at all dose levels. This effect was viewed as adaptive, because no clinical chemistry alterations or adverse histological lesions (such as hyperplasia, necrosis or degeneration) were found. Congestion of the spleen was observed in some rats at 10 and 20 mg/kg bw and in all rats dosed at 50 mg/kg and 250 mg/kg bw. This effect was considered to be compound-related. Table 12 summarises the results:

Table 12: Effects of the subacute study

Endpoints/dose	**0**		**2.0**		**10**		**20**		**50**		**250 mg/kg bw/d**	
sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Body weight								↓5-6%	↓%4-11%	↓4-12%	↓%11-16%	↓5-10%
Relative organ weight:												
Liver					↑8%	↑10%	↑10%	↑15%	↑24%	↑24%	↑60%	↑64%
Spleen					↑13%	↑22%	↑51%	↑33%	↑22%	↑28%	↑23%	↑20%
Thyroid									↑25%		↑75%	
Kidney						↑10%			↑9%	↑9%	↑13%	↑12%
Testes									↑13%		↑15%	↓25%
Adrenals								↓15%	↑15%	↓16%		↓16%
Brain									↑7%		↑5%	
Histopathology:												
Liver: hepatocyte enlargement centrilobular/midzonal/periportal												
minimal					4/5	2/5	0/5	0/5	1/5	4/5	0/5	4/5
moderate									4/5	1/5	5/5	1/5
Spleen:												
congestion												
minimal					3/5	3/5	5/5	5/5	4/5	1/5	2/5	2/5
moderate					0/5	2/5	0/5	0/5	1/5	4/5	3/5	3/5

A NOAEL of 2 mg/kg bw/d is proposed, based on increased relative spleen weight associated with minimal splenic congestion at 10 mg/kg bw/d.

2. Subchronic study (Bayer AG 1979)

The study is summarised in the Draft Assessment Report – Anthraquinone (2006), which was the base for the following paragraph.

In a 90-day dietary study with Wistar rats, males and females were exposed to 0, 15, 150, 1,500 ppm AQ. Clinical observations showed no difference in appearance, behaviour or mortality. Food consumption was reduced for males and females at 150 ppm and 1,500 ppm. Body weight was decreased in female rats at all doses and in male rats at 1,500 ppm. Reticulocyte counts raised and RBC counts were slightly reduced at all concentrations, suggesting haemolytic anaemia. However, these effects were seen at week 6 of exposure, but decreased in males at week 13, while still being increased in females. At doses of 150 ppm and 1,500 ppm blood cholesterol and albumin were increased in males and females. Albumin was also increased in urine at these doses, whereas reduced urea was measured at all doses levels. Liver weight was increased in male rats at 150 ppm and 1,500 ppm. Relative thyroid and liver weight was increased in males at 1,500 ppm. No evidence of pathological organ changes was revealed in the histological investigations**.** A summary of the results is given in table 13.

Table 13: Effects of the subchronic study

Endpoints/dose	**0**		**15**		**150**		**1,500 ppm**	
sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
mortality								
Body weight			↓5%	↓5%*	↓6%	↓7%*	↓16%*	↓15%*
Food consumption					↓10%	↓10%	↓14 %	↓11 %
Hematology								
Week 6								
RBC count			↓6%	↓5%	↓6 %	↓6 %*	↓6%	↓4%
Reticulocyte count			↑108%*	↑92%*	↑149%*	↑92%*	↑191%*	↑128%*
Week 13								
RBC count			↓2%	↓3%	↓5 %*	↓6 %	↓5%	↓4%
Reticulocyte count			↑11%	↑54%*	↑99%*	↑136%*	↑16%	↑218%*
Clinical chemistry wk 13:								
Albumin						↑7%*		↑15%*
Cholesterol							↑41%*	↑88%*
Urinalysis wk 13								
Albumin					↑29%*	↑39%*	↑64%*	↑86%*
Organ weight								
Rel. liver weight								
Abs. Thyroid weight							↑42%	
Abs. Liver weight					↑22%		↑33%	

* Statistically significantly different from control.

A NOAEL of 15 ppm (1.36 mg/kg bw/d) was proposed, not taking into account the slightly reduced body weight, the increased reticulocyte counts and slightly, non-significantly decreased RBC counts, which could be considered as not adverse at that dose level due to the slight decreases in RBC counts (less than 10% level).

3. 14 week study in rats (NTP 2005)

This abstract has been extracted from the NTP Technical Report 494 - Anthraquinone (NTP 2005).

Groups of 10 male and 10 female F344/N rats were fed diets containing 0, 1,875, 3,750, 7,500, 15,000 or 30,000 ppm AQ (equivalent to average daily doses of approximately 135, 275, 555, 1,130 or 2,350 mg AQ/kg bw) for 14 weeks. All rats survived until the end of the study. Mean body weights of females were significantly lower in the exposed groups than in the control group. Feed consumption by the exposed and control groups was similar at the end of the study. Liver and kidney weights of exposed groups were greater than those of the controls, as were testis weights of males exposed to 7,500 ppm or greater. A minimal, responsive anaemia was apparent in groups of male and female rats exposed to 3,750 ppm or greater by day 26 of the study. The (regenerative haemolytic) anaemia persisted and involved all exposed groups of rats at the end of the study. Renal function was also affected by AQ exposure as demonstrated by increases in urine protein and glucose concentrations and aspartate aminotransferase and N-acetyl-ß-D-glucosaminidase activities. Estrous cycles were longer in 15,000 and 30,000 ppm females than in the controls. Groups of exposed rats showed liver hypertrophy (M/F ≥1875 ppm); eosinophilic hyaline droplets in the kidney (M/F ≥1875 ppm); congestion, hematopoietic cell proliferation, iron-positive pigmentation of the spleen (indicative of hemosiderin deposition; /F ≥1875 ppm); and bone marrow hyperplasia (M ≥3,750 ppm,F ≥1,875 ppm). The incidences of nephropathy in 15,000 and 30,000 ppm females were significantly greater than those in the controls, and the severities of nephropathy were increased in exposed groups of males and in 30,000 ppm females. The concentrations of α2u-globulin in the kidneys were significantly greater in all exposed groups of males. Thyroid gland follicular cell hypertrophy was present in all males and females exposed to 3,750 ppm or greater. Incidences of inflammation and transitional cell hyperplasia in the urinary bladder of 30,000 ppm females were greater than those in the controls. No NOAEL could be derived.

4. 14 week study in mice (NTP 2005)

This abstract has been extracted from the NTP Technical Report 494- Anthraquinone (NTP 2005).

Groups of 10 male and 10 female B6C3F1 mice were fed diets containing 0, 1,875, 3,750, 7,500, 15,000 or 30,000 ppm AQ (equivalent to average daily doses of approximately 0, 250, 500, 1,050, 2,150 or 4,300 mg/kg in males and 0, 300, 640, 1,260, 2,600 or 5,300 mg/kg in females) for 14 weeks. All mice survived until the end of the study. Mean body weights and feed consumption were similar among exposed and control groups. A responsive anaemia occurred in exposed mice at week 14 (M ≥7,500 ppm, F ≥1,875 ppm; in both sexes without unequivocal dose-response relationship). Liver weights of exposed groups of mice were significantly greater than those of the control groups. The incidences of centrilobular hypertrophy in the liver of mice exposed to 3,750 ppm or greater were significantly greater than those in the controls, and the severities increased with increasing exposure concentration. Cytoplasmic alteration of the urinary bladder was observed in all exposed mice, and the severities increased with increasing exposure concentration. The incidences of hematopoietic cell proliferation were increased in all exposed groups of males and females, and pigmentation was observed in the spleen of all exposed mice (except one male and one female in the 30,000 ppm groups). No NOAEL could be set.

Repeated dose toxicity: inhalation

Different experiments (chronic inhalation toxicity, acute and subacute oral toxicity, eye irritation, tests for photodynamic effects) were conducted in a study by Volodchenko et al. (1970). Test animals were 96 rats, 10 rabbits and 50 mice. A not specified number of animals (probably rats) was exposed to doses of 5.2±0.8 and 12.2±0.7 mg/m^3 (analytical) dust concentrations of AQ for 6-8 hours daily in the 4 month repeated dose inhalation toxicity study. At 12.2 mg/m^3 the following symptoms occurred: Body weight loss and changes in blood parameters, i.e. lowered levels of haemoglobin (76.2% after two months, 74.4% after 4 months), erythrocytopenia (7.5 million vs 9.08 million in the control animals), relative reticulopenia. The blood parameters normalized during the experimental period. Histopathological investigations showed several effects in the lungs: emphysema, atelectasis, cellular proliferation, in particular perivascular hyperaemia of the capillaries and exsudation in the alveolar lumen. The microstructural changes of the lung regenerated within the first month after termination of the experiment. No severe effects were observed in the 5.2 mg/m³ dose groups. This study is considered as not valid, because of the scarce information available.

Repeated dose toxicity: dermal

No information are available.

Repeated dose toxicity: other routes

No information are available.

Human information

No information is available.

Other relevant information

No information is available.

Summary and discussion of repeated dose toxicity

Four oral toxicity studies with repeated doses were performed. In two of them, both conducted by NTP (2005) no NOAEL can be derived. In these studies the doses were much higher than in the two older studies, performed by the Bayer AG (1976 and 1979). Target organs were the liver, kidneys, urinary bladder and the haematopoietic system.

The 28 day study with rats performed by Bayer AG (1976) included AQ doses from 2 up to 250 mg/kg bw/d given by gavage. A lowered general health condition of the laboratory animals was reported at 10 mg/kg bw/d onwards. They showed body weight decreases for doses beginning with 20/50 mg/kg bw/d (F/M). In addition, altered organ weights were seen, but these were considered to be related to the former effect (decreased body weight). Minimal congestion and increased weight was reported for the spleen. Hepatocyte enlargement (liver cell hypertrophy) and increased liver weights were reported. This effect was more severe in male rats and was not found to be associated with alterations in clinical chemistry. Probably this effect indicates an adaptive response.

A 90-day dietary rat study (Bayer AG 1979) was conducted at doses of 15, 150 and 1,500 ppm (ca. 1.4, 13 and 175 mg/kg bw/d). Reduction in body weight and food consumption was observed

beginning at 15 ppm. Increased reticulocytes and slightly decreased RBC count were measured from doses of 15 ppm onwards, indicative of haemolytic anaemia. However, after weighing and histological examination of the spleen (and other organs) no haemosiderin depositions or other related effects were seen in this study, the red blood cell reduction was quite low and no firm explanation could be given for the highly altered reticulocyte count. A NOAEL of 1.36 mg/kg bw/d is proposed due to the fact that the observed effects on body weight and blood count at this doses level were considered as minimal and non-adverse.

In the two 14-week NTP studies in rats (NTP 2005) and mice (NTP 2005) a mild, responsive anaemia was seen beginning with doses of 1,870 ppm (rat) and 3,750 ppm (mouse) and starting at day 26 of the study. Also, altered renal function and thyroid gland follicular cell hypertrophy were apparent in rats. Additionally, centrilobular hypertrophy of liver cells was observed in mice and rats at doses of 1,875 ppm and above. All exposed mice showed cytoplasmic alteration of the urinary bladder. Inflammation & transitional epithelial hyperplasia of the urinary bladder was observed in female rats at 30000 ppm. Hematopoietic cell proliferation and pigmentation in the spleen of mice and rats are effects that corresponded to the haemolytic and regenerative nature of the anaemia.

Induction of regenerative anaemia is consistent in studies conducted by NTP (2005) and the Bayer AG (1976 and 1979). Mild regenerative anaemia has been induced at doses of 1,875 ppm and above in rats and mice in the NTP studies (2005). Reductions in Hct, Hb and RBC did not exceed the 10% level in rats at 30,000 ppm and reached maximum -13% in mice at this dose. Anaemia observed in rats and mice is consistent with the findings in the 90 day study in rats conducted by the Bayer AG (1979). The lowest dose where anaemic effects have been observed was 150 ppm in the 3 month study of the Bayer AG (1979). However, no special staining on haemosiderin depositions were conducted in any of these studies and overall anaemic effects were weak. The NTP studies (2005) reported organ effects secondary to the aenemia such as spleen congestion, iron-positive pigmentation (hemosiderin desposition) as well as increased (extramedullar) hematopoietic cell proliferation, and bone marrow hyperplasia, whereby the overall effect was considered to be a regenerative haemolytic anaemia.

One study on chronic inhalation toxicity in rats is available (Volodchenko et al. 1970). The study has various limitations as it is only available in summary, only two dose groups were tested, number and species of exposed animals is not clear and it is not specified, which organs were examined in histological investigations. Hence, it consists of less valid information and is just reported for the sake of completeness. Effects on body weight, blood parameters and the lungs were observed, however the study documentation is insufficient to allow any conclusion from these data.

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

The presented studies on repeated dose were reported for purpose of background information only. The findings in repeated dose studies are of relevance to identify toxic effects and possible precursor lesions with regard to the hazard class carcinogenicity.

4.9 Germ cell mutagenicity (Mutagenicity)

Mutagenicity of AQ is discussed controversially in literature, mainly due to conflicting data from bacterial testing systems, where contamination of AQ samples with 9-NA play a major role. With regard to the carcinogenicity study of the National Toxicology Program (NTP 2005), especially the contaminant 9-NA and also metabolites of AQ have to be examined concerning their mutagenicity. AQ is produced by different production methods, which generate varying grades of purity. The

most common methods for AQ production are Oxidation of anthracene (AQ-OX), Friedel-Crafts technology (AQ-FC) and Diels-Alder chemistry (AQ-DA) (Butterworth et al. 2001).

Table 14: Summary table of relevant in vitro and in vivo mutagenicity studies with AQ

Method	Results	Remarks	Reference
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvr A (with and without S9-mix) Test concentrations: 0, 30, 60, 125, 250, 500, 1,000 and 2,000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: with and without S9-mix at the highest tested dose of 2,000 µg/plate negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'Purified NTP AQ-OX' without 9-nitroanthracene as impurity purity: 99 %	Butterworth et al. 2001
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvr A (with and without S9-mix) Test concentrations: 0, 30, 60, 125, 250, 500, 1,000 and 2,000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results: positive without and with S9-mix without S9-mix: - TA 98 (≥ 125 µg/plate); dose-dependent effect - TA 1537 (≥ 250 µg/plate); dose-dependent effect - TA 100 (2,000 µg/plate) with S9-mix: - TA 98 (≥ 1,000 µg/plate); dose-dependent effect - TA 1537 (≥ 1,000 µg/plate); dose-dependent effect cytotoxicity: no precipitations: with and without S9-mix at the highest tested dose of 2,000 µg/plate negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'NTP AQ-OX' with about 0.1 % 9-nitroanthracene as impurity purity: 99 %	Butterworth et al. 2001

Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvr A (with and without S9-mix) Test concentrations: 0, 30, 60, 125, 250, 500, 1,000 and 2,000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: with and without S9-mix at the highest tested dose of 2,000 µg/plate negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'AQ-FC' without 9-nitroanthracene as impurity purity: 99 %	Butterworth et al. 2001
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvr A (with and without S9-mix) Test concentrations: 0, 30, 60, 125, 250, 500, 1,000 and 2,000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: with and without S9-mix at the highest dose tested of 2,000 µg/plate negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'AQ-DA' without 9-nitroanthracene as impurity purity: 99 %	Butterworth et al. 2001
Mouse lymphoma assay (L5178Y cells; TK locus) Test concentrations (with and without S9-mix): 0, 1.57, 3.13, 6.25, 12.5, 25, 37.5 and 50 µg/mL GLP: yes Equivalent to OECD Guideline 476	Evaluation of results: negative cytotoxicity: no negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'AQ-DA' without 9-nitroanthracene as impurity purity: 99 % The highest tested concentration is about twice the solubility limit in medium.	Butterworth et al. 2001

Chromosome aberration test (CHO cells) Test concentrations (with and without S9-mix.): 0, 12.5, 25, 37.5 and 50 µg/mL GLP: yes Equivalent to OECD Guideline 473	Evaluation of results: negative cytotoxicity: no negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'AQ-DA' without 9-nitroanthracene as impurity purity: 99 % Solubility considerations determined the highest dose to be 50 µg/mL.	Butterworth et al. 2001
Micronucleus test (bone marrow) Mice: Crl:CD-1 (ICR) BR mice (5 males and 5 females for each dose and each harvest time) Test concentrations: gavage of 250, 2,500 and 5,000 mg/kg bw Sampling times: 24, 48 and 72 h after administration GLP: yes Equivalent to OECD Guideline 474	Evaluation of results: negative toxicity: 'lack of significant toxicity' (only information) negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation 'AQ-DA' without 9-nitroanthracene as impurity purity: 99 %	Butterworth et al. 2001

4.9.1 Non-human information

By means of a weight of evidence approach the existing information for the toxicological endpoint mutagenicity is sufficient for the conclusion that AQ is neither mutagenic in vitro nor in vivo.

In tests with positive results AQ is generally not highly purified or definitively burdened with mutagenic 9-NA as contamination. On the other hand, the metabolites 1-OH-AQ and 2-OH-AQ show mutagenic properties in vitro.

For justification of classification/non-classification of AQ only those mutagenicity studies are of major relevance, which were carried out in accordance with the corresponding OECD test guideline. Mutagenicity tests whose test performance was carried out similar to the corresponding OECD test guideline should be also considered for the description of mutagenic effects.

In vitro data

Bacterial gene mutation tests with AQ

Table 15: Summary table of bacterial gene mutation tests with AQ

Method	Results	Remarks	Reference
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvrA (with and without S9-mix) Test concentrations: 0, 30, 60, 125, 250, 500, 1,000 and 2000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: with and without S9-mix at the highest tested dose of 2,000 µg/plate negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'Purified NTP AQ-OX' without 9-nitroanthracene as impurity purity: 99 %	Butterworth et al. 2001
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvrA (with and without S9-mix) Test concentrations: 0, 30, 60, 125, 250, 500, 1,000 and 2,000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results: positive without and with S9-mix without S9-mix: - TA 98 (≥ 125 µg/plate); dose-dependent effect - TA 1537 (≥ 250 µg/plate); dose-dependent effect - TA 100 (2,000 µg/plate) with S9-mix: - TA 98 (≥ 1,000 µg/plate); dose-dependent effect - TA 1537 (≥ 1,000 µg/plate); dose dependent effect cytotoxicity: no precipitations: with and without S9-mix at the highest tested dose of 2,000 µg/plate negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'NTP AQ-OX' with about 0.1 % 9-nitroanthracene as impurity purity: 99 %	Butterworth et al. 2001

Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvr A (with and without S9-mix) Test concentrations: 0, 30, 60, 125, 250, 500, 1,000 and 2,000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: with and without S9-mix at the highest tested dose of 2,000 µg/plate negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'AQ-FC' without 9-nitroanthra-cene as impurity purity: 99 %	Butterworth et al. 2001
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvrA (with and without S9-mix) Test concentrations: 0, 30, 60, 125, 250, 500, 1,000 and 2,000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: with and without S9-mix at the highest dose tested of 2,000 µg/plate negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'AQ-DA' without 9-nitroanthra-cene as impurity purity: 99 %	Butterworth et al. 2001
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98, TA 100 and TA 102 (with and without S9-mix) Test concentrations: 0, 100, 333, 1,000, 3,333 and 10,000 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: with and without S9-mix ≥ 1,000 µg/plate vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: 100 %	NTP 2005 (Table E2)

Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98, TA 100 and TA 1537 (with and without S9-mix) Test concentrations: 0, 30, 100, 300, 1,000, 3,000 and 10,000 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: without S9-mix ≥ 30 µg/plate; with S9-mix ≥100 µg/plate vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: 99.8 %	NTP 2005 (Table E3)
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98 and TA 100 (S9-mix: with and without) Test concentrations: 0, 33, 100, 333, 1,000 and 2,500 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results: positive without and with S9-mix without S9-mix: - TA 98 (≥ 33 µg/plate); dose-dependent effect - TA 100 (≥ 1,000 µg/plate); dose-dependent effect with S9-mix: - TA 98 (≥ 333 µg/plate); dose-dependent effect - TA 100 (≥ 1,000 µg/plate); dose-dependent effect precipitations: without and without S9-mix at 2,500 µg/plate cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: 97 %	NTP 2005 (Table E1) (primary literature: Zeiger et al., 1988)
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98 and TA 100 (with and without S9-mix) Test concentrations: 0, 30, 100, 300, 1,000, 3,000 and 10,000 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results: negative precipitations: with and without S9-mix ≥ 1,000 µg/plate cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study preparation: A 65343 purity: not known	NTP 2005 (Table E4)

Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98 and TA 100 (with and without S9-mix) Test concentrations: 0, 30, 100, 300, ,1000, 3,000 and 10,000 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results: negative precipitations: with and without S9-mix ≥ 1,000 µg/plate cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study preparation: A 54984 purity: not known	NTP 2005 (Table E5)
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98 and TA 100 (with and without S9-mix) Test concentrations: 0, 30, 100, 300, 1,000, 3,000 and 10,000 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results: weakly positive in TA98 without S9-mix (all other positive results with and without S9-mix have been induced by concentrations, which also induced precipitations) without S9-mix: - TA 98 ≥ 100 µg/plate; dose-dependent effect - TA 100 = 10,000 µg/plate with S9-mix: - TA 98 ≥ 1,000 µg/plate ; dose-dependent effect - TA 100 = 10,000 µg/plate precipitations: with and without S9-mix ≥ 300 µg/plate cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: 99.4 %	NTP 2005 (Table E6)
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535, TA 1537, TA 1538 and TA1538 (with and without S9-mix) Test concentrations: 0, 0.2, 2.0, 10 and 20 µg/plate GLP: not reported Similar to OECD Guideline 471	Evaluation of results: weakly positive without S9-mix: - TA 98 ≥ 10 µg/plate - TA 1537 ≥ 10 µg/plate - TA 1538 ≥ 10 µg/plate with S9-mix: - TA 100 ≥ 20 µg/plate - TA 1537 = 20 µg/plate cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: not known	Liberman et al. 1982

Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvrA (with and without S9-mix) Test concentrations: 0, 1.5, 5.0, 15, 50, 150 and 1,000 µg/plate GLP: yes Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: equivocal with S9 mix cytotoxicity: not determined vehicle controls valid: yes positive controls valid: yes	4 (not assignable) purity: not known (no detailed description of experimental results)	Täublová 2009
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 97, TA 98, and TA 100 (with and without S9-mix) Test concentrations: 0, 5.0, 10, 50 and 200 µg/plate GLP: not reported Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: negative cytotoxicity: yes - without S9-mix: TA 98 at 200 µg/plate - with S9-mix: TA 97 at 200 µg/plate vehicle controls valid: yes positive controls valid: not reported	4 (not assignable) purity: not known	Sakai et al. 1985
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100 and TA 2637 (with and without S9-mix) Test concentrations: “0.1 to 1,000” µg/plate (only range reported) GLP: not reported Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: negative cytotoxicity: not reported vehicle controls valid: yes positive controls valid: yes	4 (not assignable) purity: not known	Tikkanen et al. 1983

Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535, TA 1537 and TA 1538 (with and without S9-mix) Test concentrations: "0.1 to 1,000" µg/plate (only range reported) GLP: not reported Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: negative cytotoxicity: not reported vehicle controls valid: not reported positive controls valid: not reported	4 (not assignable) purity: not known	Salamone et al. 1979
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 1535, TA 1537 and TA 1538 (with and without S9-mix) Test concentrations: "104 to 520" µg/plate (only range reported) GLP: not reported Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: negative cytotoxicity: no vehicle controls valid: not reported positive controls valid: not reported	4 (not assignable) purity: not known	Gibson et al. 1978
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) Test concentrations: not reported GLP: not reported Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: negative cytotoxicity: not reported vehicle controls valid: yes positive controls valid: yes	4 (not assignable) purity: not known	Anderson and Styles 1978

Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535, TA 1537 and TA1538 (with and without S9-mix) Test concentrations: not reported GLP: not reported Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: negative cytotoxicity: not reported vehicle controls valid: yes positive controls valid: yes	4 (not assignable) purity: not known	Brown and Brown 1976

Overall, AQ induced negative responses in bacterial tests.

Four bacterial gene mutation tests are available whose test performance is in accordance with OECD guideline 471 (Butterworth 2001). AQ samples with high purity (samples AQ-FC and AQ-DA; purity: 99 %; without 9-NA as impurity) were clearly negative. One sample of AQ (AQ-OX; purity 99 %) with about 0.1 % 9-NA as impurity induced positive effects with and without S9-mix. After the removal of this impurity AQ (sample AQ-OX purified; purity: 99 %) induced no mutagenic effects in bacteria. This suggests the conclusion that 9-NA affects the outcome of the bacterial experiments. For more information on 9-NA see section 4.9.3.

Tests whose test performance is closely related to OECD Test Guideline 471 support the negative results of the guideline-conform bacterial gene mutation tests (NTP 2005, tables E2, E3, E4 and E5). The results of two positive bacterial gene mutation tests (NTP 2005, table E1; Liberman et al. 1982) must be subjected a critical evaluation, because the tested AQ sample has only a purity of 97 % or is unknown and it is not known if e.g. mutagenic 9-NA was present as impurity. For another positive result (NTP 2005, table E6) the biological relevance is questionable, because the weak effect was induced only in one tester strain without S9-mix at a concentration near the solubility limit. Apart from that, further effects with and without S9-mix were induced only at concentrations, which also induced precipitations.

Mammalian cell tests with AQ

Table 16: Summary table of in vitro soma cell tests with AQ

Method	Results	Remarks	Reference
Mammalian cell gene mutation test (V79 cells) Test concentrations: 0, 1.25, 2.5, 5, 10, 20 µg/mL (with and without S9-mix) GLP: yes Due to the lack of information, no statement of compliance with OECD Guideline 476 is possible.	Evaluation of results: negative cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	4 (not assignable) purity: not known	Bednáriková 2010
Mouse lymphoma assay (L5178Y cells; TK locus) Test concentrations (with and without S9-mix): 0, 1.57, 3.13, 6.25, 12.5, 25, 37.5 and 50 µg/mL GLP: yes Equivalent to OECD Guideline 476	Evaluation of results: negative cytotoxicity: no negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation: 'AQ-DA' without 9-nitroanthra-cene as impurity purity: 99 % The testing limit is about twice the solubility limit in medium.	Butterworth et al. 2001
Gene mutation test (h1A1v2 cells; TK locus) Test concentrations: 0, 1, 10, 100, 250 and 5,000 ng/mL GLP: no information Not in accordance with OECD Guideline 476	Evaluation of results: negative cytotoxicity: no negative controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: 97 % The test was only done with metabolic activation. Cells of the hlAlv2 cell line constitutively express cytochrome P4501Al, which is known to be necessary for the metabolism of many promutagens. Insufficient data presentation (only pooled data of two independent experiments were given).	Durant et al. 1996
Chromosome aberration test	Evaluation of results: negative	1 (reliable without	Butterworth et

(CHO cells) Test concentrations (with and without S9-mix.): 0, 12.5, 25, 37.5 and 50 µg/mL GLP: yes Equivalent to OECD Guideline 473	cytotoxicity: no negative controls valid: yes positive controls valid: yes	restrictions) key study preparation: 'AQ-DA' without 9-nitroanthra-cene as impurity purity: 99 % Solubility considerations determined the highest dose to be 50 µg/mL.	al. 2001
Chromosome aberration test (V79 cells) Test concentrations: 0, 1.25, 2.5, 5.0, 10 and 20 µg/mL (with and without S9-mix) GLP: yes Not in accordance with OECD 473	Evaluation of results: negative cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	4 (not assignable) purity: not known	Lazová 2010
Micronucleus test (SHE cells) Test concentrations: 3.13, 6.25, 12.5 and 25 µg/mL GLP: no information Not in accordance with OECD Guideline 487 (SHE cells are not included in the guideline)	Evaluation of results: weakly positive metabolically active SHE cells: - weakly positive effect at the highest tested concentration of 25 µg/mL (doubling of the micronucleus frequency compared to the negative control) cytotoxicity: no negative controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: not known	Gibson et al. 1997

AQ does not induce mutagenic effects in mammalian cell cultures.

AQ (without 9-NA as impurity) induces neither a mutagenic effect in a mouse lymphoma assay (Butterworth et al. 2001) nor in a chromosome aberration test (Butterworth et al. 2001). Both test performances are equivalent to OECD Test Guideline 476, respectively OECD Test Guideline 473.

A gene mutation test with h1A1v2 cells is also negative (Durant et al. 1996). In a micronucleus assay in SHE cells (Gibson et al. 1997) AQ induced a weak positive effect (doubling of the micronucleus frequency compared to the negative control) at the highest tested concentration of 25 µg/mL. At both tests there exist methodological limitations. However, the biological meaning of the micronucleus test in SHE cells remains questionable, because the weak positive effect is induced by a charge of AQ with unknown purity, and it is not known whether e. g. mutagenic 9-NA was contained as an impurity in the tested sample.

In vivo data

Table 17: Summary table of in vivo mutagenicity studies with AQ

Method	Results	Remarks	Reference
Micronucleus test (bone marrow) Mice: Crl:CD-1 (ICR) BR mice (5 males and 5 females for each dose and each harvest time) Test concentrations: gavage of 250, 2,500 and 5,000 mg/kg bw Sampling times: 24, 48 and 72 h after administration GLP: yes Equivalent to OECD Guideline 474	Evaluation of results**:** negative toxicity: 'lack of significant toxicity' (only information) negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study preparation 'AQ-DA' without 9-nitroanthra-cene as impurity purity: 99 %	Butterworth et al. 2001
Micronucleus assay (bone marrow) Mice: B6C3F_1 mice (5 males only for each harvest time) Test concentrations: i.p. of 500, 1,000 and 2,000 mg/kg bw; each dose was injected three times at 24-hour intervals Sampling time: 24 h after third injection GLP: no information Similar to OECD Guideline 474	Evaluation of results: negative toxicity: no information negative controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: not known	NTP 2005 (Table 12)
Micronucleus assay (peripheral blood erythrocytes) Mice: B6C3F1 mice (5 males and 5 females for each concentration) Test concentrations: 1,875, 3,750, 7,500, 15,000 and 30,000 ppm for 14 weeks in the feed GLP: no information Not in accordance with OECD Guideline 474	Evaluation of results: positive Weak positive effect in males at the highest dose tested (doubling of the micronucleus frequency compared to the negative control) and positive effects in females at all doses tested without a clear dose-dependency. negative controls valid: yes positive controls valid: a positive control is lacking	4 (not assignable because of the lack of a positive control) purity: 99.8 %	NTP 2005 (Table 13)

DNA damage (alkaline elution technique coupled with a microfluorimetric method) Mice: Swiss CD1 Test concentration: i.p. of 250 mg/kg bw Sampling time: 4 h after administration GLP: no information Not in accordance with a OECD guideline; lack of a positive control; screening test without detailed information.	Evaluation of results: positive in cells of liver and kidney A three-fold increase of single-strand DNA-breaks was observed in liver and kidney cells compared to the solvent control. negative control: valid positive control: a positive control is lacking	4 (not assinable) purity: not known	Cesarone et al, 1982

No induction of mutagenic effects was observed in soma cells.

In a bone marrow micronucleus assays whose test performance is equivalent to OECD Test Guideline 474 a negative result was observed in male and female rats after oral administration of AQ (Butterworth et al. 2001).

A negative bone marrow micronucleus assay after i.p. injection of AQ (NTP 2005, table 12) has only limited evidence, because the test performance is not equivalent to OECD Test Guideline 474. There is no justification why only males were tested and not also females.

4.9.2 Human information

No data available.

4.9.3 Other relevant information

The description of mutagenicity of metabolites and impurities of the AQ sample used for the carcinogenicity testing of the NTP (2005) should be taken into account with regard to the discussion on induction of carcinogenic effects.

Of the impurities only the mutagenicity data of the 9-NA were considered. For other impurities such as anthrone, phenanthrene, and anthracene it is stated that they are not or only very weakly genotoxic (NTP 2005).

The mutagenicity of the main metabolite 2-OH-AQ as well as of the metabolite 1-OH-AQ were considered with regard to the carcinogenicity. Anthrone is also reported to be a minor metabolite, but its role in carcinogenicity is expected to be low and its mutagenicity is considered to be equivocal (NTP 2005).

With 9-NA, 2-OH-AQ and 1-OH-AQ only a few mutagenic studies were performed.

<u>Impurity (9-NA)</u>

Table 18: Summary table of bacterial gene mutation tests with 9-NA

Method	Results	Remarks	Reference
Bacterial reverse mutation test (Ames test; gene mutation) TA 98 and TA 100, (with and without S9-mix) Test concentrations: 0, 2.9, 11.4, 45.6 and 182.5 nmol/plate (equivalent to: 0, 0.6, 2.3, 9.4 and 37 µg/plate) GLP: no information Not in accordance with OECD Guideline 471	Evaluation of results: weakly positive without and with S9-mix without S9-mix: - TA 98 (≥ 45.6 nmol/plate) with S9-mix: - TA 98 (at182.5 nmol/plate) cytotoxicity: no vehicle controls valid: yes positive controls valid: no	4 (not assignable) purity: > 99%	Fu et al. 1985
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98 and TA 100 (with and without S9-mix) Test concentrations: 0, 0.1, 0.3, 1.0, 3.0 and 10 µg/plate GLP: yes Similar to OECD Guideline 471	Evaluation of results: positive without S9-mix; negative with S9-mix without S9-mix: - TA 98 (≥ 0.3 µg/plate); dose-dependent effect (Fmax.= 3.5) - TA 100 (10 µg/plate; Fmax = 2.0) cytotoxicity: no negative controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: 97 %	Butterworth et al. 2004
Bacterial reverse mutation ytest (Ames test; gene mutation) TA 98 and TA 100 (with and without S9-mix) Test concentrations: 0, 100, 333, 1,000, 3,333 and 6,667 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results: positive without and with S9-mix without S9-mix: - TA 98 (≥ 100 µg/plate); dose-dependent effect (Fmax = 5.2) - TA 100: negative with S9-mix: - TA 98 (≥ 100 µg/plate); dose-dependent effect (Fmax = 5.2) - TA 100 (≥ 100 µg/plate); dose-dependent effect (Fmax = 8.2) precipitations: with and without S-9 mix ≥ 1,000 µg/plate cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: not known	NTP 2005 (Table 11)
Bacterial reverse mutation test (Ames test; gene mutation)	Evaluation of results: weakly positive without and with S9-mix without S9-mix:	4 (not assignable) purity: 'highly purified'	Pitts et al. 1982

TA 98 and TA 98R (with and without S9-mix) Test concentrations: not specified GLP: no information Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	- TA 98: 0.30 rev/µg - TA 98NR: 0.14 rev/µg with S9-mix: - TA 98: 0.32 rev/µg - TA 98NR: 0.13 rev/µg cytotoxicity: not reported vehicle controls valid: not reported positive controls valid: yes	TA 98R is a nitro-reductase deficient strain	
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98 and TA 100 (with and without S9-mix) Test concentrations: not clearly specified GLP: no information Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: positive cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	4 (not assignable) purity: not known	Zeiger et al. 1988

Table 19: Summary table of in vitro soma cell tests with 9-NA

Method	Results	Remarks	Reference
Mouse lymphoma assay (L5178Y cells) Test concentrations without S9-mix: 0, 0.1, 1.0, 10, 20 and 30 µg/mL Test concentrations with S9-mix: 0, 0.1, 1.0, 5.0, 10 and 50 µg/mL GLP: yes Similar to OECD Guideline 476	Evaluation of results**:** equivocal with S9-mix - with S9-mix: questionable effect ≥ 5.0 µg/mL; although the weak dose-dependent increase in small colonies suggesting predominance of a clastogenic mechanism this effect is associated with increasing strong toxicity cytotoxicity: - without S9-mix: strongly toxic ≥ 20 µg/mL - with S9-mix: strongly toxic ≥ 5.0 µg/mL negative controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: not known	Butterworth et al. 2004
Gene mutation test (h1A1v2 cells; TK locus) Test concentrations: 0, 1, 10, 100, 1,000 and 6,000 ng/mL GLP: no information Not in accordance with OECD Guideline 476	Evaluation of results: weakly positive (doubling of the mutant frequency at the highest test concentration of 6,000 ng/mL) cytotoxicity: no negative controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study purity: > 99 % Cells of the hlAlv2 cell line constitutively express cytochrome P4501Al, which is known to be necessary for the metabolism of many promutagens.	Durant et al. 1996

9-NA induced positive effects in bacterial gene mutation tests and was weakly positive in mammalian cell cultures.

Two positive bacterial gene mutation tests were carried out similar by OECD Test Guideline 471 (Butterworth et al. 2004; NTP 2005).

Two mammalian cell gene mutation tests support the conclusion on mutagenicity, but indicate only a weak potency. Durant et al. (1996), found a weak positive result at the TK-locus in h1A1v2 cells. The result of a mouse lymphoma assay in L5178Y cells, carried out similar by OECD Test Guideline 476, should be considered as equivocal, because with S9 mix positive effects were only observed at concentrations that induced strong to extreme toxic effects (Butterworth et al. 2004).

Metabolites (1-OH-AQ; 2-OH-AQ)

Table 20: Summary table of bacterial gene mutation tests with two in vivo metabolites of AQ (1-OH-AQ / 2-OH-AQ)

Method	Results	Remarks	Reference
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvr A (with and without S9-mix) Test concentrations: 0, 3.3, 10, 33.3, 100, 333 and 1,000 µg/plate GLP: yes Equivalent to OECD Guideline 471	Evaluation of results**:** positive without and with S9-mix without S9-mix: - TA 1537 (≥ 3.3 µg/plate); without dose-dependency with S9-mix: - TA 1537 (≥ 3.3 µg/plate); without dose-dependency cytotoxicity: no negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study 1-hydroxyanthraquinone purity: not known	Butterworth et al. 2004
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98, TA 100 and TA 102 (with and without S9-mix) Test concentrations: 0, 100, 333, 1,000, 3,333 and 10,000 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results: negative cytotoxicity: no precipitations: with and without S9-mix ≥ 1,000 µg/plate vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study 1-hydroxyanthraquinone purity: not known	NTP 2005 (Table E7)
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA 1535 and TA 1537 (with and without S9-mix) E. coli WP2 uvr A (with and without S9-mix) Test concentrations: without S9-mix: 0, 1.0, 3.3, 10, 33.3, 100, 333 and 1,000 µg/plate with S9-mix: 0.3, 1.0, 3.3, 10, 33.3, 100 and 1,000 µg/plate GLP: yes	Evaluation of results: positive with S9-mix with S9-mix: - TA 1537 (≥ 0.3 µg/plate); dose-dependent effect cytotoxicity: - with S9-mix: increased toxicity from 33 µg/plate upwards negative controls valid: yes positive controls valid: yes	1 (reliable without restrictions) key study 2-hydroxyanthraquinone purity: not known	Butterworth et al. 2004

Equivalent to OECD Guideline 471			
Bacterial reverse mutation test (Ames test; gene mutation) S. typhimurium TA 98 and TA 100 (with and without S9-mix) Test concentrations: 0, 3.3, 10, 33, 100, 200, 333 and 450 µg/plate GLP: no information Similar to OECD Guideline 471	Evaluation of results**:** positive without and with S9-mix without S9-mix: - TA 98 (≥ 10 µg/plate); dose-dependent effect with S9-mix: - TA 98 (≥ 33 µg/plate); dose-dependent effect precipitations: with and without S9-mix ≥ 333 µg/plate cytotoxicity: no vehicle controls valid: yes positive controls valid: yes	2 (reliable with restrictions) support study 2-hydroxy-anthraquinone purity: not known	NTP 2005 (Table 8)
Bacterial reverse mutation assay (Ames test; gene mutation) S. typhimurium TA 98, TA 100, TA and TA 2637 (with and without S9-mix) Test concentrations: 0.1 to 1,000 µg/plate (only range reported) GLP: not reported Due to the lack of information, no statement of compliance with OECD Guideline 471 is possible.	Evaluation of results: positive with S9-mix: - TA 100 (3.4 rev/nmol) - TA 2637 (9.6 rev/nmol) cytotoxicity: not reported vehicle controls valid: yes positive controls valid: yes	4 (not assignable) 2-hydroxy-anthraquinone purity: not known	Tikkanen et al. 1983

2-OH-AQ is positive in bacterial gene mutation tests.

2-OH-AQ was positive with S9-mix in a bacterial gene mutation test that was carried out equivalent to OECD Test Guideline 471 (Butterworth et al. 2004). In a further study, whose test performance was similar to OECD Test Guideline 471 (NTP 2005, table 8) positive results were induced with and without S9 mix.

1OH-AQ is equivocal in bacterial gene mutation tests.

With respect to the induction of gene mutations in bacteria, results from two available studies give no firm conclusion for 1-OH-AQ. In a bacterial gene mutation test that meets the requirement of OECD guideline 471 (Butterworth et al. 2004), mutagenic effects were induced with and without S9-mix. In contrast, another bacterial gene mutation test, whose test performance was similar to OECD guideline 471 (NTP 2005, table E7) exhibited negative results with and without S9 mix.

4.9.4 Summary and discussion of mutagenicity

For the justification of classification only data of studies are to be considered whose test performance is in accordance with the corresponding OECD test guideline. All studies with AQ that meet this requirement showed negative findings in vitro (bacterial gene mutation test, mouse lymphoma assay, chromosome aberration test) and in vivo (micronucleus test).

4.9.5 Comparison with criteria

According to the ECHA guidance to CLP (point 3.5.2.2) substances shall be classified as germ cell mutagens, when:

Category 1A: there is positive evidence from human epidemiological studies,

Category 1B: there are positive results from in vivo heritable germ cell mutagenicity tests in mammals or in vivo somatic germ cell mutagenicity tests in mammals (in combination with evidence that the substance has potential to cause mutations to germ cells) or tests showing mutagenic effects in germ cells of humans, without demonstration of transmission to progeny,

Category 2: there is positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, e.g.: somatic cell mutagenicity tests in vivo, in mammals or other in vivo somatic cell genotoxicity tests, which are supported by positive results from in vitro mutagenicity assays.

Relevant mutagenicity studies with AQ were negative in vitro (bacterial gene mutation test, mouse lymphoma assay, chromosome aberration test) and in vivo (micronucleus test). Accordingly no mutagenicity was induced in somatic cells (criterion for Category 2). Data for induction of mutagenic effects in germ cells (criterion for Category 1B) or epidemiological studies (criterion for Category 1A) are not available.

4.9.6 Conclusions on classification and labelling

With regard to mutagenicity no classification is proposed for AQ.

RAC evaluation of mutagenicity

Summary of the Dossier submitter's proposal

(1) Introduction. The DS reported that anthraquinone has been tested and reported in the open literature in a wide battery of *in vitro* studies along with a few *in vivo* studies. The CLH report details each specific study (tables 14, 15, 16, 17, 18, 19 and 20). Genotoxicity results for anthraquinone and many of its derivatives were mixed, with conflicting reports from different laboratories (see Table on the next page summarising genotoxicity tests with AQ). It is stated by various authors that these differences are due to the variability in contaminants arising from the different anthraquinone manufacturing processes. Therefore, the available data should be evaluated with care and distinguished by the substance's origin where possible. It is important to point out that there is no data to allow an assessment of the relative contribution of the different manufacturing processes to the mutagenicity and/or carcinogenicity potential of anthraquinone.

According to the open literature, commercial anthraquinone is generally produced by three different production methods worldwide:

(1) Oxidation of anthracene distilled from coal tar (AQ-OX),

(2) Friedel–Crafts technology (AQ-FC) and,
(3) Diels–Alder chemistry (AQ-DA).

The difficulty with many published reports lies in trying to establish the process used for the production of the test article. This is a critical point. In many cases there is simply insufficient data to establish the reliability of a particular study in assessing the potential genotoxicity activity of preparations of AQ. The production process considered to be most problematic is the nitric acid mediated oxidation of anthracene derived from coal tar (AQ-OX process). Distillate moieties that carry over in the same fraction as anthracene often remain as contaminants in the final anthracene-derived AQ-OX product. These include varying amounts of polycyclic aromatic hydrocarbon (PAH) contaminants, particularly nitroanthracene isomers which are considered to be mutagenic but this cannot be substantiated because of the absence of definitive data to support these claims.

Production of AQ by the Friedal-Crafts process (AQ-FC) and the Diels-Alder reaction (AQ-DA) are essentially free of PAH contaminants and nitroanthracenes.

The DS reported several studies, some positive and some negative for genotoxicity but many of these had significant limitations. Classification criteria were considered primarily based upon data from the studies performed according to OECD test guidelines under GLP conditions. According to the DS, most studies with AQ that met this requirement showed negative findings *in vitro* (bacterial gene mutation test, mouse lymphoma assay, chromosome aberration test) and *in vivo* (micronucleus test). There were no studies for unscheduled DNA synthesis or for germ cell mutagenicity.

Table: Summary of Genotoxicity tests with AQ

Study	Material and process	Result*	Methods and acceptability	Reference
In vitro				
Bacterial mutagenicity	NTP AQ-OX (purified) (nitric acid)	negative	GLP, OECD TG 471, acceptable	Butterworth *et al.* 2001
	NTP AQ-OX (A07496) (nitric acid)	positive with and without S9-mix	GLP, OECD TG 471, sample A07496 was used in the NTP 2 year studies, acceptable	Butterworth *et al.* 2001
	AQ-FC technical	negative	GLP, OECD TG 471, acceptable	Butterworth *et al.* 2001
	AQ-DA technical	negative		Butterworth *et al.* 2001
	100% AQ process unspecified	negative	GLP and OECD unknown, acceptable	Zeiger *et al.*, 1992 Table E2 NTP 2005
	97% AQ process unspecified	positive, with and without S9-mix	GLP and OECD unknown, acceptable	Zeiger *et al.*, 1988 Table E1 NTP 2005
	NTP AQ-OX (A07496) (nitric acid)	negative	GLP unknown, sample A07496 was used in the NTP 2 year studies, acceptable	Table E3 NTP 2005

	AQ-DA (A65343)	negative	GLP unknown, acceptable	Table E4 NTP 2005
	AQ-FC (A54984)	negative		Table E5 NTP 2005
	AQ-DA (A40147)	weakly positive		Table E6 NTP 2005
	97% AQ process unspecified	positive, with and without S9-mix	GLP unknown, OECD TG 471, acceptable	Liberman *et al.*, 1982
	AQ-OX** (air)	negative with S9 mix	GLP, OECD TG 471, acceptable	Täublová 2009
	AQ unspecified	negative	GLP and OECD unknown, lack of details, supportive	Sakai *et al.* 1985
		negative, with S9-mix only		Tikkanen *et al.* 1983
		negative		Salamone *et al.* 1979
		negative		Gibson *et al.* 1978
		negative		Anderson and Styles 1978
		negative		Brown and Brown 1976
Mammalian cell mutagenicity	AQ-OX** (air)	negative	V79 cells, GLP, OECD TG 476, acceptable	Bednáriková 2010
	AQ-DA technical	negative	mouse lymphoma assay, acceptable	Butterworth *et al.* 2001
	AQ unspecified	negative	h1A1v2 cells (TK locus), GLP unknown, acceptable	Durant *et al.* 1996
Clastogenicity	AQ-DA technical	negative	CHO cells, GLP, OECD TG 473, acceptable	Butterworth *et al.* 2001
	AQ-OX (nitric acid)	positive	micronucleus test (SHE cells, 2xctrl), GLP and OECD unknown, acceptable	Gibson *et al.* 1997
	AQ-OX** (air)	negative	V79 cells, GLP, OECD TG 473, acceptable	Lazová 2010
UDS		No data		
In vivo				
Micronucleus	AQ-DA technical	negative	mouse (Swiss CD-1) bone marrow, GLP, OECD TG 474, acceptable	Butterworth *et al.* 2001
	AQ unspecified	negative	Mouse ($B6C3F_1$) acute bone marrow, ip	NTP 2005 (Table E12)

			injection, GLP unknown, OECD TG 474, acceptable	
	AQ-OX (nitric acid)	positive	micronucleus test (3×ctrl), dietary 14-wk, (peripheral blood erythrocytes), supportive	NTP 2005 (Table E13)
DNA damage	AQ unspecified	positive (liver and kidney)	mouse (Swiss CD-1) acute, ip injection, sacrifice at 4 hour time point. 3 fold increase of elution rate of DNA from support matrix; indicator of increased single-strand DNA breaks compared to solvent control. Negative control: valid?; no positive control, no GLP, no OECD, supportive	Cesarone *et al.* 1982.

Shaded cells are negative with respect to genotoxicity and acceptable from a regulatory point of view.

* Acceptable studies imply Klimisch scores of 1 – 2, supportive implies a Klimisch score of 4.

** Batch V1161, AQ-OX (anthracene oxidation with air in the vapour phase). AQ purity 98.9%.

The production process for AQ is important because many toxicology studies used anthracene to producing the AQ-OX type, including the National Toxicology Program (NTP) 2-year cancer bioassay that reported a weak to modest increase in tumours in the kidney and bladder of male and female F344/N rats and in the livers of male and female B6C3F1 mice in studies dating from the mid 1990's (NTP 2005).

Key studies were conducted by Butterworth *et al.*, in both 2001 (AQ from different processes, Table 1 in the CLH report) and 2004 (AQ metabolites or 9-nitroanthracene (9-NA), Tables 2 and 3 in the CLH report) and served to outline the importance of the quality, purity and source (manufacturing process) of AQ used in the different investigations.

In these studies a sample of the AQ-OX used in the NTP bioassay was shown to be mutagenic in the Ames test strains TA98, TA100 and TA1537 (see relevant Table on activity of NTP AQ-OX in the Background Document (BD)). Addition of an S9 metabolic activation system decreased or eliminated the mutagenic activity. Furthermore, the NTP AQ-OX was shown to contain 9-NA at a concentration of 1200 ppm as well as other polycyclic aromatic hydrocarbons. When this test material was purified of 9-NA and the other contaminants, the presumably pure AQ as well as the technical grade samples of AQ-FC and AQ-DA were found to be without mutagenic activity (See the Table in the section for additional key elements). Therefore, these studies implied that contaminants, derived from the anthracene oxidation production process (AQ-OX), may be responsible for the positive genotoxicity results observed by Butterworth *at al.,* (2001; 2004).

(2) Mutagenicity of metabolites and impurities.

Butterworth *et al.* (2001) reported that the anthraquinone used for the 2-year bioassay was mutagenic in bacteria and attributed the mutagenicity to the 0.1% 9-nitroanthracene (9-NA) contaminant. When considering the impurities, only the mutagenicity of 9-NA was evaluated. Most of these studies were *in vitro* but an *in vivo* study by Delgado-Rodriguez *et al.* (1995) using the wing spot test of *Drosophila melanogaster* was also evaluated. Other impurities such as anthrone, phenanthrene, and anthracene were stated to be in very low abundance with mixed results with regard to negative and weakly positive results in genotoxicity studies (NTP 2005). The NTP (2005) report concluded that the evidence available for anthrone, phenanthrene, and anthracene suggest that these compounds are not genotoxic or very weakly genotoxic.

Subsequently, Butterworth *et al.* (2004) reported that a commercial sample of 9-nitroanthracene was mutagenic in TA98 and TA100 in the absence of S9 but not mutagenic in the presence of S9. 9-NA induced 53 revertants/µg in TA98 without S9 while the positive control, 2-nitrofluorene, induced 370 revertants/µg. Based on the assumption that neither anthraquinone nor its metabolites made any contribution to the carcinogenic response, Butterworth *et al.* (2004) indicated 9-NA would have to be a carcinogen with the potency of benzo[a]pyrene to produce the observed carcinogenic responses in the long term rodent studies. However, the mutagenicity data summarised by the NTP refuted this claim and the NTP report indicated that metabolites of AQ (and thus independent from the production process employed) are as likely to be carcinogenic as 9-NA. The DS also postulated that these metabolites may be responsible for the carcinogenetic effects seen in the NTP studies.

Both 1-Hydroxyanthraquinone (1-OH-AQ) and 2-hydroxyanthraquinone (2-OH-AQ) are metabolites of AQ (NTP 2004) with 2-OH-AQ present in significantly greater abundance than 1-OH-AQ. Indeed, about 20% of the administered dose may be found in urine as conjugated 2-OH-AQ. The biological activities of 1-OH-AQ and 2-OH-AQ are relevant because Blomeke *et al.* (1992) reported that 1-OH-AQ was positive in the Ames mutagenicity bacterial tester strain TA1537 without S9 and Tikkanen *et al.,* (1983) showed that hydroxyl substituents are necessary for mutagenicity in anthraquinone derivatives. However, some studies show conflicting results with respect to the mutagenicity profile of these two metabolites. The NTP reported that 2-OH-AQ was a more potent mutagen in *S. typhimurium* TA98 than 9-nitroanthracene. In contrast, 2-hydroxyanthraquinone was negative in TA98 but gave positive results in TA100 in the presence of metabolic activation (Tikkanen *et al.*, 1983; Butterworth *et al.*, 2004). 1-OH-AQ reportedly induced tumours of the liver, stomach and large intestine in rats (Mori *et al.*, 1990). Highly purified 1-hydroxyanthraquinone was negative in the absence of and positive in the presence of metabolic activation in TA1537 (Butterworth *et al.*, 2004).

The level of 2-OH-AQ present in exposed rats is significant. It may be at least 5.8-fold that of 9-nitroanthracene. Because anthraquinone is metabolised to at least one mutagenic metabolite with greater mutagenic potency than 9-NA, the NTP (2005) concluded that the carcinogenic activity of anthraquinone may occur via a mutagenic mechanism regardless of the presence of contaminants.

The DS described studies investigating the mutagenicity of the main contaminant (9-NA) present in the AQ-OX studies reported by NTP in their 2005 report. Mutagenicity studies investigating the main metabolites of AQ were also accessed (Table below). The DS concluded that 9-NA and 2-OH-AQ were positive in the bacterial gene mutation tests (9-NA was also positive in mammalian cell gene mutation assays) and that the results for 1-OH-AQ were equivocal.

Table: Summary of Genotoxicity tests on impurities/metabolites of AQ

Study	Material	Result*	Reference
In vitro			
Bacterial mutagenicity	9-NA (purity unknown)	weakly positive with and without S9-mix, GLP unknown, supportive	Fu *et al.* 1985
	9-NA (Aldrich 97%)	positive without S9-mix, GLP, OECD TG 471, acceptable	Butterworth *et al.* 2004
	9-NA (purity unknown)	positive with and without S9-mix, GLP unknown, OECD TG 471, acceptable	NTP 2005 (Table 11)
	9-NA (purity unknown)	weakly positive with and without S9-mix, GLP unknown, guideline unknown, supportive	Pitts *et al.* 1982
	9-NA (purity unknown)	positive, GLP unknown, guideline unknown, supportive	Zeiger *et al.* 1988
	1-OH-AQ (highly pure)	positive with and without S9-mix, GLP, OECD TG 471, acceptable	Butterworth *et al.* 2004
	1-OH-AQ (purity unknown)	negative, GLP unknown, OECD TG 471, acceptable	NTP 2005 (Table E7)
	2-OH-AQ (highly pure)	positive with S9-mix, GLP, OECD TG 471, acceptable	Butterworth *et al.* 2004
	2-OH-AQ (purity unknown)	positive with and without S9-mix, GLP unknown. OECD TG 471, acceptable	NTP 2005 (Table 8)
	2-OH-AQ (purity unknown)	positive with S9-mix, GLP unknown guideline unknown, supportive	Tikkanen *et al.* 1983
Mammalian cell mutagenicity	9-NA (Aldrich 97%)	equivocal with S9, GLP, OECD TG 476, acceptable	Butterworth *et al.* 2001
	9-NA (>99%)	weakly positive, h1A1v2 cells; TK locus, GLP unknown, acceptable	Durant *et al.* 1996
Clastogenicity		No data	
UDS		No data	
In vivo			
Micronucleus		No data	

* Acceptable studies imply Klimisch scores of 1 – 2, supportive implies a Klimisch score of 4.

(3) Summary.

AQ has been reported to be negative in a variety of genotoxicity tests including numerous Ames mutagenicity assays. In addition, it is reported that AQ-DA is negative in the *Salmonella-Escherichia coli* reverse mutation assays, the L5178Y mouse lymphoma forward mutation assay, for inducing chromosomal aberrations, polyploidy or endoreduplication in Chinese hamster ovary cells, and in the *in vivo* mouse micronucleus assay (Butterworth *et al.*, 2001).

The DS concluded from the tests available, that AQ without 9-NA is negative for mutagenicity. Some indications on a mutagenic potential from positive bacterial tests are given for the metabolites, but no follow-up testing has been conducted, the mutagenicity database is severely limited. The impurity 9-NA was also positive in bacterial tests but the mammalian cell

tests are not so clear and indicate weakly positive or equivocal results.

Based on the available positive *in vitro* data the level of concern for a mutagenic potential is roughly comparable for both the metabolites (1-OH-AQ and 2-OH-AQ) and 9-NA. A final conclusion on the mutagenic potential is not possible for any of these substances due to the lack of reliable data from further *in vivo* testing.

The DS concluded that anthraquinone was not mutagenic *in vitro* or *in vivo*. Classification was not proposed.

Comments received during public consultation

There were extensive comments from 6 industry representatives. All agreed that AQ originating solely from the nitric acid oxidation of anthracene process was mutagenic. However, since this process is no longer employed, all current AQ in commerce is free from 9-NA and should be exempt from classification for mutagenicity (and carcinogenicity) since there is little or no data to substantiate a cause for concern. The DS has responded in detail in the RCOM document and these comments are fully supported by RAC.

One response from an academic institution supplied 3 recent studies; a bacterial reverse mutation test (Täublová 2009), an *in-vitro* mammalian cell gene mutation test (Bednáriková, 2010) and an *in-vitro* mammalian chromosome aberration test (Lazová, 2010). All were judged to be of high quality and used AQ derived from the vapour-phase oxidation of anthracene with air. Results were negative for mutagenicity and are also included in table 1.

One Member State explicitly stated that there should be no classification for mutagenicity.

Additional key elements

Table: Activity of *NTP AQ-OX in the Ames and *E. coli* mutagenicity assays (Butterworth *et al., 20*01)

AQ-OX µg/plate	**9-NA[a] µg/plate**	**Average revertants per plate ± SD**				
Without liver S9		**TA98**	**TA100**	**TA1535**	**TA1537**	**WP2uvrA**
0	0	18 ± 4	89±9	12±2	7±3	15±4
30	0.04	20±3	107±14	12±6	10±1	14±2
60	0.07	25±10	113±15	12±1	10±3	13±5
125	0.15	**42±6**	113±18	10±4	8±4	10±3
250	0.3	**62±5**	127±18	16±7	**21±10**	12±6
500	0.6	**116±16**	142±4	17±9	**26±5**	20±1
1000	1.2	**213**	131±21	11±1	**40±2**	10±2
2000	2.4	**433±40**	**220±6**	18±3	**95±6**	16±6
Positive control**		**193±23**	**617±6**	**589±96**	**503±21**	**145±6**
With liver S9						
0	0	30±8	149±4	20±1	10±4	15±3
30	0.04	33±4	140±19	19±3	12±2	14±3
60	0.07	30±9	138±15	25±4	11±3	14±1

125	0.15	32±4	134±3	19±1	14±1	20±5
250	0.3	37±4	127±2	20±4	11±4	12±5
500	0.6	52±5	130±9	18±5	18±2	12±4
1000	1.2	**102±11**	147±21	20±6	20±4	16±4
2000	2.4	**162±13**	164±22	21±1	32±8	15±4
Positive control***		**373±17**	**534±81**	**78±5**	**111±12**	**245±21**

* sample A07496, used in the 2 year carcinogenicity studies

** Positive controls: TA98 1.0µg 2-nitrofluorene; TA100 / TA1535 2.0µg Na azide; TA1537 2.0µg ICR-191; WPuvrA 1.0µg 4-nitroquinoline-N-oxide.

*** Positive controls: TA98 2.5µg B[α]P; TA100 / TA1535 / TA1537 2.5µg 2-aminoanthracene; WPuvrA 25µg 2-aminoanthracene.

[a] NTP AQ-OX contains 9-NA at a level of 1200 ppm. This column shows the calculated amount of this contaminant on the plate.

The bolded values are judged as positive responses by RAC.

Table: Activity of purified NTP AQ-OX in the Ames and *E. coli* mutagenicity assays (Butterworth *et al., 20*01)

AQ-OX µg/plate	**Average revertants per plate ± SD**				
Without liver S9	**TA98**	**TA100**	**TA1535**	**TA1537**	**WP2uvrA**
0	14 ± 4	75±4	10±4	6±2	15±5
30	16±3	87±8	15±7	9±1	18±4
60	20±1	81±11	6±5	6±3	15±4
125	15±4	80±4	8±3	8±4	13±3
250	10±3	83±12	9±1	5±4	10±2
500	11±2	80±9	13±4	6±1	12±1
1000	15±2	95±2	13±3	7±2	14±5
2000	22±3	86±8	13±5	6±1	13±3
Positive control	121±10	533±8	418±17	657±38	132±26
With liver S9					
0	21±3	93±15	13±1	8±2	14±3
30	23±3	96±14	13±6	10±5	18±2
60	32±3	88±17	8±2	10±3	20±5
125	31±6	84±10	11±1	9±2	19±2
250	26±5	75±14	10±4	9±1	18±4
500	28±5	79±6	6±0	9±3	19±4
1000	28±2	87±10	9±2	9±5	17±2
2000	29±3	86±7	14±4	7±2	22±6
Positive control	321±18	582±22	135±12	565±105	350±23

Comments on individual studies:
(1) Sato *et al.* (1959) Metabolism of anthraquinone. II. Sulfate Conjugate of 2-hydroxyanthraquinone. J. Biochem. 46 (8) 1097-1099.

This publication cites previous investigations of anthraquinone metabolism (Sato *et al.*, 1956), in which AQ was fed to rats and 2-OH-AQ was recovered from the urine. "However, its quantity was found to be very small when freshly voided urine was examined...". The actual constituent of rodent urine as it is excreted was found via paper chromatography to be the sulphate conjugate of 2-OH-AQ which decomposes on standing to sulphate and 2-OH-AQ.

(2) Tikkanen *et al.* (1983) Mutagenicity of anthraquinones in the Salmonella preincubation test. Mutat. Res. 116 (3-4) 297-304.

This publication tested 15 naturally occurring anthraquinones using strains TA98, TA100 and TA2637 with and without S9-mix. Key information about methodology is missing and overall the study could only be considered as supportive at best. Only the results with S9-mix are clearly tabulated and presented. All compounds tested were negative with and without S9-mix for strain TA98 (including AQ and 2-OH-AQ) but 2-OH-AQ was positive with S9-mix in TA100 and TA2637 (AQ was negative in all strains with S9-mix).

Industry highlighted a point about storage conditions for the AQ material reported in the NTP studies and raised concern over the stability of AQ and/or its contaminants and asserted that the NTP peer review process was flawed. However, studies addressing storage conditions for the material indicate it is stable for many years at room temperature if stored in a dry environment.

(3) Graves *et al.* (2004) Abstract no. 113. Determination and quantitation of anthraquinone urinary metabolites. Toxicologist 78 (1-S): 23. Original unpublished Battelle study reports G004110-BUT and G004110-BXH Analysis of urine samples for 1- and 2-hydroxyanthraquinone (2003).

Graves showed that the metabolites 1-OH-AQ and 2-OH-AQ are common to all batches of AQ ingested in an animal metabolism study no matter what synthetic process was employed. Some quantitative differences in the production of these metabolites were observed with different synthetic origins. The metabolism study was conducted using male Fischer 344 rats fed formulations of 4 lots of anthraquinone, produced by three different synthetic routes: (AQ-OX, AQ-DA and AQ-FC). 1-OH-AQ and 2-OH-AQ and anthraquinone were found in all samples from the dosed animals, with 2-OH-AQ typically found at ten-fold the concentration of 1-OH-AQ.

(4) Delgado-Rodriguez *et al.* (1995). Genotoxic activity of environmentally important polycyclic aromatic hydrocarbons and their nitro derivatives in the wing spot test of Drosophila melanogaster. Mutat. Res. 341 (4) 235-247.

The results for 9-NA in both the standard cross and the high bioactivation cross (a surrogate index for metabolic activation) were inconsistent and did not show a dose response. This paper does not provide much evidence for genotoxic activity for 9-NA; at some test concentrations there were positive results, at others negative results for small single spots. The test substance naphthalene was also tested in this study (along with positive controls) and showed clear positive responses especially following metabolic activation thus illustrating that the test system was able to detect mutagenic substances.

Is it possible to compare the relative potencies of the two main metabolites and 9-NA?

The genotoxicity packages for 9-NA and the two hydroxyl anthraquinone derivatives are not complete. There is insufficient data to conclude on the relevance of these chemical species in terms of mutagenicity, or to compare their potential relative mutagenic potencies or to compare their potencies with those of known mutagenic exemplars that are used as positive control substances. Some attempts have been made in the NTP report to illustrate the relative potential potency of these compounds to induce mutagenicity but these quantitative steps must be assessed with caution.

Assessment and comparison with the classification criteria

The DS assessment of the genotoxicity and mutagenicity profile of anthraquinone (AQ) is comprehensive and rigorous. The primary material under assessment in this section is AQ. An extensive base of toxicity studies is available and is sufficient to make a determination of its mutagenic potential. The following are some of the factors impacting on the assessment of the biological activity of AQ:

1. Industry argued (from a classification point of view) that there are different types of anthraquinone depending on the source production process employed. There are 2 × oxidation of anthracene processes (i. using nitric acid and ii. vapor-phase oxidation of anthracene with air), 1 × Friedel-Crafts Reaction (synthesis from phthalic anhydride and benzene) and 1 × Diels–Adler reaction (naphthalene process).
2. The different production processes give rise to highly pure AQ with differences in the composition of very minor contaminants.
3. The AQ derived from nitric acid oxidation of anthracene gives highly pure AQ but with 0.1% 9-nitroanthracene (9-NA) which was weakly positive for mutagenicity in some *in vitro* bacterial assays. It is not known if 9-NA contributes to the positive in vitro results obtained with the NTP AQ-OX material.
4. There are many different types of studies, many of which are not compliant with current regulatory standards and in the case of 9-NA and the AQ hydroxyl metabolites, there are no complete mutagenicity profiles. These contaminants/metabolites are considered relevant in the context of the long term animal studies and the potential carcinogenicity of AQ.
5. AQ from all processes is metabolised to 1-OH-AQ and 2-OH-AQ which are also considered to be mutagenic.
6. The Butterworth *et al.* studies (2001 and 2004) show that contaminants in the AQ material in the NTP bioassays account for the positive mutagenicity results with some AQ investigations and that mutagenic activity is associated with the presence of 9-NA.

Comparison with the classification Criteria:

No human data are available for anthraquinone, therefore classification as Muta. 1A is not justified. Anthraquinone is negative in acceptable *in vivo* somatic cell mutagenicity guideline tests in mammals and data indicating induction of mutagenic effects in germ cells (criterion for Category 1B) were not available. Thus there is no justification for classification as Muta. 1B.

Most of the relevant mutagenicity studies (i.e. OECD guideline & GLP compliant) with anthraquinone were negative *in vitro* (bacterial gene mutation test, mouse lymphoma assay, chromosome aberration test) and *in vivo* (micronucleus test). No mutagenicity was induced in

two of the three acceptable *in vivo* somatic cell tests (positive results are normally a criterion for Category 2). A third, repeat dose integrated micronucleus study using both male and female mice from the 14-week dietary study treated with the same anthraquinone technical material as used in the NTP carcinogenicity bioassays was weakly positive if not equivocal (Table E13, NTP, 2005). The results in males were unconvincing with no dose response and a marginal positive result for the highest dose (4300 mg/kg bw/day) which exceeded the limit dose for the assay. The results in females were more sensitive with a weak response but without a clear dose-response relationship in the range 300 to 2600 mg/kg bw/day. The positive result in the female high dose group (5300 mg/kg bw/day) clearly exceeded the limit dose. There was no data reported for positive controls; the data did not demonstrate a direct correlation between percent PCEs and micronucleus frequency except in the high exposure concentration groups; and there was a clear increased rate of erythropoiesis as seen from increased reticulocyte counts but with an unclear dose-related response (M: 12-17-17-16-20-20 × 10^4/µL and F: 10-16-19-20-19-26 × 10^4/µL), as shown for the frequency of micronuclei in peripheral blood erythrocytes. It is unclear how relevant these results may be in light of the lack of a dose-response relationship and the very high concentrations of test material used. An additional positive *in vivo* study by Cesarone *et al.*, (1982) was also briefly mentioned by NTP (2005) and the DS. However, it was of questionable significance, the result indicating an increased level of DNA single strand breaks relative to solvent controls but it is not possible to put the result into context. Unspecified AQ at 250 mg/kg bw was administered as a single i.p. dose in a non-guideline, non GLP study. The elution rate of alkali soluble DNA fragments was measured and compared to a solvent control along with several other compounds including confirmed carcinogens (benzidine, 2-aminonaphthalene). The difficulty is that there is no way to determine the trigger value in this assay that coincides with a positive mutagenic response. Since caffeine gives a value > 2 x solvent control, AQ > 3 x solvent control, and benzidine at very high concentrations gives a value of >5 x solvent control, the sensitivity of this assay is questionable. This is not a validated assay for *in vivo* mutagenicity and it is not possible to conclude if the 'positive' result is significant. Anthraquinone, where identified as being contaminated with potentially mutagenic 9-nitroanthracene and other impurities resulting from manufacture using the nitric acid oxidation of anthracene process, was also shown to be positive in a variety of *in vitro* tests. However, not all of these studies have been shown to be of an appropriate regulatory standard and indeed when the NTP tested the AQ-OX material, they found it to be negative in tests for *in vitro* mutagenicity in TA100, TA1537 and TA98 tester strains (table E3, NTP 2005).

The positive results observed for the hydroxyl metabolites of anthraquinone and the 9-nitroanthracene contaminate are perhaps more relevant for the discussions on carcinogenicity and do not influence the proposal for no classification with respect to germ cell mutagenicity. However care must be taken not to impart too much importance to 9-NA and the hydroxyl metabolites because of the limited amount of data available to RAC at this time.

On the basis of a weight of evidence approach where *in vitro* and *in vivo* tests generally indicate no effect on mutation frequency, the RAC agrees with the conclusion of the DS. **No classification of anthraquinone for mutagenicity is required**.

4.10 Carcinogenicity

Table 21: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference
$B6C3F_1$ Mice 50M/50F 2-year study Test concentrations in diet: 0, 833, 2,500, or 7,500 ppm (eq. to average daily doses of app. 90, 265 or 825 mg/kg bw to M and 80, 235 or 745 mg/kg bw to F) GLP conform	AQ caused liver cancer in M/F; and thyroid gland tumours in mice may have been related to AQ. No NOAEL can be proposed.	1 (reliable without restriction) key study Test material (EC name): Anthraquinone purity: 99.8 %	NTP 2005
F344/N Rats 50M/50F 2-year study Test concentrations in diet: 469, 938, 1,875 or 3,750 ppm (eq to average daily doses of approximately 135, 275, 555, 1,130 or 2,350 mg /kg bw) GLP conform	AQ caused cancer of the kidney and urinary bladder in M/F rats and of the liver in F rats. The occurrence of some liver tumours in M rats may have been related to AQ exposure. No NOAEL can be proposed.	1 (reliable without restriction) key study Test material (EC name): Anthraquinone purity: 99.8 %	NTP 2005

4.10.1 Non-human information

Carcinogenicity: oral

Table 22: Summary table of carcinogenicity studies

Method	Results	Remarks	Reference
$B6C3F_1$ Mice 50M/50F 2-year study Test concentrations in diet: 0, 833, 2,500, or 7,500 ppm (eq. to average daily doses of app. 90, 265 or 825 mg/kg bw to M and 80, 235 or 745 mg/kg bw to F) GLP conform	AQ caused liver cancer in M/F; and thyroid gland tumours in mice may have been related to AQ. No NOAEL can be proposed.	1 (reliable without restriction) key study Test material (EC name): Anthraquinone purity: 99.8 %	NTP 2005
F344/N Rats 50M/50F 2-year study Test concentrations in diet: 469, 938, 1,875 or 3,750 ppm (eq to average daily doses of approximately 135, 275, 555, 1,130 or 2,350 mg /kg bw) GLP conform	AQ caused cancer of the kidney and urinary bladder in M/F rats and of the liver in F rats. The occurrence of some liver tumours in M rats may have been related to AQ exposure. No NOAEL can be proposed.	1 (reliable without restriction) key study Test material (EC name): Anthraquinone purity: 99.8 %	NTP 2005

B6C3F1 mice, 18 M/18F B6AKF1 mice, 18 M/18F 464 mg/kg bw/d AQ in 0.5% gelatin on days 7-28 followed by 1206 ppm in diet (ad libitum) after day 28 Oral (gavage/diet) study, 18 mo, started at the age of 7 days AQ and 119 other test compounds tested Histologic examination of major organs (except cranium) (no data on organs included)	No significant increase in tumour incidence (concluded in the IUCLID dataset and Innes, 1969) Original data from NTP report PB-223 159, 1968: pulmonary adenoma in 2 F B6C3F1 and in 2F B6aKF1 mice, 1 reticulum cell sarcoma in F B6AKF1, 2 hepatoma in M B6C3F1) 4 M + 4F B6C3F1 mice and 2 F B6AKF1 mice died during experiment.	4 (not assignable) (available information is not sufficient for a relevant evaluation) Test material (EC name): Anthraquinone purity: not known	Innes et al, 1969
ACI/N Rats, 60M 480 d Test concentrations: basal diet as control and basal diet containing 1-Hydroxyanthraquinone at a concentration of 1 % no standardised test protocol	Evaluation of results: positive Test results: Positive: intestinal and liver neoplasms (adenoma, adenocarcinoma, carcinoma) as well as benign stomach neoplasm	4 (not assignable) (no standardised test protocol) Test material (EC name): 1-Hydroxyanthraquinone	Mori et al. 1990

The paragraphs below are in accordance with the summaries of the two carcinogenicity assays of NTP (2005) and were adapted, where necessary.

F344/N rats (groups of 50 male and 50 female) were fed diets containing 469, 938, 1,875, or 3,750 ppm AQ (equivalent to average daily doses of approximately 135, 275, 555, 1,130, or 2,350 mg/kg bw) for 105 weeks (2 years). Survival of the exposed females was greater than that of the control group, whereas the survival of all males was similar. Mean body weights were lower in exposed females throughout the major part of the study (89%, 84%, 83% and 81% of controls at the end of the study for 469, 938, 1,875 and 3,750 ppm, respectively; NTP 2005, table 6) and were lower in exposed males during the later part of the study (97%, 95%, 96% and 94% of control for 469, 938, 1,875 and 3,750 ppm, respectively; NTP 2005, table 5). The feed consumption of controls and exposed animals was similar in males and females.

Pathological investigations revealed an increased occurrence of renal tubule adenoma and renal tubule adenoma or carcinoma (combined) in all exposed females. Also, the incidences of renal tubule adenoma in all exposed males exceeded the historical control range and the incidence was significantly increased in the male 938 ppm group. AQ exposure was associated with an increased incidence of non-neoplastic lesions of the kidney including hyaline droplet accumulation, pigmentation, and mineralization in the renal medulla and transitional epithelial hyperplasia in males and females, as well as renal tubule hyperplasia in females. Exposed females exhibited an increased incidence of nephropathy and exposed males showed an increased severity of nephropathy. Thereby the concentration of α2u-globulin of males exposed to 3,750 ppm exceeded the value of the control group at 3 months, but the overall evaluation did not point to male rat-specific α2u nephropathy (see 4.10.4.).

Compared to the controls, a significantly increased incidence of urinary bladder transitional epithelial papilloma was observed in the 1,875 ppm male group. Furthermore, the incidences in males exposed 938 ppm or greater to exceeded the historical control range. A positive trend was demonstrated in the incidences of transitional epithelial hyperplasia and papilloma or carcinoma (combined) of the urinary bladder in females.

The incidences of hepatocellular adenoma or carcinoma (combined) were slightly increased in exposed males and females and at the upper end of the historical control range. Thereby hepatocellular adenomas occurred in all groups of exposed female rats, and the incidence in the 938 ppm group was significantly greater compared to the control incidence. The incidences of hepatocellular adenoma in 938, 1,875, and 3,750 ppm female rats exceeded the historical control range. Hepatocellular adenomas were also present in all exposed groups of male rats, and hepatocellular carcinoma was present in two males (one in the 1,875 ppm dose group and the other in the 3,750 ppm dose group). However, (as noted in NTP 2005) the occurrence of hepatocellular neoplasms was considered an uncertain finding (Dossier Submitter, DS: and not a negative one due to the low incidences), because of the increased incidences of several non-neoplastic liver lesions, the presence of hepatocellular carcinomas in the two highest dose groups and the increased incidences of hepatocellular adenomas in females.

Congestion, pigmentation, and hematopoietic cell proliferation of the spleen occurred at an elevated incidence in exposed males and females. In most groups of exposed rats the incidences of bone marrow hyperplasia (DS: assumed to be erythopoietic) were increased, and exposed females exhibited also an increased incidence of bone marrow atrophy (DS: assumed to be a lymphopoietic atrophy, which might occur in parallel).

In comparison to the control group the incidences of mononuclear cell leukaemia were significantly reduced in all exposed rats, and the incidences were less than the historical control ranges.

B6C3F_1 mice (groups of 50 males and 50 females) were fed diets containing 0, 833, 2,500, or 7,500 ppm (equivalent to average daily doses of approximately 90, 265, or 825 mg/kg to males and 80, 235, or 745 mg/kg to females) for 105 weeks (2 years). In the 7,500 ppm male group less animals survived than in controls. Mean body weights were lower compared to the control group in 7,500 ppm females at the end of the study (96% of the controls, NTP 2005, table 16) and in 7,500 ppm males during the last 6 study months (95% of the controls, NTP 2005, table 15). The feed consumption of controls and exposed animals was similar in males and females.

Pathological investigations showed an increase with a positive trend in incidences of hepatocellular neoplasms (including multiple neoplasms) in males and females, whereby the incidences were increased in all exposed groups. Significantly increased incidences of hepatoblastoma were observed in males exposed to 2,500 or 7,500 ppm. In exposed mice there were increased incidences of non-neoplastic lesions of the liver. Incidences of neoplasms of thyroid gland follicular cells in males and females were marginally increased. There were higher incidences of intracytoplasmic inclusion bodies of the urinary bladder in nearly all of the exposed males and females in all dose groups, hematopoietic cell proliferation of the spleen in males and females in all groups, as well as higher incidences of thyroid gland follicular cell hyperplasia and kidney pigmentation compared to controls in exposed males.

The following table summarizes the findings of NTP (copied from the report):

Table 23: Summary of the 2-Year Carcinogenesis Studies

	Male F344/N Rats	Female F344/N Rats	Male B6C3F1 Mice	Female B6C3F1 Mice
Concentrations in feed	0, 469, 938, 1,875 or 3,750 ppm	0, 469, 938, 1,875 or 3,750 ppm	0, 833, 2,500 or 7,500 ppm	0, 833, 2,500 or 7,500 ppm
Body weights	Exposed groups less than control group	Exposed groups less than control group	7,500 ppm group less than control group	7,500 ppm group slightly less than control group
Survival rates	22/50, 23/50, 22/50, 26/50, 22/50	23/50, 40/50, 35/50, 37/50, 40/50	45/50, 41/50, 43/50, 23/50	35/50, 42/50, 35/50, 42/49
Nonneoplastic effects	Kidney: hyaline droplet accumulation (3/50, 14/50, 10/50, 16/50, 16/50); severity of nephropathy (2.2, 3.1, 3.1, 3.0, 3.0); pigmentation (25/50, 31/50, 36/50, 38/50, 33/50); medulla, mineralization (30/50, 42/50, 46/50, 47/50, 49/50); transitional epithelium, hyperplasia (28/50, 45/50, 44/50, 48/50, 48/50) Liver: centrilobular hypertrophy (0/50, 4/50, 21/50, 13/50, 29/50); cystic degeneration (9/50, 31/50, 36/50, 28/50, 29/50); inflammation (13/50, 30/50, 28/50, 30/50, 27/50); eosinophilic focus (9/50, 22/50, 30/50, 29/50, 20/50); mixed cell focus (4/50, 12/50, 15/50, 13/50, 10/50); cytoplasmic vacuolization (5/50, 18/50, 23/50, 17/50, 23/50) Spleen: congestion (6/50, 35/50, 37/50, 30/50, 31/50); pigmentation (12/50, 36/50, 38/50, 33/50, 28/50); hematopoietic cell proliferation (37/50, 45/50, 44/50, 43/50, 39/50)	Kidney: hyaline droplet accumulation (33/50, 48/50, 45/50, 44/50, 44/49); nephropathy (39/50, 49/50, 47/50, 49/50, 49/49); pigmentation (27/50, 50/50, 48/50, 50/50, 47/49); medulla, mineralization (17/50, 25/50, 27/50, 28/50, 20/49); renal tubule, hyperplasia (0/50, 12/50, 13/50, 15/50, 11/49); transitional epithelium, hyperplasia (0/50, 5/50, 12/50, 3/50, 10/49) Liver: centrilobular hypertrophy (0/50, 18/50, 23/50, 19/50, 26/49); cystic degeneration (0/50, 5/50, 10/50, 10/50, 6/49); inflammation (25/50, 46/50, 44/50, 38/50, 46/49); eosinophilic focus (8/50, 32/50, 34/50, 39/50, 34/49); mixed cell focus (3/50, 30/50, 20/50, 23/50, 13/49); angiectasis (3/50, 15/50, 18/50, 15/50, 21/49) Spleen: congestion (1/50, 46/50, 42/50, 44/50, 45/49); pigmentation (33/50, 45/50, 48/50, 48/50, 47/49); hematopoietic cell proliferation (39/50, 50/50, 47/50, 47/50, 46/49)	Liver: centrilobular, hypertrophy (24/50, 34/50, 41/50, 33/49); degeneration, fatty, focal (0/50, 7/50, 6/50, 0/49); hepatocyte, erythrophagocytosis (1/50, 9/50, 13/50, 6/49); eosinophilic focus (14/50, 17/50, 24/50, 20/49); focal necrosis (2/50, 3/50, 3/50, 8/49) Urinary Bladder: intracytoplasmic inclusion body (0/50, 46/49, 46/49, 42/45) Thyroid Gland: follicular cell hyperplasia (7/50, 10/50, 15/49, 21/46) Spleen: hematopoietic cell proliferation (12/50, 14/50, 12/49, 30/42) Kidney: pigmentation (0/50, 2/50, 2/50, 18/47)	Liver: centrilobular hypertrophy (1/49, 27/50, 22/50, 39/49); degeneration, fatty, focal (2/49, 3/50, 1/50, 9/49); eosinophilic focus (6/49, 15/50, 11/50, 22/49) Urinary Bladder: intracytoplasmic inclusion body (0/44, 40/48, 43/46, 46/48) Spleen: hematopoietic cell proliferation (9/45, 17/49, 17/48, 26/48)

Nonneoplastic effects (continued)	Bone Marrow: hyperplasia (25/50, 28/50, 37/50, 36/50, 33/50)	Bone Marrow: hyperplasia (19/50, 31/50, 28/50, 19/50, 23/50); atrophy (4/50, 13/50, 13/50, 11/50, 13/50)		
Neoplastic effects	Kidney: renal tubule adenoma (1/50, 3/50, 9/50, 5/50, 3/50); transitional epithelial papilloma (0/50, 0/50, 2/50, 0/50, 1/50) Urinary Bladder: transitional epithelial papilloma (0/50, 1/50, 3/50, 7/50, 3/49)	Kidney: renal tubule adenoma (0/50, 4/50, 9/50, 7/50, 12/49); renal tubule adenoma or carcinoma (0/50, 6/50, 9/50, 8/50, 14/49) Urinary Bladder: transitional epithelial papilloma or carcinoma (0/49, 0/49, 0/49, 1/50, 2/49) Liver: hepatocellular adenoma (0/50, 2/50, 6/50, 4/50, 3/50)	Liver: hepatocellular adenoma (21/50, 32/50, 38/50, 41/49); hepatocellular carcinoma (8/50, 13/50, 17/50, 21/49); hepatoblastoma (1/50, 6/50, 11/50, 37/49); hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma (26/50, 35/50, 43/50, 48/49)	Liver: hepatocellular adenoma (6/49, 28/50, 27/50, 40/49); hepatocellular carcinoma (2/49, 3/50, 8/50, 8/49); hepatocellular adenoma or carcinoma (6/49, 30/50, 30/50, 41/49)
Equivocal findings	Liver: hepatocellular adenoma or carcinoma (1/50, 3/50, 4/50, 5/50, 3/50) Liver: hepatocellular carcinoma (0/50, 0/50, 0/50, 1/50, 1/50)	None	Thyroid Gland: follicular cell adenoma (0/50, 0/50, 2/49, 2/46)	Thyroid Gland: follicular cell adenoma (1/45, 1/48, 2/48, 2/48); follicular cell carcinoma (0/45, 0/48, 0/48, 2/48); follicular cell adenoma or carcinoma (1/45, 1/48, 2/48, 4/48)
Decreased incidences	Mononuclear Cell Leukemia: (25/50, 2/50, 1/50, 5/50, 7/50)	Mononuclear Cell Leukemia: (18/50, 1/50, 1/50, 2/50, 0/50)	None	None
Level of evidence of carcinogenic activity	**Some evidence**	**Clear evidence**	**Clear evidence**	**Clear evidence**

Carcinogenicity: inhalation

No data available.

Carcinogenicity: dermal

No data from guideline compliant studies available. No robust information can be taken from other non-guideline studies using non-standard routes and test design:

In a skin painting study 15 male and 15 female mice with 0.1% or 0.25% solutions of AQ (ICI 1991). No information was given on test substance purity, vehicle, strain, duration/frequency of treatment, ('every day or every other day' reported), parameters examined at study end or numbers of survivors/sex/group at day 200. The results of 38 mice surviving over 200 d were reported as 1/38 papilloma, 2/38 lung cancer without any test group in which the findings were observed and 1/46 papilloma in the control group (benzene). The information is only given in the IUCLID dataset

(IUCLID 2000) and is too limited to identify the study type (the benzene only control group could indicate that this was a initiation-promotion study).

A single subcutaneous injection of 1000 mg/kg bw AQ was administered to 18 male and female mice. After 18 months observation period no significant increase in tumours were reported (Bionetics Research Laboratories 1968; cited from IUCLID 2000). A study on subcutaneous implantation of discs of 13 mm diameter with pore size of 0.22 µm containing AQ and assessment of the implantation site after 3 months did not reveal tumours at the implantation site in 10 male and 10 female Swiss mice (Longstaff 1978; cited from IUCLID 2000).

4.10.2 Human information

There is no sound information available on the exposure of humans with AQ and its effects on cancer *per se*. But, IARC (2012) summarised several studies from the dye and resin industry in the USA, where workers can potentially be exposed to AQ during the production process. The results are often equivocal, because in these industrial branches a 5 year period of co-exposure with epichlorohydrin, a chemical which is associated with an increased risk of lung cancer could confound the findings and no data on exposure levels were available.

An initial retrospective cohort study conducted by Delzell et al. (1989) found excess of lung cancer and central nervous system tumours in certain subgroups of dye and resin workers (all 2642 male employees were included in the study) employed at the investigated plant. In the two follow-up nested case-control studies (Barbone et al. 1992 and 1994) a more detailed evaluation of both cancer types was performed (IARC 2012). The lung cancer investigation (Barbone et al. 1992) showed significantly elevated risks for lung cancer among workers in the AQ and epichlorohydrin production area (Odds Ratio (OR): 2.4; 95% confidence interval (CI) 1.1-5.2; 21 exposed cases, 24 exposed controls) and, within this area, for AQ production (OR: 12; 95%; CI: 1.4-99; six exposed cases, one exposed controls) and AQ dye standardisation (OR: 3.3; 95% CI: 1.0-11; eight exposed cases, six exposed controls). In the AQ intermediate dye production the odds ratio among workers was 1.8 (95% CI: 0.6-5.1; eight exposed cases, 10 exposed controls). The second study (Barbone et al. 1994) further investigated associative of tumours of the central nervous system (11 cases, eight decedents, three living) and epichlorohydrin exposure. Here the authors found statistically significantly increased risks for central nervous system tumours among workers in the AQ dye production, but there were no controls (OR: ∞, 95% CI: 1.7-∞; three exposed cases). Also a statistically significantly increased odds ratio was reported for acute and routine epichlorohydrin exposure (OR: ∞, 95% CI: 1.5-∞; three exposed cases; and OR: 4.2; 95% CI: 0.7-26; four exposed cases). Therein rested a positive, statistically significant association with 'cumulative potential exposure' (P_{trend} = 0.11) and 'duration of routine potential exposure' (P_{trend} = 0.11). IARC (2012) stated that three of the four epichlorohydrin-exposed cases also worked in the AQ intermediated dye or azo dye areas. In later years a follow up from the original cohort study (Delzell et al. 1989) conducted by Sathiakumar and Delzell (2000) included an expanded cohort and all workers of the evaluated plant (3266, men and women; average follow up 27 years). An increased but not statistically significant mortality for several cancers (including lymphosarcoma, cancer of the colon, lung, liver, genital tissue, bladder and central nervous system) was reported. A statistically significant elevated risk for mortality of lung cancer was in association with working in the AQ production area (standardised mortality ratio (SMR): 1.68; 95% CI: 1.15-2.37; relative risk for ever versus never exposure, 1.7; 95% CI: 1.1-2.6; 32 exposed cases for both analyses). The risk was higher in long term than in short term workers (up to 20 years, thereafter the risk did no rise further).

The studies above summarised showed an excess risk of mortality from lung cancer in workers in the dye and resin processing in a single facility in the USA who were potentially exposed to AQ, AQ dye intermediates, anthracene, vanadium pentoxide, and epichlorohydrin. There was a 12-fold increased risk for lung cancer for workers involved in the production of AQ, but this was based on only few exposed cases. Even fewer cases were the basis for an excess incidence of central nervous system tumours. There are three major limitations of these studies: 1. risks were estimated with the employment in AQ and AQ dye production, but no exposure to AQ *per se* was analysed; 2. due to the low number of cases only a limited statistical power (to detect effects for specific cancers) emerges; 3. there is a limited ability to evaluate the potential confounding factors form other occupational exposures. Therefore IARC concluded there is inadequate evidence in humans for the carcinogenicity of AQ.

4.10.3 Other relevant information

Data from toxicokinetic as well as repeated dose studies and genotoxicity experiments should be taken into account. In brief, AQ was found with the highest concentrations in toxicokinetic studies in the adipositas (fat) tissue (NTP 2005) and the liver and the kidney (Bayer AG 1983). Main metabolites were 1-OH-AQ and 2-OH-AQ. In repeated dose experiments hypertrophic effects in the liver were seen in rats and mice in the 14 day studies of the NTP (2005) as well as in the 28 and 90 day studies conducted by Bayer AG (1976 and 1979). Additionally, the subchronic NTP studies showed histological lesions in kidney, liver, spleen, bone marrow and thyroid glands of male and female rats and the urinary bladder of female rats. AQ showed some positive evidence for genotoxicity, but there is no sufficient evidence to classify AQ as genotoxic taken into account all information available. More information on these issues is described in detail in the sections above.

A mammalian cell transformation test in Chang (human liver) cells and BHK-21 C13 (baby Syrian hamster kidney) cells were incubated separately with solutions of 120 chemicals including AQ in DMSO or water (no detailed data for AQ), with and without metabolic activation by rat liver postmitochondrial supernatant (concentrations of AQ (0.08, 0.4, 2, 10, 50, 250 µg/ml of the cell suspension) was negative (Styles 1978).

4.10.4 Summary and discussion of carcinogenicity

In the two 2-year carcinogenicity studies conducted by NTP (2005), AQ was carcinogenic in rats and mice. Increased tumour rates were seen in the kidney, urinary bladder, thyroid gland and liver. The only organ with consistent increased tumour rates in both species was the liver; however, the increase in liver tumours was rather weak in rats.

In male F344/N rats there was some evidence of carcinogenic activity of AQ based on elevated incidences of renal tubule adenoma and of transitional epithelial papillomas of the kidney and urinary bladder. In addition, hepatocellular neoplasms were observed, which may have been associated with AQ exposure. In female F344/N rats there was clear evidence of carcinogenic activity of AQ based on increased incidences of renal tubule neoplasms. Also, elevated incidences of urinary bladder transitional epithelial papilloma or carcinoma (combined) and of hepatocellular adenoma in female rats were related to exposure to AQ. In male and female $B6C3F_1$ mice there was clear evidence of carcinogenic activity based on elevated incidences of liver neoplasms. Weaker, but some evidence was seen for an association between thyroid gland follicular cell neoplasms in male and female mice and AQ exposure.

Renal tubule adenomas are rare in male rats and even more uncommon in female rats. In feeding studies by NTP they occurred with a historical control incidence of 7/902 in males and had not been

observed in any control female rats before (NTP 2005). Papillomas of the transitional epithelium of the kidney in male rats and the transitional epithelium of the urinary bladder in male and female rats are also uncommon neoplasms (NTP 2005). The increased incidences of renal tubule hyperplasia and hyperplasia of the transitional epithelium of the kidney and urinary bladder, as well as the increased incidences of rare neoplasms in these tissues, are clearly associated with chemical exposure. Concerning the presence of protein droplets in the kidney of male and female rats it is not sure, if they were involved in the induction of renal neoplasms. Despite the increased α2u-globulin concentration in male rat kidneys, no renal tubule cell hyperplasia has been observed at the 3- or 12-month interim evaluations of the 2 years study. Also, no elevated (proliferation) labelling indices were found in the kidneys of males or females after BrdU incorporation during a 32-day study (NTP 2005). Hence, the expanded evaluation did not point to male rat-specific α2u nephropathy. These findings and the fact, that kidney tumours and tubular lesions were also observed in female rats, are in agreement with the NTP conclusion that α2u nephropathy cannot be considered as the mode of carcinogenic action for the kidney tumours. Overall, the dossier submitter agrees on the inter-pretation of the NTP authors.

The NTP report further stated that the incidences of renal tubule hyperplasia in males and females, renal tubule adenomas in males, most non-neoplastic lesions in the liver of males and females and hepatocellular adenomas in females were all nonuniform dose response relationships (DS: in the meaning that incidences were elevated in all dose groups compared to controls, but no clear monotonic dose-related increase in incidences was seen). Overall, female rats were more responsive to AQ exposure than male rats. This can be explained by the different internal doses of AQ measured in the NTP toxicokinetic experiments, which was in females approximately twice the concentration as in male rats (at 3, 6, 12, and 18 months and at each exposure concentration). In both species, rats and mice, exposure to AQ produced similar responses in the liver, which included significant increases in liver weights and incidences of centrilobular hypertrophy characterized by increased amounts of eosinophilic cytoplasm.

The reported target organs of the NTP carcinogenicity study are in accordance with the results from repeated dose studies, where (minimal to moderate centrilobular/midzonal/periportal) hypertrophy of liver cells was observed in a 28-day oral rat study (Bayer AG 1976). In a 14 week study in mice histological investigations of NTP found minimal to moderate centrilobular hypertrophy of liver cells with enlarged nuclei. Nevertheless, information on subcellular structural changes with regard to the pronounced centrilobular enlargement of hepatocytes (hypertrophy) was lacking in the 2 year study. The liver weight of all treated male and female mice was increased in the 14 week NTP study as well as the liver weight of male and female rats in the 28 day and 90 day study performed by the Bayer AG (1976 and 1979). The subchronic NTP studies showed histological lesions in kidney, liver, spleen, bone marrow and thyroid glands of male and female rats and the urinary bladder of female rats. Thereby, eosinophilic hyaline droplets of variable size occurred within the renal tubules in exposed males and females, with α2u-globulin increases of the same degree in all treated male rats. In female rats a similar phenotype is reported, albeit weaker in severity. Furthermore, toxicokinetic studies demonstrated that the liver and the kidneys were the organs with the highest relative concentrations (Bayer AG 1983). Last, in a 32-day study (NTP 2005) cell proliferation was increased in the urinary bladder but not in the kidney of male and female rats.

According to the Draft Assessment Report - Anthraquinone (2006) Doi et al. (2005) stated that despite the mechanism for AQ carcinogenicity in the liver, kidney and urinary bladder remains unclear, different modes of action can be proposed, which do not include mutagenicity of AQ itself. These comprise i. a. the intercalative binding to DNA or the electron reduction to semiquinone radicals. The first involves sliding of the planar AQ ring between adjacent stacked base pairs (Neidle and Abraham 1984). The latter functions via catalysation processes (many enzymes are able

to do this) and ultimately results in peroxidative DNA damage due to reoxidation to quinones in the presence of oxygen species, while these transform to superoxides and other reactive oxygen species (Hartman and Goldstein 1989; Fisher et al. 1992; Barasch et al. 1999). Another alternative is that AQ undergoes ring hydroxylation or is reduced to hydroquinones, and is subsequently conjugated. Thereby, DNA affinity and redox activity of AQ derivates depend on the pattern and type of substitution. AQ induces CYP2B1 as well as CYP1A2 activity (in the liver, but not in intestine mucosa (Longo et al. 2000)) in Fisher rats. The NTP (2005) conducted a 32 day study in F344/N rats, where treatment- and dose-related elevations in the liver levels of CYP1A1 (two to threefold increase above control) and CYP2B1 (80-fold above controls in males, 40-fold above control in females) were observed. Hence, cytochrom P-450 enzymes possibly play a role in formation of active metabolites. In the NTP 32-day study, 8-hydroxy-2'-deoxyguanosine and 2'-deoxyguanosine concentrations in the kidney were decreased (at the same time point as the increased cytrochrome levels), whereas in the liver variable results were seen.

The cancer results of NTP are controversially discussed in literature, especially due to contamination of the NTP samples with 0.1% 9-NA, which is a mutagen, while AQ showed only equivocal mutagenic activity. Butterworth, 2004, claimed that 9-NA was solely responsible for the positive carcinogenicity findings, based on the mutagenicity of 9-NA and non-mutagenicity of AQ, and it was stated that neither AQ nor its metabolites contribute to the positive cancer results. The concentration of 9-NA in the test substance administered to rats and mice in the NTP studies was below 0.1%. If a concentration of 0.1% 9-NA was solely responsible for the positive mutagenic findings in the NTP sample tested by Butterworth et al 2001 (for which the NTP was unable to confirm the results), it would be more potent than benzo[a]pyrene (B[a]P) and about equally as potent as the positive control 2-nitrofluorene (based on the revertants per microgram) (Butterworth et al. 2001). This is not reflected by the following experimental data. Butterworth et al. 2004 tested the mutagenicity of 9-NA and found that it was mutagenic in TA98 and TA100 (in TA100 only at the highest tested dose) in the absence of S9 and not mutagenic in the presence of S9. In the experiment 9-NA induced 53 revertants/µg in TA98 without S9 while the positive control, 2-nitrofluorene, which was stated to have an equally potency, induced 370 revertants/µg. Pitts et al. (1982) demonstrated with purified compounds that 9-NA is a weak mutagen in TA98 (0.3 revertants/µg, without S9) in comparison to 2-nitrofluorene (417 revertants/µg, without S9) and significantly weaker than benzo[a]pyrene (700 revertants/µg, with S9). These findings are in concordance with Fu et al. (1985), who found 9-NA to be a weak mutagen in TA98 and TA100. NTP (2005) reported results with a potency of 0.315 revertants/µg for 9-NA in TA98 without S9 - similar to that found by Ptts et al. (1982). Furthermore, the potency of 9-NA in an in vitro forward mutation assay in h1A1v2 human lymphoblastoid cells (these cells constitutively express CYP1A1) was only 0.0032 times that of the positive control, B[a]P (Durant et al. 1996). In conclusion the experimental mutagenicity data suggest that 9-NA is a substantially weaker mutagen than it is proposed by Butterworth et al. (2001).

In addition, the lack of activity in mutagenicity assays does not give proof of non-carcinogenicity of AQ, because carcinogenicity of AQ could also be mediated by other mechanisms. Also metabolites appear in situ (regardless of the method of manufacture) can evoke carcinogenicity. At least one of those, namely 2-OH-AQ, is a bacterial mutagen. Experimental data indicated that 2-OH-AQ exhibits a clearly greater mutagenic potency than 9-NA: Butterworth et al. (2004) estimated that 2-OH-AQ is a bacterial mutagen twice as potent as the impurity 9-NA. The data of NTP (2005) provide further confirmatory evidence that the mutagenic potency of 2-OH-AQ in TA98 is higher than that of 9-NA. Moreover, measurements in the male rat urine showed, that 2-OH-AQ is systemically present at several-fold higher amounts than it is theoretically possible for the 0.1% 9-NA contamination, even if the latter was 100% bioavailable (NTP 2005, p. 92 table 23). The NTP concluded that 2-OH-AQ is an in situ metabolite of AQ that, based on experimental mutagenicity

data, is as likely to be of comparable carcinogenicity or (with higher probability) more carcinogenic than 9-NA. Hence, AQ has the potential to act through a mechanism involving mutagenicity, and 9-NA is not a necessary component of this action. Last, it is neither demonstrated that 9-NA is an in vivo carcinogen nor that it has a potential to be carcinogenic at the proposed contamination concentration. In fact, it should be taken into account further that carcinogenicity has not been examined for 100% pure AQ or AQ-DA or AQ-FC. Thus, it is to conclude that AQ as tested in the NTP studies (containing 0.1% 9-NA) was carcinogenic. A possible contribution of the impurity cannot be assessed.

Addressing structure-activity relationships, as stated in the NTP report, both AQ and substituted AQs are able to interact directly with DNA via intercalation, because of the size and planarity of their ring system (Islam et al. 1985; Tanious et al. 1992). Looking at NTP data for similar structures it becomes apparent that several substituted AQs are carcinogenic in long-term animal studies (see NTP 2005, table 21). While the planar ring system endows AQs with the capability of intercalating with the DNA, its substituents determined the metabolism, the ultimate mutagenicity, the carcinogenic response and the target organs involved. Most of the AQ compounds of the NTP data base carried halogen, amino, or nitro substitutions. The bladder, kidney, and liver of rats and liver of mice were the major sites of tumourigenesis (similar to the pattern observed for AQ) but also tumours in the gastrointestinal tract in rats and mice and the kidney and urinary bladder in rats were seen. 1-amino-2,4-dibromoanthraquinone was the only compound administered in feed other than AQ that produced renal neoplasms in both male and female rats. Looking at hydroxylated compounds 1,8-dihydroxyanthraquinone and 1-OH-AQ administered in feed targeted clearly the large intestine, while investigations with emodin (1,3,8-trihydroxy-6-methylanthraquinone) in female rats and male mice only exhibited equivocal results (NTP 2005). Other literature (Mori et al. 1990) reported induction of tumours of the liver, stomach and large intestine in male ACI/N rats for 1-OH-AQ after dietary administration.

In conclusion, the available carcinogenicity studies on AQ as tested by the NTP (2005) showed that there was clear evidence of carcinogenic activity of AQ in female F344/N rats as well as clear evidence of carcinogenic activity in male and female $B6C3F_1$ mice. The tumour findings are consistently observed in target organs showing toxic effects and precursor lesions in repeated dose studies and in organs with highest tissue concentrations (see toxicokinetic data). Additionally, the target organs of carcinogenic responses are in accordance with those of other AQs. It is not possible to determine to what extent, if any, 9-NA has influenced the outcomes of the NTP carcinogenicity study on AQ. The low exposure level, the bioavailability, and the weak mutagenicity make it unlikely and implausible that 9-NA was solely and totally responsible for the carcinogenic response. Due to biotransformation processes mutagenic metabolites of AQ appear, which are at least five times more potent and present at systemically higher concentration than 9-NA. Therefore, the results are attributable to AQ (and its active metabolites) and AQ is considered to be carcinogenic. AQ was not clearly demonstrated as mutagenic in the available tests, however, a contribution of mutagenicity (by mutagenic metabolites) cannot be excluded.

4.10.5 Comparison with criteria

According to the CLP directive a substance shall be classified as carcinogenic if:

Category 1A

‘It is known to have carcinogenic potential for humans; classification is largely based on human evidence.’

Based on the results given above, classification as Category 1A is not appropriate, because there is insufficient evidence from the few indirect epidemiological studies with confounding effects from co-exposure to other (carcinogenic) substances.

Category 1B

'It is presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.'

This category depends on the strength of evidence, which consists of animal experiments for which there is sufficient evidence to demonstrate animal carcinogenicity. This means a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in

(a) two or more species of animals or in two or more independent studies in one species carried out at different times or in different laboratories or under different protocols;

(b) in both sexes of a single species;

(c) occurrence of malignant neoplasm to an unusual degree with regard to the incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

Concerning animal evidence the NTP studies showed some evidence for carcinogenicity of AQ in male F344/N rats, clear evidence for carcinogenicity of AQ in female F344/N rats as well as clear evidence of carcinogenic activity in male and female $B6C3F_1$ mice. In comparison to the given criteria for the CLP regulation AQ fulfils the criteria for category 1B with regard to

(a) two positive species,

(b) both sexes, and

(c) (rare) tumours types above the background incidence as well as several target organs of tumour response. In additional considerations there are structurally related substances (from the NTP data base) with shown carcinogenic potential as well as a possible contribution of mutagenicity (in particular of metabolites) as a mode of action. Furthermore, other conclusive modes of tumourigenic action like DNA intercalation, occurrence of semiquinone radicals and reactive oxygen species cannot be excluded.

Category 2

'It is a suspected human carcinogen, but the evidence is not sufficient for Category 1A or 1B.'

Following consideration would lead to classification as category 2:

(a) the evidence is limited to a single experiment;

(b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies;

(c) the agent increases the incidence only of benign neoplasm or lesions of uncertain neoplastic potential; or

(d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

Category 2 is not appropriate, because the criteria are not fulfilled. The evidence is

(a) neither limited to a single experiment (two species were tested in the NTP study)

(b) nor limited with regard to benign neoplasms (there was clear evidence for renal tubule carcinoma as well as transitional epithelial urinary bladder carcinoma in female rats, hepatocellular carcinoma in male and female mice, and, in addition, there was some evidence for hepatocellular carcinoma in male rats and follicular cell carcinoma in female mice)

(c) nor limited to only promoting activity.

With regard to (b) the impurity 9-NA is discussed extensively in the literature. Concerns about misleading results due to contamination issues with low levels of 0.1% 9-NA measured in the NTP material are rebutted by considerations regarding the mutagenic potential of the impurity as well as the potency of the metabolites of AQ and calculations concerning the systemically present in vivo concentrations of the compounds. In addition, it is not conclusively demonstrated that 9-NA is a carcinogen or that the occurring concentrations are sufficiently high to cause the noted tumourigenic effects. 9-NA is judged to be neither strong enough mutagenic nor abundant enough to solely and totally cause the observed carcinogenicity.

Therefore, the NTP results are valid; there is sufficient evidence for carcinogenicity and classification of AQ as carcinogenic Category 1B with the hazard phrase H350: *May cause cancer* is proposed.

This classification is in accordance with the Draft Assessment Report - Anthraquione (2006), which classified AQ according to the DSD as carcinogenic category 2 with the assigned symbol T and the risk phrase R45. It is also in accordance with the classification of IARC, who ranked AQ in group 2B (possibly carcinogenic to humans).

4.10.6 Conclusions on classification and labelling

Due to clear evidence of carcinogenic activity in animals the following classification and labelling is proposed according to the Classification, Labelling and Packaging Regulation (CLP):

Category 1B carcinogen, H 350 (May cause cancer).

RAC evaluation of carcinogenicity
Summary of the Dossier submitter's proposal
Background: No new long-term toxicity studies have been conducted on anthraquinone since 1996 when the two bioassays were finalised for the NTP. The DS has re-evaluated the studies that were described in the NTP (2005) report for carcinogenicity classification in the context of the CLP Regulation. According to Butterworth *et al.* (2001, 2004), the presence of 9-nitroanthracene used in the NTP cancer bioassays was responsible for the carcinogenic effects as a result of its genotoxic activity. As noted by the DS, the carcinogenicity of 9-NA has not been demonstrated, therefore it is not possible to estimate the contribution of this impurity to the observed tumour incidences. In the two 2-year carcinogenicity studies reported by the NTP (2005), AQ was found to be tumorigenic in rats and mice. AQ increased the incidences of tumours of the kidney and urinary bladder in male and female rats and of the liver in female rats. In male and female mice given AQ, the incidences of liver tumours were greatly increased, and a few of these

animals developed thyroid gland tumours. The NTP acknowledged the presence of mutagenic contaminants in the AQ technical material at low concentrations but also concluded that the metabolism of AQ to mutagenic metabolites makes AQ potentially carcinogenic (regardless of the production methods employed). The NTP summarised the animal data thus:

- There was *some evidence of carcinogenic activity of anthraquinone in male F344/N rats* (increased incidences of renal tubule adenoma and of transitional epithelial papillomas of the kidney and urinary bladder).
- There was *clear evidence of carcinogenic activity of anthraquinone in female F344/N rats* (increased incidences of renal tubule neoplasms).
- There was *clear evidence of carcinogenic activity in male and female B6C3F1 mice* (increased incidences of liver neoplasms).

The International Agency for Research on Cancer (IARC, 2012) summarised several studies from the dye and resin industry in the USA, where workers were potentially exposed to AQ during the production process. These studies however had major limitations and IARC concluded there was inadequate evidence in humans for the carcinogenicity of AQ but there was *sufficient evidence in the experimental animals* for carcinogenicity. The overall evaluation (using the same studies as reported by the NTP in 2005) concluded that AQ was possibly carcinogenic to humans (Group 2B).

In 2013, the German Federal Institute for Risk Assessment (BfR) removed AQ from its list of recommendations for food packaging (BfR opinion no. 005/2013). It reassessed the use of AQ in the manufacture of paper intended for food contact following the publication of an expert opinion of the European Food Safety Agency (EFSA) in 2012. In its opinion on AQ as an active pesticide ingredient, the EFSA concluded that carcinogenic effects cannot be ruled out for AQ and that the hazard potential for mammals cannot be determined unequivocally from the limited dataset available.

Manufacturing methods for Anthraquinone (AQ):

Anthraquinone in commercial use is produced by several different methods worldwide:

1. Friedel-Crafts Reaction: (AQ-FC), synthesis from phthalic acid anhydride and benzene.
2. Diels–Adler reaction: (AQ-DA), condensation of 1,4-naphthoquinone with butadiene followed by oxidative dehydrogenation.
3. Dimerization of styrene to 1-methyl-3-phenylindane using phosphoric acid as a catalyst, followed by catalytic vapour-phase oxidation to anthraquinone.
4. Oxidation of anthracene with air in the vapour phase (AQ-OX).
5. Oxidation of anthracene with chromic acid in 48% sulphuric acid (AQ-OX).
6. *Oxidation of anthracene with nitric acid (AQ-OX, no longer used)*.

The AQ used in the bioassays reported in the NTP 2005 study (AQ-OX) was purchased from Zeneca Fine Chemicals and produced with the nitric acid oxidation of anthracene, a method that in general produces AQ of the highest purity. This was contaminated with trace amounts of 9 nitroanthracene which is a potential mutagen. The nitric acid oxidation method is no longer used for production of commercially available AQ. There was no cancer studies on AQ derived from the other production processes.

Relevance of Repeated dose toxicity data:
The main target organs (liver, kidney, urinary bladder, thyroid and the haematopoietic system) were identified in repeated dose experiments. Dramatic hypertrophic effects in the liver were seen in rats and mice in the 14 day studies (NTP, 2005) as well as in the 28 and 90 day studies conducted by Bayer AG (1976 and 1979). Additionally, the sub-chronic NTP studies showed histological lesions in kidney, liver, spleen, bone marrow and thyroid glands of male and female rats and the urinary bladder of female rats. The 32-day rat feeding study described in the NTP (2005) report provided some basic mechanistic information such as a strong induction of CYP2B and proliferative activity in the urinary bladder (see later).

Summary of the Tumour Profiles Observed in Rodents:
In the two 2-year carcinogenicity studies conducted by NTP (2005), AQ was carcinogenic in rats and mice. Increased tumour rates were seen in the kidney, urinary bladder, thyroid gland and liver. A summary is provided below for rats and mice. Additional details are provided in the section Additional Key Elements of the Background Document.

In conclusion, the available carcinogenicity studies on AQ showed that there was clear evidence of carcinogenic activity in female F344/N rats as well as clear evidence of carcinogenic activity in male and female B6C3F1 mice. The tumour findings were consistently observed in target organs showing toxic effects and precursor lesions in repeated dose studies and in organs with the highest tissue concentrations of AQ. Additionally, the target organs with carcinogenic responses are consistent with those of other AQ derivatives that were briefly mentioned in the CLH report and outlined in Table 21 of the NTP final report (2005).

Relevance of anthraquinone metabolism and the contaminant 9-nitroanthracene.
There has been extensive discussion on the relevance of the contaminant 9-nitroanthracene (9-NA) and the metabolites 1-hydroxyanthraquinone (1-OH-AQ) and 2-hydroxyanthraquinone (2-OH-AQ) in both the CLH report and the RCOM document in relation to the carcinogenicity of the tested material in the NTP bioassays.

Industry representatives contend that the results of the NTP anthraquinone 2-year bioassays are not valid because the technical material tested does not represent the technical material commercially available today. However, the material tested was in fact of very high purity (99.8%) with only trace amounts of contaminants in the range of 0.1% to 0.65%, depending on the analytical testing laboratory and the study report. One of those components was 9-nitroanthracene, which was shown to be mutagenic in a series of well-designed *in vitro* studies by Butterworth *et al.* (2001, 2004). Comparisons with 2-nitrofluorene (see the CLH report, section 4.10.4) suggest that 9-NA is a substantially weaker mutagen than originally proposed by Butterworth *et al.* (2001).

Further characterisation of the contaminants in the technical material used in the NTP bioassays (figure below) was performed by the NTP and Butterworth *et al.*, (2001, 2004). Levels of contamination in the bioassay material were reported to be from 0.2% (NTP) up to 0.65% (Butterworth *et al.*, 2004). The identified component breakdown from these studies was as follows:

- 9-NA: 0.09 – 0.11%
- polycyclic aromatic hydrocarbons[1]: 0.06 - 0.09%
- nitrobenzene[2]: 0.05%
- unidentified organics and nitro-organics[2]: 0.40%

[1] anthracene (0.05%), anthrone (0.008%), phenanthrene (0.002%), and dibenz[a,h]anthracene (concentration unknown, %)
[2] Butterworth *et al.* (2004)

AQ-OX production begins with anthracene produced from coal tar and according to industry, different lots can contain variable levels of different contaminants. An analysis of AQ-OX produced with the vapour oxidation method (confidential) confirmed the presence of similar components to those found in the AQ-OX from the nitric acid oxidation process but without 9-NA.

There is no proof or evidence that 9-NA was the only carcinogenic substance in the NTP studies. 9-NA has not been tested in a carcinogenicity study. Overall, it is considered unlikely that the carcinogenic response could solely be attributed to 9-NA. The minor occurrence of this contaminant (0.1%) in the tested technical material makes it unlikely that 9-NA was the only component that was responsible for the carcinogenic response. The DS correctly interpreted the available data in concluding that it is not possible to determine to what extent, if any, 9-NA (or any other component) influenced the carcinogenic response in the NTP 2-year rodent studies.

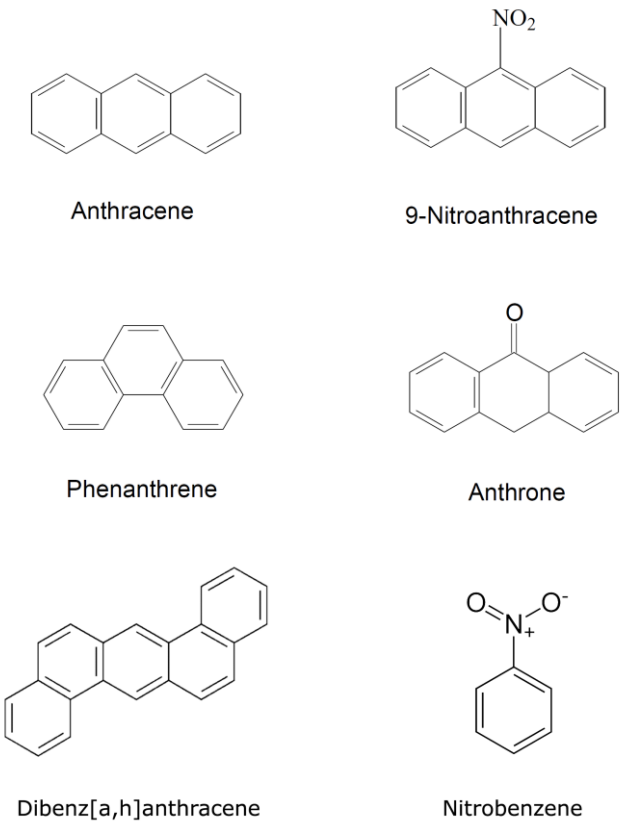


Figure: Chemical structures of some of the contaminants identified in the anthraquinone technical material used in the NTP bioassays.

Anthraquinone was metabolised extensively after absorption, giving rise to 1-hydroxyanthraquinone (1-OH-AQ) and much greater quantities of 2-hydroxyanthraquinone (2-OH-AQ). The metabolic studies for AQ are very limited but it seems that the bulk of 2-OH-AQ was excreted via the urine in the form of its sulphate conjugate. Levels of circulating or systemically available 2-OH-AQ prior to metabolic conjugation are unknown. In a report of the International Agency for Research on Cancer (IARC, 2002), the carcinogenicity of 1-OH-AQ was evaluated as Group 2B (possibly carcinogenic to humans). Mori *et al.* (1990) found that 1-OH-AQ induced tumours in the large bowel, liver, and stomach of treated male ACI/N rats. The

NTP reported that 2-OH-AQ was a bacterial mutagen in strain TA98 without S9 (NTP, 2005). Butterworth *et al.* (2004) observed weak responses in strains TA100 and TA1537 but only in the presence of S9-mix. According to this author, literature reports of potent mutagenic activity for 1-OH-AQ and 2-OH-AQ in bacteria without S9 were due to the presence of contaminants in the tested samples.

The hydroxyl metabolites of AQ are common to all sources of AQ regardless of the production process and are therefore relevant in considering the NTP studies as valid investigations of AQ carcinogenicity. Urine samples from F344 male rats dosed with AQ for 7 and 9 days at different feed concentrations were analysed for 1- and 2-OH-AQ. The metabolic studies by Graves (2003) showed that 2-OH-AQ is the major AQ metabolite present in urine with lesser amounts of 1-OH-AQ also being present regardless of the method of AQ synthesis.

The hydroxyl metabolites of AQ is not systematically more potent with some tester strains in the bacterial mutagenicity assay than 9-NA. However, what relevance mutagenic potency in *in vitro* tests of these substances may have to the development of neoplasms *in vivo* is unknown. It has been shown that AQ alone does not have mutagenic properties in somatic cells. The lack of AQ activity in mutagenicity assays does not equate to non-carcinogenicity, because AQ is metabolised *in situ* to at least one mutagen that is as likely to be as carcinogenic as 9-NA. The carcinogenic activity resulting from administration of AQ and arising from its subsequent metabolism and/or impurities cannot be therefore excluded.

The DS concluded, in agreement with the original NTP (2005) report, that the relative contribution of AQ, its metabolites or the impurity 9-NA to carcinogenicity cannot be ascertained. All, some or a single component (whether known or unknown) could be responsible for the tumour response.

Mode of Action. Although the mechanisms underlying anthraquinone carcinogenicity in the liver, kidney, and urinary bladder are unclear, a few modes of action may be proposed. These include:

(1) intercalative binding to DNA,
(2) reduction to semiquinone radicals that result in peroxidative damage,
(3) reactive metabolites that may interact with DNA/protein complexes,
(4) direct cytotoxicity stimulating a sustained regenerative response,
(5) nuclear receptor activation, e.g. CAR and the aryl hydrocarbon receptor (AhR).

The induction of hepatic CYP2B1 activity (PROD with smaller contributions from CYP1A1 evidenced from increased EROD activity) was demonstrated in rats fed AQ and suggests cytochrome P-450 may play a role in the formation of active metabolites. Studies examining the metabolism of AQ have reported the presence of 2-OH-AQ, 1-OH-AQ, 9,10-dihydroxyanthracene and 2,9,10-trihydroxyanthracene, and conjugates in the urine of Fischer, Chester Beatty, and another unspecified strain of rat fed AQ (Sato *et al.* 1956; Sato *et al.* 1959; Sims, *1964*; Sipes *et al.* 1993; Graves, 2003).

A 32-day feeding study with AQ in F344/N rats was described in the NTP (2005) report which examined cytochrome P450 activity in the liver (Table 9), 8-hydroxy-2'-deoxyguanosine and 2'-deoxyguanosine concentrations in the liver and kidney, and cell proliferation in the liver, kidney, and urinary bladder (Table 10). Doses for males and females were 0, 40, 80 and 320 mg/kg bw/day. Key results indicate:

- 1.7 – 3.0 fold increase in CYP1A1 activity (EROD) indicating a small amount of AhR activation (or CAR transactivation of CYP1A1), but no dose response.
- 14 – 78 fold increase in CYP2B1 activity (PROD) indicating strong CAR activation, with a robust dose response in both sexes with consistently higher responses in males relative to females.

- 8-hydroxy-2'-deoxyguanosine concentrations in the kidney and liver were not markedly different from controls indicating a low concern for oxidative stress in these tissues.
- Significant increases in cell proliferation were observed in the urinary bladder.

Additional details are provided in the section Additional Key Elements of the Background Document.

Little further information is available regarding the mode of action for AQ induced carcinogenicity. Also, there were no other carcinogenicity or long-term bioassays with AQ synthesised from processes other than the nitric acid oxidation of anthracene. Studies on several AQ derivatives such as emodin indicate a variety of possible DNA interactions including inhibition and stabilisation of the topoisomerase II DNA cleavage complex leading to an increased incidence of DNA double strand breaks (Li *et al.* 2010). While the planar ring system endows AQs with the potential capability of intercalating with DNA, it is well recognised that the type and degree of substitution on the anthracene nucleus of AQ derivatives are a major determining factor with regards to mutagenic and carcinogenic potential and site of action so that simple read-across from these compounds warrants caution. What is clear, however, is that substituted anthraquinones display significant carcinogenic potential in long term rodent studies as a common group effect, often with liver and kidney and sometimes with urinary bladder involvement amongst others. These substituted anthraquinone derivatives are very closely related in chemical structure and include: 1-amino-2-methylanthraquinone, 1,4,5,8-tetraaminoanthraquinone and 1-amino-2,4-dibromoanthraquinone.

Comments received during public consultation

There were extensive comments from 6 industry representatives. All argued that AQ originating solely from the nitric acid oxidation of anthracene process was no longer available and that classification for carcinogenicity does not apply to the current commercially available technical grades of AQ because mutagenic 9-NA is not present in these preparations. The DS responded in detail in the RCOM document. In the absence of new data, the lack of 9-NA in the technical material is not sufficient to invalidate the tumour responses observed in the NTP bioassays.

Two Member States agreed with Carc. 1B – H350
One Member State suggested Carc. 2 – H351

Additional key elements

Summary of the Tumour Profiles Observed in Rodents (NTP, 2005):
In the two 2-year carcinogenicity studies conducted by NTP (2005), AQ was carcinogenic in rats and mice. Increased tumour rates were seen in the kidney, urinary bladder, thyroid gland and liver.

The four Tables below present respectively (i) significant Neoplasms (Male Rats) in a 2-Year Feed Study of AQ. Dose mg/kg bw/day, (ii) significant Neoplasms (Female Rats) in a 2-Year Feed Study of AQ. Dose mg/kg bw/day, (iii) significant Neoplasms (Male mice) in a 2-Year Feed Study of AQ. Dose mg/kg bw/day and (iv) significant Neoplasms (Female mice) in 2-Year Feed Study of AQ. Dose mg/kg bw/day.

Table: Significant Neoplasms (Male Rats) in a 2-Year Feed Study of AQ. Dose mg/kg bw/day.

Tumour	HCD	0	20	45	90	180
Liver: Hepatocellular Adenoma Overall incidence	21/902 (2.3%) 0-10%	1/50 (2%)	3/50 (6%)	4/50 (8%)	4/50 (8%)	2/50 (4%)
Liver: Hepatocellular Carcinoma Overall incidence	7/902 (0.8%) 0-6%	0	0	0	1/50 (2%)	1/50 (2%)
Liver: Hepatoblastoma Overall incidence	no data	no data	no data	no data	no data	no data
Kidney: Renal tubule Adenoma Overall incidence	7/902 (0.8%) 0-4%	1/50 (2%)	3/50 (6%)	**8**/50 (**16%**)	5/50 (10%)	3/50 (6%)
Kidney: Renal Trans. Ep. papilloma Overall incidence	1/902 (0.1%) 0-2%	0	0	2/50 (4%)	0	1/50 (2%)
Urinary Bladder: Trans. Ep. Papilloma Overall incidence	2/891 (0.2%) 0-2%	0	1/50 (2%)	3/50 (6%)	**7**/50 (**14%**)	3/50 (6%)
Systemic: Leukaemia Mononuclear Overall incidence	494/904 (55%) 32-74%	25/50 (50%)	**2**/50 (**4%**)	**1**/50 (**2%**)	**5**/50 (**10%**)	**7**/50 (**14%**)

N.B. No neoplasms observed in these tissues at the 12 month interim sacrifice. HCD is Historical Incidence at Battelle Columbus Laboratory, data as of 1998. There were no significant effects on the male thyroid. Values in bold are statistically significant ($p < 0.05$, poly-3 test). Pre-neoplastic lesions appear biologically significant:

(1) renal transitional epithelium hyperplasia (28/50, 45/50, 44/50, 48/50, 48/50; 0→180 mg/kg bw/day)

(2) renal tubule hyperplasia (3/50, 7/50, 3/50, 9/50, 9/50; 0→180 mg/kg bw/day)

(3) hepatic eosinophilic foci (9/50, 22/50, 30/50, 29/50, 20/50; 0→180 mg/kg bw/day)

Table: Significant Neoplasms (Female Rats) in a 2-Year Feed Study of AQ. Dose mg/kg bw/day.

Tumour	HCD	0	25	50	100	200
Liver: Hepatocellular Adenoma Overall incidence	4/901 (0.4%) 0-4%	0	2/50 (4%)	**6**/50 (**12%**)	4/50 (8%)	3/50 (6%)
Liver: Hepatocellular Carcinoma Overall incidence	0/901	1/50 (2%)	0	0	0	0
Liver: Hepatoblastoma Overall incidence	no data	no data	no data	no data	no data	no data
Kidney: Renal tubule Adenoma Overall incidence	0/901	0	4/50 (8%)	**9**/50 (**18%**)	**7**/50 (**14%**)	**12**/50 (**24**%)
Kidney: Renal tubule Carcinoma Overall incidence	0/901	0	2/50 (4%)	0	1/50 (2%)	2/50 (4%)

Kidney: Renal Trans. Ep. papilloma Overall incidence	0/1348*	0	0	0	0	0
Urinary Bladder: Trans. Ep. Papilloma Overall incidence	2/891 (0.2%) 0-2%	0	0	0	1/50 (2%)	1/50 (2%)
Urinary Bladder: Trans. Ep. Carc. Overall incidence	0/891	0	0	0	0	**1**/50** (**2%**)
Systemic: Leukaemia Mononuclear Overall incidence	261/901 (29%) 14-42%	18/50 (36%)	**1**/50 (**2%**)	**1**/50 (**2%**)	**2**/50 (**4%**)	**0**

N.B. No neoplasms observed in these tissues at the 12 month interim sacrifice. HCD is Historical Incidence at Battelle Columbus Laboratory, data as of 1998. There were no significant effects on the female thyroid. Values in bold are statistically significant ($p < 0.05$, poly-3 test). Pre-neoplastic lesions appear biologically significant:

(1) renal transitional epithelium hyperplasia (0/50, 5/50, 12/50, 3/50, 10/50; 0→200 mg/kg bw/day)

(2) renal tubule hyperplasia (0/50, 12/50, 13/50, 15/50, 11/49; 0→200 mg/kg bw/day)

(3) hepatic eosinophilic foci (8/50, 32/50, 34/50, 39/50, 34/49; 0→200 mg/kg bw/day)

* From total number of females in feeding studies from the NTP historical control database dated Jan 1997.

** A single tumour in an exposed group may or may not have resulted from the chemical under study. However these tumours are very rare, none recorded in 891 females examined.

Table: Significant Neoplasms (Male mice) in a 2-Year Feed Study of AQ. Dose mg/kg bw/day.

Tumour	HCD	0	90	265	825
Liver: Hepatocellular Adenoma Overall incidence	333/850 (39%) 20-60%	21/50 (42%)	**32**/50 (**64%**)	**38**/50 (**76%**)	**41**/50 (**82%**)
Liver: Hepatocellular Carcinoma Overall incidence	166/850 (20%) 10-29%	8/50 (16%)	**13**/50 (**26%**)	**17**/50 (**34%**)	**21**/50 (**42%**)
Liver: Hepatoblastoma Overall incidence	0/850	1/50 (2%)	**6**/50 (**12%**)	**11**/50 (**22%**)	**37**/49 (**76%**)
Kidney: Renal Tubule Adenoma Overall incidence	4/1700 (0.2%) 0-2%*	0	1/50 (2%)	2/50 (4%)	0
Thyroid Gland: Follicular cell Adenoma Overall incidence	12/846 (1.4%) 0-4%	0	0	2/50 (4%)	2/50 (4%)

HCD is Historical Incidence at Battelle Columbus Laboratory, data as of 1998. There were no significant tumour incidences for the male thyroid. Values in bold are statistically significant ($p < 0.05$, poly-3 test). Pre-neoplastic lesions appear biologically significant:

(1) thyroid gland follicular cell hyperplasia (7/50, 10/50, 15/49, 21/46; 0→825 mg/kg bw/day)

(2) hepatic eosinophilic foci (14/50, 17/50, 24/50, 20/49; 0→825 mg/kg bw/day)

* Historical data taken from Eustis *et al.*, (1994) The utility of multiple-section sampling in the histopathological evaluation of the kidney for carcinogenicity studies. Toxicol. Pathol. 22(5):457-72.

Table: Significant Neoplasms (Female mice) in 2-Year Feed Study of AQ. Dose mg/kg bw/day.

Tumour	HCD	0	80	235	745
Liver: Hepatocellular Adenoma Overall incidence	203/852 (24%) 12-50%	6/49 (12%)	**28**/50 (**56%**)	**27**/50 (**54%**)	**40**/49 (**82%**)
Liver: Hepatocellular Carcinoma Overall incidence	98/852 (12%) 6-20%	2/49 (4%)	3/50 (6%)	8/50 (16%)	**8**/49 (**16%**)
Liver: Hepatoblastoma Overall incidence	2/852 (0.2%) 0-2%	0	0	0	1/49 (2%)
Thyroid Gland: Follicular cell Adenoma Overall incidence	13/847 (1.5%) 0-6%	1/45 (2%)	1/48 (2%)	2/48 (4%)	4/48 (8%)

N.B. No neoplasms observed in these tissues at the 12 month interim sacrifice. HCD is Historical Incidence at Battelle Columbus Laboratory, data as of 1998. There is a slight rise in thyroid tumour incidence with dose which may be related to treatment. Values in bold are statistically significant ($p < 0.05$, poly-3 test). Pre-neoplastic lesions appear biologically significant:

(1) thyroid gland follicular cell hyperplasia (10/45, 14/48, 16/48, 15/48; 0→745 mg/kg bw/day)

(2) hepatic eosinophilic foci (6/49, 15/50, 11/50, 22/49; 0→745 mg/kg bw/day)

In conclusion, the available carcinogenicity studies on AQ showed that there was clear evidence of carcinogenic activity in female F344/N rats as well as clear evidence of carcinogenic activity in male and female B6C3F1 mice. The tumour findings were consistently observed in target organs showing toxic effects and precursor lesions in repeated dose studies and in organs with the highest tissue concentrations of AQ. Additionally, the target organs with carcinogenic responses are consistent with those of other AQ derivatives that were briefly mentioned in the CLH report and outlined in Table 21 of the NTP final report (2005).

Although the mechanisms underlying anthraquinone carcinogenicity in the liver, kidney, and urinary bladder are unclear, a few modes of action were be proposed. The two Tables below present respectively (i) liver Cytochrome P450 Activities for Rats and (ii) kidney, liver, and urinary bladder cell proliferation data for rats in the 32-day feed study of anthraquinone.

Table: Liver Cytochrome P450 Activities for Rats at the 8-Day Interim Evaluation in the 32-Day Feed Study of Anthraquinone. Doses in mg/kg bw/day. N = 10

	0	40	80	320
Male:				
CYP1A1 (EROD)	19.4 ± 1.6	58.0 ± 4.0	56.3 ± 5.8	52.1 ± 4.4
CYP2B1 (PROD)	3.3 ± 0.3	131 ± 13	215 ± 10[b]	257 ± 8
Female:				
CYP1A1 (EROD)	25.3 ± 2.4	55.7 ± 5.8	42.5 ± 6.1	46.8 ± 4.6
CYP2B1 (PROD)	3.0 ± 0.4	41.4 ± 4.8	96.2 ± 9.6	143 ± 15

Data are given as pmol/minute per mg protein (mean ± standard error).

[b] n=9

Table: Kidney, Liver, and Urinary Bladder Cell Proliferation Data for Rats in the 32-Day Feed Study of Anthraquinone. Doses in mg/kg bw/day. N = 10

	0	40	80	320
Male:				
Kidney	9.39 ± 0.72	7.73 ± 0.87	8.58 ± 0.56	10.4 ± 1.2
Liver	5.81 ± 0.88	5.68 ± 1.06	6.38 ± 0.95	7.56 ± 1.05
Urinary bladder	0.495 ± 0.077	0.840 ± 0.233[b]	0.532 ± 0.087	**1.50 ± 0.24***
Female:				
Kidney	4.75 ± 0.27	5.22 ± 0.40	4.82 ± 0.45	4.47 ± 0.35
Liver	3.45 ± 0.47	5.57 ± 1.22	6.54 ± 0.87	3.85 ± 0.66
Urinary bladder	1.07 ± 0.13[b]	2.60 ± 0.43[b]	**4.96 ± 0.81***	**3.15 ± 0.44***

* Significantly different (P≤0.05) from the control group by Dunnett's one-tailed t-test.

Data are given as the percentage of mean labelled cells (mean ± standard error).

[b] n=9

Assessment and comparison with the classification criteria

The DS proposal for CLP classification of AQ as category 1B carcinogen, H350 (May cause cancer) is based on three main factors:

(1) Tumour response in two rodent species in multiple organ systems,

(2) The potential for mutagenic metabolites to be generated from AQ,

(3) That 9-NA is not solely responsible for the observed tumours.

Industry representatives have argued against classification based on several points:

(1) The AQ-OX used to generate data for the NTP (2005) report contained mutagenic substances, especially 9-NA.

(2) That the tested AQ-OX was produced via the nitric acid oxidation of anthracene, a process no longer in use, therefore classification as Carc. 1B is only suitable for this material exclusively.

(3) AQ manufactured using other processes (e.g. Friedel-Crafts Reaction, Diels–Adler reaction, vapor-phase oxidation of anthracene with air) are not mutagenic *in vitro* and do not contain the 9-NA contaminant. Pure AQ is non-mutagenic.

(4) The main metabolite of AQ is sulphated 2-OH-AQ and not free 2-OH-AQ. Any mutagenic or carcinogenic concern over 2-OH-AQ is negligible because most of it is found in a conjugated form that is rapidly excreted in the urine.

(5) Comparison with AQ structural analogues in the NTP final report (2005) is not valid because these contain known structural alerts for carcinogenicity (halogen, amino or nitro-substituted functional groups).

(6) A negative *in vitro* mutagenicity test on a sample of AQ-OX from the original NTP bioassays was confounded by doubts over the stability of contaminating components because of misleading information given to the NTP regarding storage conditions and identification of the tested material.

(7) Boobis *et al.* (2009) stated "The data for anthraquinone are considered suspect because other carcinogenicity studies were negative, and…" the AQ tested by NTP was contaminated with 9-NA. (N.B. there are no references to these "other studies" to substantiate the statement for other negative carcinogenicity studies with AQ. The NTP report (2005) briefly refers to negative results in two mouse

strains in a paper by Innes *et al.* (1969), but details were lacking).

(8) Further investigations are required before proceeding with any decision on classification of AQ.

RAC is of the opinion that the following observed tumours are of relevance for human hazard assessment:

(1) Kidney tubular tumours in F344/N rats. The rodent urinary tract appears to be an uncommon site for spontaneous tumour formation and renal tubule adenomas are rare in male rats and even more uncommon in female rats (table 6). Inducible α2μ-globulin nephropathy giving rise to renal tubular tumours can be discounted in this case because the tumour response is observed in both females and males. Despite the increased α2μ-globulin concentration in male rat kidneys, no renal tubule cell hyperplasia has been observed at the 3- or 12-month interim evaluations in the 2 year study. Also, no elevated labelling indices (proliferation) were found in the kidneys of males or females after BrdU incorporation during a 32-day study (NTP 2005). Chronic progressive nephropathy (CPN) exacerbation by chemical exposure is often associated with a generally small increase in the incidence of renal tubular adenomas in 2-year carcinogenicity bioassays. Because CPN is a rodent-specific entity the finding of a small significant increase in renal tubular tumours, linked to exacerbation of CPN is usually considered to be of little relevance to human hazard assessment. However, CPN is not an established mode of action or mechanism of renal carcinogenicity. In a recent investigation into 60 NTP carcinogenicity studies in F344 rats, it was found there were inconsistent relationships between chemically exacerbated CPN and kidney tumour incidences (Melnick *et al.*, 2012). In the NTP studies, while there was a definite exacerbation of CPN by AQ (males; 2.2% vs. 3.1%, 3.0%, and 3.0% in the treated groups, respectively) and there were increases in hyaline droplet incidence in the sub-chronic (90-day) and 2-year studies in both males and females, the tumour response was of such a large magnitude that it is considered highly relevant for human hazard assessment. Moreover, the more notable increase in renal tubule tumours observed in female rats exposed to anthraquinone (0% vs. 18%, 16%, and 29% in the treated groups, respectively) did not correspond to an effect on CPN severity (1.2% vs. 1.4%, 1.3%, and 1.5% in the treated groups, respectively), thus demonstrating a clear lack of association between exposure-related increases in severity of CPN and renal tubular tumours. With reference to CPN in rats, the aetiology of this disease and its exacerbation by chemicals are unknown and so it cannot be argued as a mode-of-action for renal tumours specific to rodents in the present context.

(2) Liver tumours in B6C3F1 mice. The B6C3F1 mouse is a hybrid originating from crossing C57BL/6 with C3H mice. The C3H parental strain is known to manifest a high rate of spontaneous liver tumours, especially in males, and appears to be highly susceptible to tumorigenic agents. This trait is also present in the B6C3F1 hybrid strain. Indeed, a high background incidence for hepatic tumours is evident in both the historical control data and concurrent controls (both adenomas and carcinomas, tables 7 & 8). In addition, evidence from the 32-day dietary study in the NTP report infers extensive CAR activation (through increases in PROD enzymatic activity, indicative of CYP2B induction) with exposure to AQ (in rats) which is known to be associated with increased rodent hepatic tumour induction. The involvement of CAR was not investigated *per se* and very little mechanistic or mode of action information is available except for the enzymatic and proliferative investigations in the 32-day rat dietary study.

A clear treatment related tumour response is evident from the NTP long term dietary mouse study in both sexes. Treated groups are significantly above the

concurrent controls and outside of the historical control incidence range with a very strong tendency to aggressive malignancy as evident by the progression of hepatocellular carcinomas to hepatoblastomas in the male mouse at all tested concentrations. The occurrence of hepatoblastomas is particularly noteworthy and the large increase in incidence with dose (2%-12%-22%-76%, respectively) is of concern. Hepatoblastomas are considered to be poorly differentiated, highly malignant and occur both in rodents and humans. They are usually of low spontaneous occurrence, appear to arise both in adenomas and carcinomas in mice, and a variety of different chemicals can induce them (Turusov *et al.*, 2002). Their relevance to human hazard assessment cannot be discounted.

(3) Thyroid tumours in female B6C3F1 mice. Typically thyroid tumours in rodents, especially if mediated by UDP-glucuronyltransferase (UGT) induction are not relevant for humans. Evidence from the 32-day dietary study in the NTP report suggests extensive CAR activation with exposure to AQ (in rats) which is known to be linked to UGT induction. There is no specific data to indicate whether this mechanism is in operation and it remains speculative at best but the indirect evidence for CAR activation with AQ treatment may lower the level of concern for human hazard assessment.

(4) Urinary bladder transitional epithelial papillomas in male rats. This is of very low background incidence in F344/N rats but there is a strong treatment response with AQ in males. The mechanism of urinary bladder tumorigenesis induced by AQ is unknown. There is little detail concerning the histopathological effects. Isolated single instances of calculus were observed but without any indication of a treatment related effect or a dose response. A high background incidence of inflammation masked any treatment related effect. There were no tumours at the 3- and 12-month interim evaluations in either sex. Physical irritation of the urothelium in rats for a long period leads to papilloma followed by carcinoma, a mechanism of low concern to humans. AQ treatment in rats cannot be described as acting through a physical irritation of the urinary bladder from the available data and is therefore considered relevant for human hazard assessment.

In addition, RAC points out further evidence of the carcinogenicity of AQ in animals:

1. There is a causal relationship between AQ and an increased combination of benign and malignant tumours in two species of animals;
    - *malignant tumours*: Rat (F), kidney carcinomas; Mouse (M), hepatic carcinomas and hepatoblastomas.
    - *benign tumours*: Rat (F), liver adenomas, kidney adenomas; Rat (M), kidney adenomas, kidney papillomas, urinary bladder papillomas; Mouse (M) liver adenomas; Mouse (F) hepatic adenomas, thyroid follicular cell adenomas.
2. There is an increase in the incidence of tumours in both sexes of a single species in a well-conducted study; hepatic tumours in mice; renal tubule tumours in rats.
3. There is at least a single study in one species and sex where malignant neoplasms occur to an unusual degree; renal tubule carcinomas in female rats; heptoblastomas in male mice.
4. There is at least a single study in one species and sex where there are strong findings of tumours at multiple sites; renal tubule adenomas, urinary bladder papillomas in male rats; liver adenomas and renal tubule adenomas in female rats; hepatic adenomas and thyroid follicular cell adenomas in female mice.

RAC notes that there are several factors to take into account that increase the level of concern of AQ regarding human carcinogenicity:

1. Tumours occur in relevant tissues common to rodent and man (liver, kidney, urinary bladder). The tumours do not occur in tissues with no equivalent in humans.
2. Tumours occur in two rodent species.
3. Tumours occur at multiple sites.
4. Rare tumours are found (renal tubule and urinary bladder in rats, hepatoblastoma in mice).
5. There is a progression from benign to malignant tumours (renal tubule adenoma → carcinoma in female rats; hepatocellular adenoma → carcinoma → hepatoblastoma in male mice), which is supportive of Carc. 1B.
6. Tumours are observed in both sexes.
7. Close structural analogues with differences in the functional group substitutions around the aromatic rings cause a variety of tumours some of which are shared with those observed for AQ (Sendlebach *et al.* 1989; Doi *et al.* 2005).
8. Metabolism studies show the formation of 1-OH-AQ and 2-OH-AQ metabolites regardless of the source (production process) of the AQ fed to rats in the diet.
9. There are concerns regarding the mutagenicity and carcinogenicity of the two hydroxyl-AQ metabolites. There is no complete genoticity/mutagenicity toxicology dossier for these components or for 9-NA contaminant to determine to what extent, if any, they have influenced the outcomes of the NTP carcinogenicity study on AQ.
10. The incidence of many tumours is not only greater than the concurrent control group but also greater than the range of incidences noted in chronologically relevant historical controls.
11. There are a few instances where there is a small increase in a particular tumour type which historical control data shows to be very uncommon and therefore unlikely to have occurred by chance. This implies a carcinogenic response even if the findings were not statistically significant, e.g. with the following tumour types: renal tubular adenoma in male mice, urinary bladder transitional epithelial carcinoma in female rats, renal transitional epithelial adenoma in male rats.

RAC further notes that there are several factors to take into account that decrease the level of concern of AQ regarding human carcinogenicity:

1. A strong induction of mostly benign tumours.
2. Tumours only seen in one sex (two rodent species).
3. The renal tubule tumours develop coincident with increased incidence of chronic progressive nephropathy (CPN) and increased hyaline droplet formation. The renal tubule tumours are a consequence of CPN exacerbation by AQ and are not relevant to human hazard assessment. In addition, the non-neoplastic changes observed in sub-chronic studies may be suggestive of α2μ-globulin nephropathy syndrome.
4. Thyroid tumours in rodents mediated by UDP-glucuronyltransferase (UGT) induction are not relevant for humans. RAC notes that this is speculative for AQ, since there are no data to directly support if this is the operational mechanism of action in the observed follicular cell tumours.
5. Certain animal strains have a propensity to develop particular types of tumour

spontaneously with variable and potentially high incidence. Such is the case for liver tumours in B6C3F1 mice. Induction of liver tumours in this strain of mouse is of low concern for human hazard assessment.

6. Most of the tumour profiles followed a non-monotonic dose response so that the causal relationship between AQ and an increased combination of benign and malignant tumours can be challenged.
7. The technical material used in the NTP 2-year bioassays was AQ contaminated with mutagenic 9-nitroanthracene and/or other mutagenic impurities. A clean-up process on this material showed no mutagenic activity in contrast to the original unprocessed technical material.
8. Currently sourced and commercially available AQ is not produced from the nitric acid oxidation of anthracene and will not have 9-NA as a contaminant. The results of the NTP bioassays are specific only to technical material no longer under manufacture.
9. Pure AQ is not mutagenic.
10. The major metabolite produced from AQ is 2-OH-AQ as its sulphate conjugate that is rapidly excreted in the urine.

Consideration of category 1A or 2

IARC (2012) found no studies of human cancer where exposures to anthraquinone *per se* were evaluated. They did however summarise a series of publications on dye and resin workers in the USA, from a single facility in New Jersey, who were potentially exposed to AQ during its production or its use to produce AQ intermediates. The IARC Working Group noted that the major limitation of these studies was that they did not assess exposure to specific chemicals (i.e. exposure to AQ *per se* was not evaluated); risk estimates were calculated for employment in the various production areas or for different processes but the ability to evaluate potential confounding from other occupational chemical exposures (such as AQ dye intermediates, azo-dyes, anthracene, vanadium pentoxide and epichlorohydrin) was limited. These studies also had limited statistical power to detect effects for specific cancers because of the small numbers of exposed cases. IARC (2012) concluded there was inadequate evidence in humans for the carcinogenicity of anthraquinone.

According to the CLP regulation, a substance shall be classified as *carcinogenic in category 1A* if: 'It is known to have carcinogenic potential for humans; classification is largely based on human evidence.'

RAC concludes that classification of AQ as category 1A is not warranted, since there is insufficient evidence for carcinogenicity in humans from the few indirect epidemiological studies reported by IARC (2012).

According to the CLP regulation a substance shall be classified as *carcinogenic in category 2* if: 'It is a suspected human carcinogen, but the evidence is not sufficient for category 1A or 1B'. Classification with Carc. 2 is justified if any of the following considerations are true:

(a) The evidence is limited to a single experiment. In this case there were two parallel investigations.

(b) There are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies. However, concerns about misleading results due to contamination issues with low levels of 0.1% 9-NA measured in the NTP material as well as the mutagenic and carcinogenic potential of the hydroxyl-AQ metabolites are outside the scope of the 2-year bioassays. Separate studies would need to be commissioned to answer these questions.

(c) The agent increases the incidence only of benign neoplasm or lesions of uncertain

neoplastic potential. There was in fact clear evidence for renal tubule carcinoma as well as transitional epithelial urinary bladder carcinoma in female rats, and hepatocellular carcinoma in male and female mice).

(d) The evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs. This was not the case, since clear tumorigenic effects were seen following treatment with AQ.

Therefore, classification as category 2 is not supported by the RAC because the criteria for Carc 2 are not fulfilled and the whole of the available data supports a strong tumorigenic response in rodents that cannot be dismissed and is considered to be relevant to humans (see 'Factors that increase the level of concern for human carcinogenicity' above).

Consideration of category 1B

Evidence of animal carcinogenicity was presented above. According to the CLP regulation a substance shall be classified as *carcinogenic in category 1B* if: 'It is presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.'

This category depends on strength of evidence, which consists of animal experiments for which there is sufficient evidence to demonstrate animal carcinogenicity. This means a causal relationship has been established between the chemical agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in:

(a) two or more species of animals *or* in two or more independent studies in one species carried out at different times (or in different laboratories or under different protocols);

(b) both sexes of a single species;

(c) occurrence of malignant neoplasm to an unusual degree with regard to the incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

The NTP studies showed some evidence for carcinogenicity of AQ in male F344/N rats (kidney, urinary bladder), clear evidence for carcinogenicity of AQ in female F344/N rats (kidney) as well as clear evidence of carcinogenic activity in male and female B6C3F1 mice (liver). In comparison with the criteria for category 1B under the CLP regulation AQ displays the following tumour profile:

(1) positive findings in two species,

(2) positive findings in both sexes, and

(3) occurrence of (rare) tumour types at greater than background incidence in several target organs.

The mode of action for AQ has not been determined. AQ derived from processes other than nitric acid oxidation of anthracene have not been tested in 2-year bioassays. The impurity 9-NA has not been tested for carcinogenicity. The formation of hydroxyl-AQ metabolites can occur from any AQ preparation.

The DS concluded that the carcinogenic responses in animal studies are attributable to AQ (including its active metabolites and possible impurities) and thus, AQ is considered to be carcinogenic. The relative contribution to the carcinogenic response made from one or all of the components in the technical material tested by the NTP (AQ, its metabolites or possible impurities) cannot be made without further studies.

Therefore, in agreement with DS proposal, RAC concludes that **classification for carcinogenicity as category 1B (Carc. 1B, H350) is warranted**, based on animal experiments from which there is sufficient evidence to demonstrate animal carcinogenicity.

4.11 Toxicity for reproduction

This toxicological endpoint was not evaluated for this dossier.

4.12 Other effects

Not evaluated for this dossier.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Not evaluated for this report.

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