EUROPEAN CHEMICALS AGENCY

Committee for Risk Assessment

RAC

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

Adopted
4 December 2015

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EUROPEAN CHEMICALS AGENCY

4 December 2015

CLH-O-0000001412-86-86/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **anthraquinone**

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

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

The proposal was submitted by **Germany** and received by RAC on **30 January 2015.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Germany has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation*** on **18 February 2015**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **7 April 2015**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Brendan Murray**

Co-Rapporteur, appointed by RAC: **Marian Rucki**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **4 December 2015** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

Annex VI	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Entry	No current entry in Annex VI										
Dossier submitter proposal		anthraquinone	201-549-0	84-65-1	Carc. 1B	H350	GHS08 Dgr	H350			
Proposal for RAC		anthraquinone	201-549-0	84-65-1	Carc. 1B	H350	GHS08 Dgr	H350			
Resulting Entry		anthraquinone	201-549-0	84-65-1	Carc. 1B	H350	GHS08 Dgr	H350			

GROUNDS FOR ADOPTION OF THE OPINION

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.

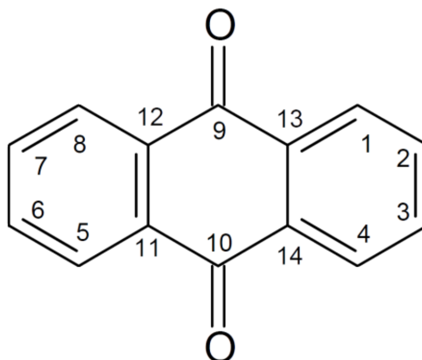

Figure: Anthraquinone: Basic structure and carbon numbering

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.

HUMAN HEALTH HAZARD ASSESSMENT

RAC evaluation of mutagenicity

Summary of the Dossier submitter's proposal

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

<table>
<tr><th>Study</th><th>Material and process</th><th>Result*</th><th>Methods and acceptability</th><th>Reference</th></tr>
<tr><td colspan="5">In vitro</td></tr>
<tr><td rowspan="6">Bacterial mutagenicity</td><td>NTP AQ-OX (purified)
(nitric acid)</td><td>negative</td><td>GLP, OECD TG 471, acceptable</td><td>Butterworth et al. 2001</td></tr>
<tr><td>NTP AQ-OX (A07496)
(nitric acid)</td><td><u>positive</u> with and without S9-mix</td><td>GLP, OECD TG 471, sample A07496 was used in the NTP 2 year studies, acceptable</td><td>Butterworth et al. 2001</td></tr>
<tr><td>AQ-FC technical</td><td>negative</td><td rowspan="2">GLP, OECD TG 471, acceptable</td><td>Butterworth et al. 2001</td></tr>
<tr><td>AQ-DA technical</td><td>negative</td><td>Butterworth et al. 2001</td></tr>
<tr><td>100% AQ process unspecified</td><td>negative</td><td>GLP and OECD unknown, acceptable</td><td>Zeiger et al., 1992
Table E2 NTP 2005</td></tr>
<tr><td>97% AQ process unspecified</td><td><u>positive</u>, with and without S9-mix</td><td>GLP and OECD unknown, acceptable</td><td>Zeiger et al., 1988
Table E1 NTP 2005</td></tr>
</table>

	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 injection, GLP	NTP 2005 (Table E12)

			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).

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 <u>as likely</u> 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.

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.

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 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**.

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.

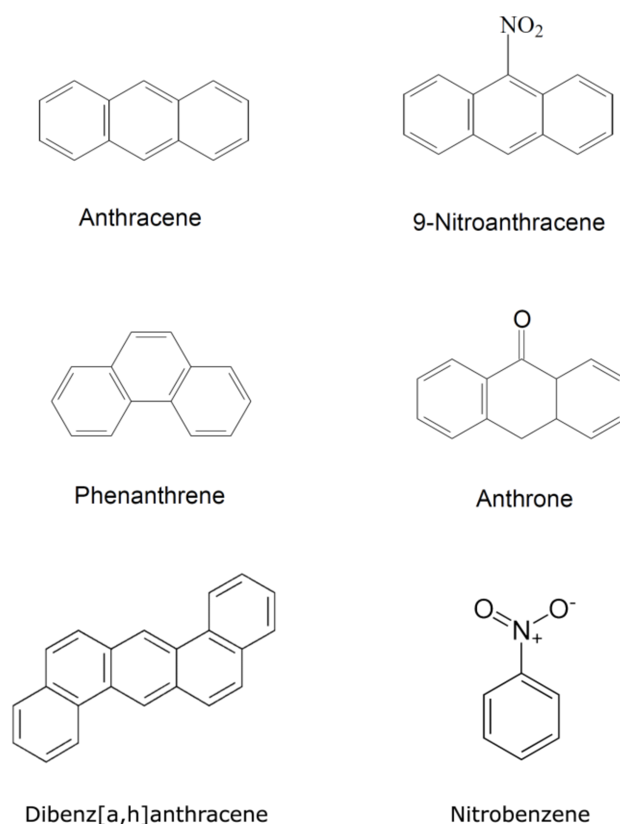

Figure: Chemical structures of some of the contaminants identified in the anthraquinone technical material used in the NTP bioassays.

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	0/1348*	0	0	0	0	0

Overall incidence						
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.

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).