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SCIENTIFIC COMMITTEE ON PLANTS **SCP/VINCLO-TER/002-Final**

OPINION OF THE SCIENTIFIC COMMITTEE ON PLANTS ON ADDITIONAL QUESTIONS FROM THE COMMISSION CONCERNING THE EVALUATION OF VINCLOZOLIN IN THE CONTEXT OF COUNCIL DIRECTIVE 91/414/EEC

(Opinion adopted by the Scientific Committee on Plants, 18 July 2002)

A. TITLE

OPINION OF THE SCIENTIFIC COMMITTEE ON PLANTS ON ADDITIONAL QUESTIONS FROM THE COMMISSION CONCERNING THE EVALUATION OF VINCLOZOLIN IN THE CONTEXT OF COUNCIL DIRECTIVE 91/414/EEC
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B. TERMS OF REFERENCE

The Scientific Committee on Plants (SCP) expressed its first opinion on vinclozolin on 28 October 1999[1]. The Committee is now requested to respond to the additional following questions in the context of the Commission's work on the implementation of Council Directive 91/414/EEC concerning the placing of plant protection products on the market.

1. Does the Committee consider that the effects observed in the fish life-cycle study with fathead minnow are of biological and ecological significance?

2. Can the Committee comment on the No Observed Effect Concentration (NOEC) suggested by the Rapporteur (50 μg/L from the 28 day rainbow trout study) with regard to its validity to protect fish from effects on reproduction?

C. OPINION OF THE COMMITTEE

Question 1.

The results of the fathead minnow life-cycle study, though not definitive, are supported by additional evidence of vinclozolin's potential to adversely affect reproduction in fish species. However, there remain substantial uncertainties associated with translating the laboratory test exposures to likely exposure levels in the field and uncertainties resulting from a high degree of variability in reproductive responses within and between species. The SCP considers that vinclozolin has the potential to have adverse effects on fish reproduction and such effects, if they occur in natural populations, could be ecologically significant.

Question 2.

The 28 day rainbow trout study did not measure or attempt to assess reproductive effects in fish. The SCP cannot determine whether the NOEC derived on the basis of effects on feeding and coloration in juvenile rainbow trout exposed for 28 days would be sufficient to protect fish from effects on reproduction.

[1] http://europa.eu.int/comm/food/fs/sc/scp/out52_en.html

A. TITLE

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C. BACKGROUND

Vinclozolin is an existing active substance in the context of Directive 91/414/EEC[2] concerning the placing of plant protection products on the market and is one of the active substances covered by the first stage of the work programme provided for under the Directive. The Committee issued two earlier opinions on the establishment of MRLs[3] (available as SCP/VINCLO/019- FINAL[4]) and on the risk assessment of birds (available as SCP/VINCLO/021-Final[5]).

A draft evaluation report (monograph) has been prepared by the Rapporteur Member State (RMS, France) on the basis of a dossier presented by the notifier (BASF). In order to prepare its opinion the Scientific Committee on Plants had access to the documents listed below.

Vinclozolin and its degradation products have anti-androgenic effects which were investigated in aquatic organisms as well as in *in vitro* studies. The lowest endpoint for aquatic organisms is based on the trout (28-day study) NOEC = 0.05 mg/L. The RMS considered that the effects observed in the fish life-cycle study with fathead minnow are not considered as biologically important in terms of fish populations and therefore proposed a NOEC of 0.05 mg/L for aquatic organisms on the basis of the most sensitive species investigated, the trout.

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[2] OJ NO L230, 19.08.91 p.1
[3] Maximum pesticide Residue Limits
[4] http://europa.eu.int/comm/dg24/health/sc/scp/out52_en.html
[5] http://europa.eu.int/comm/food/fs/sc/scp/out62_ppp_en.pdf

The Commission has asked the SCP to express an opinion on whether or not the effects observed in the available life-cycle study in fathead minnow can be considered biologically and ecologically significant. If the Committee's conclusion differs from the RMS view, the Commission would like to have the Committee's opinion on the consequences for the NOEC proposed by the RMS.

Source documents made available to the Committee:

1. Additional questions on the evaluation of Vinclozolin in the context of Council Directive 91/414/EEC: Terms of reference, submitted by DG Health and Consumer Protection, 13 November 2001 (SCP/VINCLO-TER/001).

2. Addendum to the Monograph of the active substance: Vinclozolin, (19addendum_fish_2 21.12.00), prepared by the RMS (France), submitted by DG Health and Consumer Protection, 13 November 2001 (SCP/VINCLO-TER/003).

3. Notifier position paper: Risk assessment for vinclozolin in aquatic ecosystems, BASF, 22.09.2000, BASF Doc ID 2000/1016885, submitted by DG Health and Consumer Protection, 13 November 2001 (SCP/VINCLO-TER/005).

4. Draft Review report 5038/VI/98-rev. 2, 8 September 2001, submitted by DG Health and Consumer Protection, 13 November 2001 (SCP/VINCLO-TER/004).

5. Modified life cycle study with the fathead minnnow (*Pimephales promelas*) and Vinclozolin in the presence of its metabolies B and E (Limit Test). BASF Doc ID 2000/1016916.

6. Sublethal toxic effects on rainbow trout (*Oncorhynchus mykiss* WALBAUM 1792) of BAS 352 40F in a flow-through system at pH 6.5 (OECD 204). BASF Doc ID 90/0316.

7. Bayley M, Junge M, Baatrup E. 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquatic Toxicology 56: 227-239.

8. Makynen EA, Kahl MD, Jensen KM, Tietge JE, Wells KL, Van der KraaK G, Ankley GT. 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). Aquatic Toxicology 48: 461-475.

9. Notifier position paper: Risk assessment for vinclozolin in aquatic ecosystems, BASF Doc ID 2002/1004135 (=updated version of BASF Doc ID 2000/1016885). (SCP/VINCLO-TER/008).

10. Euling SY, Kimmel CA. 2001. Developmental stage sensitivity and mode of action information for androgen agonists and antagonists. The Science of the Total Environment 274: 103-113.

11. Gray LE, Ostby J, Furr J, et al. 2001. Effects of environmental antiandrogens on reproductive development in experimental animals. Human Reproduction Update 7: 248-264.

D. SCIENTIFIC BACKGROUND ON WHICH THE OPINION IS BASED

I. QUESTION 1:

Does the Committee consider that the effects observed in the fish life-cycle study with fathead minnow are of biological and ecological significance?

Opinion:

The results of the fathead minnow life-cycle study, though not definitive, are supported by additional evidence of vinclozolin's potential to adversely affect reproduction in fish species. However, there remain substantial uncertainties associated with translating the laboratory test exposures to likely exposure levels in the field and uncertainties resulting from a high degree of variability in reproductive responses within and among species. The SCP considers that vinclozolin has the potential to have adverse effects on fish reproduction and such effects, if they occur in natural populations, could be ecologically significant.

Scientific background on which the opinion is based:

A modified life-cycle study with fathead minnow (*Pimephales promelas*) exposed to vinclozolin and its metabolites was conducted for a period of 126 days (BASF 2000, SCP/VINCLO-TER/003). Fish (F0 generation) were approximately 8 months of age at the start of the study and were not yet sexually mature. They were followed through maturity (34 days) and reproduction (over a period of 78 days). A subset of their eggs was allowed to hatch and the offspring (F1 generation) followed for an additional 42 days post hatch. Both F0 and F1 generations were exposed to either a dilution water control or one concentration of test substance (nominal = 0.1 mg/L; analytical = 0.07 mg/L vinclozolin + 0.05 mg/L metabolite BF 352-22 + 0.008 mg/L metabolite BF 352-23). This test concentration was considered to be the highest concentration which could be tested under realistic practical conditions in a long-term flow-through study without the use of solvents. On the basis of a 28-day growth test with rainbow trout no adverse effects were expected, and it was therefore decided to conduct a test with only one concentration.

The test used a flow-through design. Initially 20 fish x 4 replicate aquaria were used in control and exposure groups. On day 34 both control and exposure groups were reduced to 16 pairs each. The F1 generation was started with 4 x 50 eggs per test group that were obtained from a mixture of eggs laid by 4-5 females per F0 group.

The following endpoints were measured in the F0 fish: survival, appearance, behaviour, swimming behaviour, male coloration, time during which males stood in the brood hole, activity of males in driving females to the brood hole, number of clutches per pair, clutch size, fertility rate, body weights and lengths of males and females, and gonad histology. In the F1 fish observations were made on survival, changes in appearance and behaviour and deformations (to the extent that this was possible). Males in the exposed group were

significantly less active in directing the females to the brood hole, and there were effects observed on several measures of reproductive output (Table 1).

Table 1. Reproductive effects of vinclozolin on fathead minnow from BASF (2000).

Endpoint	% change relative to control
Total number of clutches	43% reduction
Mean number of clutches per breeding pair	55% reduction
Total number of eggs per group	0.2% reduction
Mean number of eggs per clutch	63% increase
Mean number of eggs per breeding pair	22% reduction
Mean fertility rate	6% reduction

The total and mean number of clutches were reduced significantly in the exposed group but this was to some extent offset by a significant increase in clutch size. Thus the average number of eggs produced per breeding pair was reduced by 22% in the exposed group and the mean fertility rate by 6%, neither of which were statistically significant. The RMS questioned the biological relevance of the reduction in clutch number, since it was offset by the increase in clutch size to an extent that total eggs produced per pair was not significantly reduced. However, although not statistically significant, the SCP considers that a 22% reduction in mean egg production per breeding pair potentially could have ecological significance and should not be ignored. In addition, reproductive frequency (i.e., number of clutches) and effort (i.e., clutch size) are evolved traits that in field populations may be fine-tuned to local conditions (Stearns and Hoekstra 2000). For example, under environmentally variable conditions having frequent, smaller clutches can increase the chance that at least some of the offspring are born into a favourable environment. Thus even if there is no change in total reproductive investment (i.e., total eggs per pair), disruptions in the frequency and size of clutches can have important ecological consequences.

In the F1 generation, survival was significantly reduced in the exposed group in the first three days until hatch, but the difference was not significant during the remainder of the test (Table 2).

Table 2. Effects of vinclozolin on embryo survival from BASF (2000).

Embryo survival	Control Group (%)	Exposed Group (%)
Days 78-81	84	55.5
Days 81-84	79.2	76.6
Days 84-126	69.2	62.4

A large variability in fertility rate among F0 pairs and among clutches within pairs was noted, and since a small number of pairs (i.e., 4) contributed to production of the F1 generation, the test report suggests that the lower survival rate at the start of the F1 generation ‘might be due to the normal variation between pairs and even between the spawnings of one pair’.

The results of this test cannot be considered definitive because: 1) the high variability in the reproduction endpoints means that real effects would have a low likelihood of statistical detection (i.e., high Type II error); 2) alternative (not toxicant related) explanations for observed reductions in early survival of the F1 generation cannot be

excluded; 3) the limit test design prevents evaluation of toxicant-related trends (that could help to establish whether differences among groups were or were not toxicant related).

Despite the substantial uncertainties, and for some effects lack of statistical significance, the kinds of reproductive effects observed in this study (i.e., reduced male mating activity, reduced average egg production and reduced early embryo survival) are of concern, particularly given that vinclozolin is known to show anti-androgenic effects (Euling and Kimmel 2001, Gray et al. 2001).

Makynen et al. (2000) examined the effects of vinclozolin and its metabolites on development and reproduction of fathead minnow (*Pimephales promelas*). Their first experiment was a 34-day early life-stage test (flow-through design; nominal concentrations 0, 75, 150, 300, 600, 1200 μg/L) followed by an additional 4-6 month observation period. Reduced growth was observed at the highest concentration by the end of the exposure period (30% reduction in weight compared to control on day 34) but no effects were observed on survival, gross pathology, sexual reproduction or reproductive success. Survival of juveniles over the first 10 days of the test was 84% in the control and 70% at the two highest concentrations (a 17% reduction). Survival from 10-34 days of exposure was 79% in the control and 74% at the highest concentration (a 6% reduction). Effects on fecundity (measured as eggs/spawn) are shown in Table 3. In this test viability (and thus fertility) of the eggs was generally very high with > 95% viability 48 h post-spawn irrespective of the original vinclozolin treatments. Due to the large variability among replicates (standard deviations are between 13 and 88% of the means) differences in fecundity among groups were not statistically significant. Both this high variability and the lack of a typical monotonic concentration-response relationship prevent these results from being entirely conclusive.

Table 3. Effects of vinclozolin on fathead minnow fecundity from Makynen et al. (2000).

Concentration Group	Eggs/spawn (mean ± sd)	% reduction
0	121 ± 30	
75	98 ± 13	19
150	96 ± 40	21
300	94 + 47	22
600	49 ± 43	60
1200	92 ± 52	24

Makynen et al. (2000) also performed an experiment with adults (21-day exposure to either 200 or 700 μg/L). There were no effects of either concentration on survival, weight, or reproductive (territorial behaviour) but at 700 μg/L the percent of body weight composed of gonad was significantly reduced in females and a slight increase of Beta-oestradiol was found in males. However there was little spawning throughout the test and hence it was not possible to determine the effects, if any, on fecundity or viability.

The third experiment reported by Makynen et al. (2000) measured anti-androgenic activity of vinclozolin and its metabolites M1 (BF 352-22), M2 (BF 352-23), and M3 (3,5-dichloroanilide). The results showed that vinclozolin was ineffective in competing with testosterone for binding sites in fathead minnow brain and ovary cytosolic fractions. However, Bayley et al. (2002) point out that the links between in vitro receptor binding

and in vivo activity remain unclear in teleost fish that appear to have multiple AR isomers. Therefore they argue that assessment of endocrine disrupting effects requires direct measurement of sex characters and reproduction. Bayley et al. (2002) examined the effects of vinclozolin in guppies, *Poecilia reticulata*. They found that vinclozolin was associated with delayed sexual maturation, smaller body size at maturity, a skewed sex ratio in favour of females, reductions in male courtship display behaviour, reduced sperm cell counts, and reduced reproductive output. Thus, these results indicate very clearly that vinclozolin has the potential to cause adverse effects on fish reproduction that are consistent with its anti-androgenic properties. Unfortunately exposure in this study was via contaminated food and it is not possible to make a reliable comparison of these exposures with realistic field scenarios associated with the agricultural usage of vinclozolin.

- In conclusion, the SCP considers that vinclozolin has the potential to have adverse effects on fish reproduction and such effects, if they occur in natural populations, could be ecologically significant. The results of the fathead minnow life-cycle study (BASF 2000), though not definitive, are supported by additional evidence of vinclozolin's potential to adversely affect reproduction in fish species (Makynen et al. 2000, Bayley et al. 2002). However, there remain substantial uncertainties including but not limited to: variability in measured exposure concentrations during laboratory studies (e.g., these were particularly noticeable in Makynen et al. 2000).
- The relationship between exposure via contaminated food (e.g., from Bayley et al. 2002) to exposure in relevant field scenarios.
- A very high variability in some of the fathead minnow reproductive endpoints.
- Variability among fish species in sensitivity to reproductive effects.
- The extent to which adverse effects on individual reproduction will lead to population level impacts in the field.

II. <u>QUESTION 2</u>:

Can the Committee comment on the No Observed Effect Concentration (NOEC) suggested by the Rapporteur (50 µg/L from the 28 day rainbow trout study) with regard to its validity to protect fish from effects on reproduction?

<u>Opinion:</u>

The study referred to did not measure or attempt to assess reproductive effects in fish. The SCP cannot determine whether the NOEC derived on the basis of effects on feeding and coloration in juvenile rainbow trout exposed for 28 days would be sufficient to protect fish from effects on reproduction.

<u>Scientific background on which the opinion is based:</u>

The NOEC of 0.05 mg a.i./L is based on effects on rainbow trout (*Oncorhyncus mykiss*) exposed to Ronilan DF under flow-through conditions for 28 days to nominal concentrations of 0, 0.1, 1, 5, 10, and 20 mg/L) (BASF 1990). The fish were approximately 6 months of age at the start of the study and were not sexually mature. Measured endpoints included survival, growth (weight and length), feeding, behaviour, and colour. The NOEC for this test was based on reduced feeding and discoloration observed to occur at the nominal concentration of 1 mg/L. The nominal NOEC of 0.1

mg/L was determined analytically to range between 0.03 and 0.1 (average of five measurements on different days = 0.068 mg/L). At higher concentrations "apathy", swimming at the bottom or at the surface, tumbling, no feeding, lying on the bottom, or spasms and convulsions were also observed. At the nominal concentrations 0.1 and 1 mg/L fish were significantly larger than in the control by the end of the exposure whereas fish in the 5, 10, and 20 mg/L groups were significantly smaller than the control.

This study did not measure reproductive effects and employed sexually immature fish. Therefore it cannot be determined whether the NOEC derived on the basis of effects on feeding and coloration in juvenile rainbow trout exposed for 28 days would be sufficient to protect fish from effects on reproduction.

E. REFERENCES

BASF 1990. Sublethal toxic effects on rainbow trout (*Oncorhynchus mykiss* WALBAUM 1792) of BAS 352 40F in a flow-through system at pH 6.5 (OECD 204). BASF Doc ID 90/0316.

BASF 2000. Modified life cycle study with the fathead minnnow (*Pimephales promelas*) and Vinclozolin in the presence of its metabolies B and E (Limit Test). BASF Doc ID 2000/1016916.

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Gray LE, Ostby J, Furr J, et al. 2001. Effects of environmental antiandrogens on reproductive development in experimental animals. Human Reproduction Update 7: 248-264.Makynen EA, Kahl MD, Jensen KM, Tietge JE, Wells KL, Van der KraaK G, Ankley GT. 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). Aquatic Toxicology 48: 461-475.

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